Differentiation of Osteoprogenitor Cells Is Induced by High-Frequency Pulsed Electromagnetic Fields

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Abstract: Craniofacial defect repair is often limited by a finite supply of available autologous tissue (ie, bone) and less than ideal alternatives. Therefore, other methods to produce bony healing must be explored. Several studies have demonstrated that low-frequency pulsed electromagnetic field (PEMF) stimulation (ie, 5–30 Hz) of osteoblasts enhances bone formation. The current study was designed to investigate whether a Food and Drug Administration–approved, high-frequency PEMF–emitting device is capable of inducing osteogenic differentiation of osteoprogenitor cells. Osteoprogenitor cells (commercially available C3H10T1/2 and mouse calvarial) in complete Dulbecco modified Eagle medium were continuously exposed to PEMF stimulation delivered by the ActiPatch at a frequency of 27.1 MHz. Markers of cellular proliferation and early, intermediate, and terminal osteogenic differentiation were measured and compared with unstimulated controls. All experiments were performed in triplicate. High-frequency PEMF stimulation increases alkaline phosphatase activity in both cell lines. In addition, high-frequency PEMF stimulation augments osteopontin and osteocalcin expression as well as mineral nodule formation in C3H10T1/2 cells, indicating late and terminal osteogenic differentiation, respectively. Cellular proliferation, however, was unaffected by high-frequency PEMF stimulation. Mechanistically, high-frequency PEMF-stimulated osteogenic differentiation is associated with elevated mRNA expression levels of osteogenic bone morphogenetic proteins in C3H10T1/2 cells. Our findings suggest that high-frequency PEMF stimulation of osteoprogenitor cells may be explored as an effective tissue engineering strategy to treat critical-size osseous defects of the craniofacial and axial skeleton.

Key Words: High-frequency pulsed electromagnetic fields; osteogenic differentiation; osteoprogenitor cell differentiation; pulsed electromagnetic fields

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; ERK-1, extracellular signal–regulated kinase 1; iCALs, immortalized calvarial cells; IHC, immunohistochemical; MAP, mitogen-activated protein; MSC, mesenchymal stem cell; OCN, osteocalcin; OPN, osteopontin; p38α, p38-reactivating kinase; PBS, phosphate-buffered saline; PEMF, pulsed electromagnetic field

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A major limitation in the repair of craniofacial defects lies in the finite supply of autologous tissue (ie, bone) available. Alternatives, such as demineralized bone matrix,[1] bone ceramics,[2] titanium,[3,4] and porous polyethylene implants,[5] are associated with an increased risk of infection, do not expand with a growing craniofacial skeleton (in the case of children), and can fail over time. In keeping with one of the core principles in tissue reconstruction, it is desirable to replace "like with like." To this end, engineering of bone via cells capable of expansion and differentiation into bone is a valid strategy.

Many methods have been used to engineer bony tissue. For example, a useful and effective option is the transduction of target cells with an osteoinductive cytokine such as bone morphogenetic protein (BMP).[6,7] However, this strategy is costly and can be confounded by infection risk, poor expression of the gene of interest, and the inability to modulate the gene of interest once the desired effect is achieved. An alternative approach, which avoids direct genetic alteration of target cells, is that of biophysical stimulation. Biophysical stimulation consisting of low-frequency pulsed electromagnetic fields (PEMFs) has been used clinically as adjunct treatment for processes affecting the musculoskeletal system for many years.[8–14] At the cellular level, low-frequency PEMF stimulation is thought to modulate the expression level of endogenous osteogenic cytokines and their receptors.[15–18] Nevertheless, the specific molecular mechanisms underlying the cellular response to PEMF stimulation have remained elusive.

Two key features of studies examining the osteoinductive effect of PEMFs are cell type and stimulation frequency. To this point, most scientists have evaluated the effect of PEMF stimulation on terminally differentiated osteoblasts. However, in situ cell populations at areas of bone growth are heterogeneous and contain cells at different stages of maturation. We therefore elected to investigate

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the effect of PEMFs on pluripotent cells. In addition, previous authors have generally exposed cells to low-frequency PEMF stimulation (ie, 5–30 Hz). A disadvantage of this subtype of PEMF stimulation is often the requirement for cofactors (eg, ascorbic acid, β-glycerophosphate, calcium phosphate [disks]) to induce differentiation in vitro.[15,16] In contrast, the use of high-frequency PEMFs (ie, ≥1 MHz) to induce osteogenesis has not been previously described. Therefore, we examined whether high-frequency PEMF stimulation, like its low-frequency counterpart, has the ability to stimulate bone formation.

In the current study, osteoprogenitor cells were stimulated by a high-frequency PEMF-delivery device that is Food and Drug Administration (FDA) approved to treat soft tissue discomfort and edema in the postoperative setting.[19] We show that this novel delivery system of high-frequency PEMFs is capable of augmenting osteogenic differentiation in murine progenitor cells without the aid of additional costimulants.

MATERIALS AND METHODS

Cell Culture and Chemicals

C3H10T1/2 cells were obtained from ATCC (Manassas, VA). Cells were maintained under conditions as described previously.[20–22] Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). Of note, cells were cultured in medium that did not contain pro-osteogenic factors (eg, dexamethasone, ascorbic acid, β-glycerophosphate).

Immortalized calvarial cells (iCALs) were also used for experimentation. Briefly, calvariae were isolated from 3-week-old male CD-1 mice (Charles River, Wilmington, MA). Mice were housed in standard cages in an experimental animal room (24°C, 55% humidity, 1 atm, 12-hour light-dark cycle) and were fed a standard laboratory diet and water ad libitum. This investigation was approved by the Institutional Animal Care and Use Committee of the University of Chicago (Chicago, IL), and animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines set forth by this committee. All procedures were conducted under sterile conditions.

Mice were killed, and calvariae were harvested by creating a midsagittal incision. The periosteum was incised to expose the calvarium on both sides of the midline. Soft tissue, dura, and remaining periosteum were removed. The isolated calvariae were washed repeatedly in phosphate-buffered saline (PBS) with 1% penicillin/streptomycin (p/s) solution, minced, and transferred to 10-mm^2 wells containing regular Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1% p/s solution (Sigma-Aldrich). Cultures were incubated at 37°C, 95% humidified air, and 5% CO_2. After approximately 7 days, cells grew to 80% confluency (percentage of cells covering the plate), at which point they were passaged by enzymatic digestion (0.1% trypsin; Sigma-Aldrich) to 25-cm^2 flasks containing 8 mL of Dulbecco modified Eagle medium with 10% fetal bovine serum and 1% p/s for experimentation. These cells display surface antigens found on mesenchymal stem cells (MSCs) and can differentiate into various mesenchymal tissues including bone and adipose (data not shown).

To allow for ease of culturing and preservation of cellular growth, harvested primary calvarial cells were allowed to grow in culture for 5 weeks and then underwent immortalization using a retroviral-mediated vector as previously described.[23] The immortalization process did not significantly alter the phenotype of this cell population (data not shown). Of note, early and late osteogenic differentiation and cellular proliferation of iCALs were examined, whereas all phases of differentiation (early, middle, and late) and proliferation of C3H10T1/2 cells were examined.

High-Frequency PEMF Stimulation

Pulsed electromagnetic fields were generated by the ActiPatch (BioElectronics, Frederick, MD). This device (Fig. 1A) has received FDA approval to treat soft tissue swelling, ecchymosis, and discomfort using low-energy, high-frequency PEMF technology. The nominal carrier frequency delivered is 27.1 MHz. The pulse frequency is 1000 Hz, with each pulse lasting 100 microseconds. The device produces an energy level of 50 to 100 μV/cm up to a distance of 1 cm. A distance of 5 cm or greater from the PEMF-emitting region of the device effectively reduces this energy level to 0 μV/cm. The device has a 720-hour battery life.

Unless otherwise indicated, stimulated cells were continuously exposed to PEMF stimulation (Fig. 1B). Untreated, control cells were kept at least 40 cm from the ActiPatch to ensure that they would not inadvertently be exposed to PEMF stimulation.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was assessed by a modified Great EscAPe SEAP Chemiluminescence Assay (BD Clontech, Mountain View, CA) as described previously.[20,22,24–29] Both cell lines underwent ALP activity assays. Each assay condition was performed in triplicate, and the results were repeated in at least 3 independent experiments. The activity of the well-established early osteogenic marker ALP[20,22,25–28] was measured on days 3, 5, 7, 9, and 12 of stimulation. Alkaline phosphatase activity was normalized by total cellular protein concentrations among the samples.

RNA Isolation and Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA was isolated using TRIZOL Reagents (Invitrogen, Carlsbad, CA). Total RNA was used to generate cDNA templates by reverse transcriptase (RT) reaction with hexamer and Superscript II RT (Invitrogen). The first-strand cDNA products were further diluted 5- to 20-fold and used as polymerase chain reaction (PCR) templates. Semiquantitative RT-PCR was carried out as described previously.[6,21,22,24,29–32] Polymerase chain reaction primers (Table 1) were designed using the Primer 3 program (Free Software Foundation, Inc, Boston, MA) to amplify the genes of interest (approximately 150–180 base pairs). A touchdown cycling program was as follows: 94°C for 2 minutes for 1 cycle, 92°C for 20 seconds, 68°C for 30 seconds, and 72°C for 12 cycles with a decrease in 1°C per cycle and then at 92°C for 20 seconds, 57°C for 30 seconds, and 72°C for seconds for 15 to 20 cycles, depending on the abundance of a given gene. The specificity of PCR products was confirmed by resolving PCR products on 1.0% agarose gels and visualized under a UV lamp and/or by ethidium bromide staining. All samples were normalized by the expression level of GAPDH. Reverse transcriptase–PCR data were analyzed by densitometry of gel bands

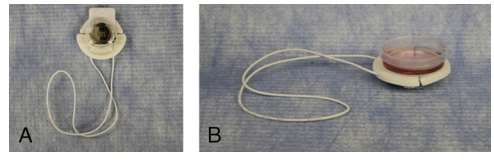

FIGURE 1. High-frequency PEMF delivery system. A, The ActiPatch is FDA approved for the treatment of soft tissue swelling, ecchymoses, and pain. This device was used as a novel delivery system of high-frequency PEMFs to evaluate the ability of high-frequency PEMF stimulation to induce osteogenic differentiation. The frequency of PEMF stimulation produced by this device is 27.1 MHz. B, Treated cells were exposed to continuous PEMF stimulation via placement on top of the PEMF-emitting region of the device for the duration of the experiment. Untreated, control cells were kept 40 cm or greater from the device, thereby precluding the possibility of PEMF stimulation.

TABLE 1. PCR Primers

Gene	Forward	Reverse
BMP-2	GCGTCAAGCCAAACACAA	CATGATAGCCCGGAGGTG
BMP-4	GCGAGCCATGCTAGTTTGA	AAGTGTCGCCTCGAAGTCC
BMP-6	GCAATCTGTGGGTGGTGAC	CTTGTCGTAAGGGCCGTCT
BMP-7	AGGAGCCAACAGACCAACC	TCACGTGCCAGAAGGAAAG
BMP-9	CGCAGCCTTAACCTCAGC	GTTGGAGGCAGGCGTAGA
ERK-1	CACGTTGGTACAGAGCTCCA	TGTGATTCAGCTGGTCAAGG
GAPDH	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
OCN	CCTTCATGTCCAAGCAGGA	GGCGGTCTTCAAGCCATAC
p38α	ACCCAGAAGACTGTGGATGG	CCCCATGAGATGGGTCAC

using ImageJ software (http://rsbweb.nih.gov/ij/download.html) and normalized to GAPDH signals obtained from the same time point. The normalized data were expressed as relative changes in mRNA levels between PEMF-treated and untreated cells.

Immunohistochemical Staining

Immunohistochemical (IHC) staining was performed for osteopontin (OPN) and osteocalcin (OCN) as previously described.[6] Cultured cells were either stimulated with high-frequency PEMFs or were left untreated. At day 10 of stimulation, cells were fixed with 10% formalin and washed with PBS. The fixed cells were permeabilized with 0.3% H_2O_2 and blocked with 10% goat serum, followed by incubation with an OPN or OCN antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. After washing, cells were incubated with biotin-labeled secondary antibodies for 30 minutes, followed by incubating cells with streptavidin–horseradish peroxidase conjugate for 20 minutes at room temperature. The presence of the expected protein was visualized by 3,3′-diaminobenzidine staining and examined under a microscope. Stains without primary antibody were used as negative controls.

Matrix Mineralization Assay (Alizarin Red S Staining)

Cells (C3H10T1/2 and iCAL) were seeded in 24-well culture plates and were either PEMF stimulated or untreated. At day 14 of stimulation, mineralized matrix nodules were stained for calcium precipitation by means of alizarin red S staining, as described previously.[6] Cells were fixed with 10% formalin at room temperature for 10 minutes. After being washed with PBS, fixed cells were incubated with 0.4% alizarin red S for 5 to 20 minutes, followed by extensive washing with PBS. The staining of calcium mineral deposits was recorded under bright-field microscopy.

Crystal Violet Viability Assay

Cellular proliferation was assessed by crystal violet staining. The crystal violet assay was conducted as described previously.[33,34] Cells were plated in 6-well culture plates at a subconfluent condition (~10^5 cells/well) and were treated with PEMF stimulation or control conditions. When cells from either group became 100% confluent, cells from both groups were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20 to 30 minutes. The stained cells were washed with tap water and air dried before macrographic images were taken.[35,36] For quantitative measurement, the stained cells were dissolved in 10% acetic acid (2 mL per well for 6-well plate) at room temperature for 20 minutes with shaking. A 500-μL portion was taken and added to 2 mL of double-distilled H_2O. Absorbance was measured at 570 to 590 nm.[37] Each assay condition was performed in triplicate and/or as 3 independent experiments.

Statistical Analysis

Microsoft Excel was used to calculate SDs and statistically significant differences between samples using the 2-tailed Student *t*-test. For all quantitative assays, each assay condition was performed in triplicate, and the results were repeated in at least 3 independent experiments. All data collected were subjected to statistical analysis. $P < 0.05$ was defined as statistically significant.

RESULTS

High-Frequency PEMF Stimulation Augments Early Osteogenic Marker ALP Activity

We first tested the effect of high-frequency PEMF stimulation on early osteogenic differentiation of osteoprogenitor cells. On days 3, 9, and 12 of stimulation, PEMF treatment induced a significant increase in ALP activity, most notably on day 12 (Fig. 2A), in C3H10T1/2 cells. Similarly, PEMF treatment induced a significant increase in ALP activity at days 3, 7, and 9 of stimulation in iCALs (Fig. 2B). These results indicate that high-frequency PEMF treatment enhances early osteogenic differentiation in C3H10T1/2 and iCAL osteoprogenitor cells in vitro.

High-Frequency PEMF Stimulation Induces Up-Regulation of Osteogenic Factor mRNA and Protein as Well as Increased Matrix Mineralization

We further determined the effect of high-frequency PEMF stimulation on the late stage of osteogenic differentiation. Both OCN and OPN are well-established markers of late-stage bone formation.[20,22,25–28] Stimulated C3H10T1/2 cells were compared with

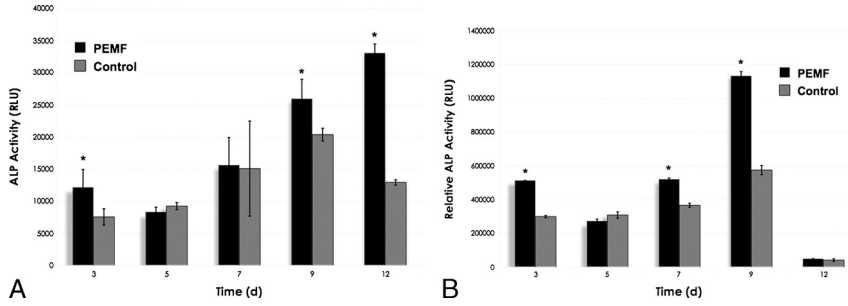

FIGURE 2. Augmentation of early osteogenic markers by PEMF stimulation. A, Alkaline phosphatase activity is a well-established marker of early osteogenic activity. Alkaline phosphatase activity was significantly elevated at days 3, 9, and 12 in PEMF-treated C3H10T1/2 cells. B, Alkaline phosphatase activity was significantly elevated at days 3, 7, and 9 in PEMF-treated iCAL cells. Data points represent the mean ± SD. Each assay was done in triplicate and/or as 3 independent experiments. *$P < 0.05$. RLU indicates relative light unit.

untreated, control cells. Semiquantitative RT-PCR of RNA extracted from treated cells demonstrated that the expression level of OCN mRNA was significantly elevated at days 7 and 10 of stimulation (Figs. 3A, B). In addition, OCN protein expression was detected in untreated cells, whereas a higher than basal level of staining was observed in PEMF-treated cells (Fig. 3C). Osteopontin expression was observed in untreated cells and to a greater extent in PEMF-treated cells (Fig. 3D). Terminal differentiation of stimulated C3H10T1/2 and iCAL cells was assessed using alizarin red S staining at day 14 of PEMF stimulation. Increased mineralization was readily detected in PEMF-treated cells (Fig. 3E). These findings suggest that high-frequency PEMF stimulation significantly promotes the late stage of osteogenic differentiation in vitro.

High-Frequency PEMF Stimulation Does Not Affect Cellular Proliferation

Previous studies investigating whether low-frequency PEMF stimulation affects cellular proliferation have reported inconsistent results.[38,39] In the current study, neither treated nor untreated C3H10T1/2 cells reached confluency at a significantly faster rate compared with the other (Fig. 4A). Similarly, no significant difference in absorbance was observed between groups (Fig. 4A). With respect to iCALs, neither treated nor untreated cells reached confluency at a significantly faster rate compared with the other (Fig. 4B). Again, no significant difference in absorbance was observed between groups (Fig. 4B). These results indicate that high-frequency PEMF stimulation does not significantly alter cellular proliferation or viability of C3H10T1/2 or iCAL cells.

High-Frequency PEMF-Induced Osteogenic Differentiation Is Not Solely Mediated by a Mitogen-Activated Protein Kinase–Dependent Pathway

Members of the mitogen-activated protein (MAP) kinase family of proteins have been implicated in signal transduction, stress-response pathways, and osteoblastic differentiation.[40–44] We analyzed whether endogenous expression of 2 members of the MAP kinase pathway, p38-reactivating kinase (p38 RK or simply, p38α) and extracellular signal–regulated kinase 1 (ERK-1), was altered in the presence of high-frequency PEMF stimulation. Using semiquantitative RT-PCR, we found that, in PEMF-treated C3H10T1/2 cells, endogenous p38α expression was elevated compared with untreated cells at days 10 and 14 of stimulation (Fig. 5A). However, using the same RT-PCR condition, endogenous expression of ERK-1 was not altered by PEMF stimulation (Fig. 5A).

Semiquantitative analysis via densitometry was performed. The relative expression level of p38α mRNA was modestly elevated in PEMF-treated cells at days 10 and 14 on stimulation (Fig. 5B). Compared with untreated cells, stimulated C3H10T1/2 cells expressed an increased p38α mRNA transcript level by 50% and 30% at days 10 and 14, respectively. There was no significant difference in the relative expression level of ERK-1 mRNA in

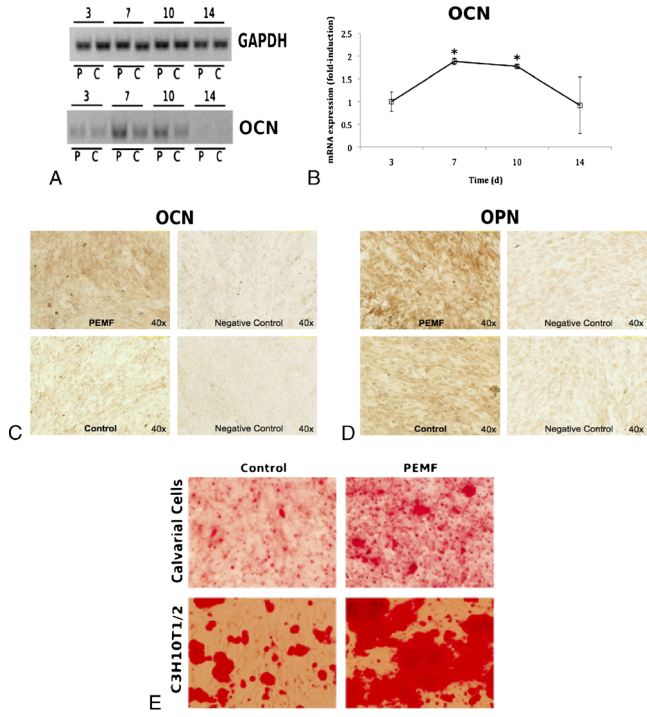

FIGURE 3. Augmentation of late osteogenic markers by PEMF stimulation. A, Endogenous expression of OCN mRNA in mesenchymal progenitor cells. Total RNA was isolated from treated and untreated C3H10T1/2 cells on days 3, 7, 10, and 14 of PEMF stimulation. Semiquantitative RT-PCR was performed using a primer pair specific for mouse OCN. The PCR products were resolved on 1% agarose gels and visualized under a UV lamp. B, Reverse transcriptase–PCR data were quantitatively analyzed by densitometry using ImageJ software and normalized to GAPDH signals obtained from the same time point. Normalized data for treated cells were then converted to fold induction by expressing the densitometric data as a ratio of treated cells to untreated cells. C, Immunohistochemical staining of OCN. C3H10T1/2 cells were stimulated by PEMFs as indicated. Expression of OCN was assessed by IHC staining analysis after 10 days of stimulation using an anti-OCN antibody (Santa Cruz Biotechnology). D, Immunohistochemical staining of OPN. C3H10T1/2 cells were stimulated by PEMFs as indicated. Expression of OPN was assessed by IHC staining analysis after 10 days of stimulation using an anti-OPN antibody (Santa Cruz Biotechnology). E, Alizarin red S staining. C3H10T1/2 cells and iCALs were stimulated using high-frequency PEMFs as indicated. Alizarin red S staining was conducted after 14 days of stimulation. Numbers in A correspond to days of stimulation. P indicates PEMF stimulated; C, control (unstimulated); Data points represent the mean ± SD. $*P < 0.05$.

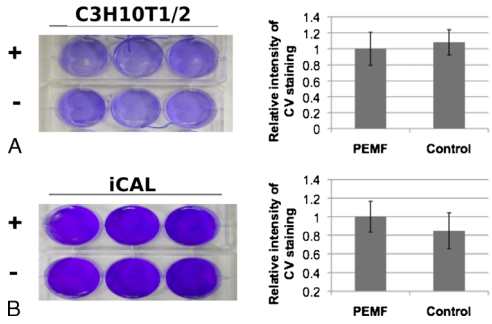

FIGURE 4. Cellular proliferation is unaffected by PEMF stimulation. A, Crystal violet viability assay. Subconfluent C3H10T1/2 cells were plated into 6-well dishes and either exposed to PEMF stimulation or not. When either PEMF-treated or untreated cells first became 100% confluent, cells from both groups were stained with crystal violet. Staining intensity was similar between both groups indicating an approximately equal number of viable cells. Crystal violet staining intensity was quantitatively assessed by dissolving the stained cells in 10% acetic acid and measuring absorbance at 570 to 590 nm. There was no significant difference in crystal violet staining intensity between the 2 groups ($P = 0.48$). B, Subconfluent iCAL cells were plated into 6-well dishes and either exposed to PEMF stimulation or not. When either PEMF-treated or untreated cells first became 100% confluent, cells from both groups were stained with crystal violet. Staining intensity was similar between both groups, indicating an approximately equal number of viable cells. Crystal violet staining intensity was quantitatively assessed by dissolving the stained cells in 10% acetic acid and measuring absorbance at 570 to 590 nm. There was no significant difference in crystal violet staining intensity between the 2 groups ($P = 0.32$). + indicates PEMF stimulated; −, unstimulated.

stimulated versus unstimulated cells (Fig. 5C). Although these results suggest that high-frequency PEMF stimulation is associated with increased p38α mRNA expression, osteogenic differentiation began well before these changes were observed as indicated by the ALP assay and IHC staining results. It is therefore unlikely that the osteoinductive effect of high-frequency PEMF stimulation is exclusively mediated by an MAP kinase–dependent signaling cascade.

High-Frequency PEMF-Induced Osteogenic Differentiation Is Associated With BMP mRNA Up-Regulation

Members of the BMP family of proteins are known inducers of osteogenic differentiation.[20,22,24–28,45] We analyzed whether endogenous expression of osteogenic members of the BMP family[46] was altered in the presence of high-frequency PEMF stimulation. The expression of BMP-2 was slightly elevated at days 7 and 10 in PEMF-stimulated versus untreated C3H10T1/2 cells (Fig. 6A). Under the same conditions, BMP-4 expression was convincingly elevated in PEMF-treated cells at days 10 and 14 of stimulation (Fig. 6A). Bone morphogenetic protein 6 expression appeared elevated at day 3 in PEMF-treated cells and at day 14 in untreated cells (Fig. 6A). The expression level of BMP-7 was elevated at days 3 and 10 of stimulation in treated cells and appeared increased at day 7 in untreated cells (Fig. 6A). Finally, we observed that, in PEMF-treated cells, the expression of BMP-9 was increased at days 3 and 10 of stimulation (Fig. 6A).

These results are quantitatively represented via densitometric analysis of relative gene expression (Figs. 6B–F). At days 7 and 10 of stimulation, there was an increase in BMP-2 mRNA expression by 25% and 37%, respectively, in treated cells (Fig. 6B). Bone morphogenetic protein 4 mRNA expression was elevated by an average of 87% and 122% in treated cells at days 10 and 14, respectively (Fig. 6C). In treated cells, BMP-6 mRNA expression was increased by 50% and decreased by 48% compared with untreated cells at days 3 and 14, respectively (Fig. 6D). With respect to BMP-7 relative mRNA expression, treated cells expressed an average of 75% more mRNA at day 3, 20% less mRNA at day 7, and 38% more mRNA at day 10 of PEMF stimulation (Fig. 6E). Finally, there was an increase in BMP-9 mRNA expression by 78% and greater than 1000% at days 3 and 10, respectively, in PEMF-treated cells (Fig. 6F). These results indicate that high-frequency PEMF stimulation is associated with significant increases of mRNA expression of numerous BMPs.

DISCUSSION

Healing of craniofacial defects, whether due to tumor, trauma, or congenital disease, presents significant reconstructive challenges to clinicians. The criterion standard material to repair such defects is autologous nonvascularized or vascularized bone derived from the cranium, rib, iliac crest, tibia, and/or fibula.[47] However, the supply of available autologous tissue is often limited, especially in the setting of a large defect. In addition, patients may experience considerable donor site morbidity. Although various therapeutic options have attempted to solve this clinical dilemma,[1–5] they are laden with

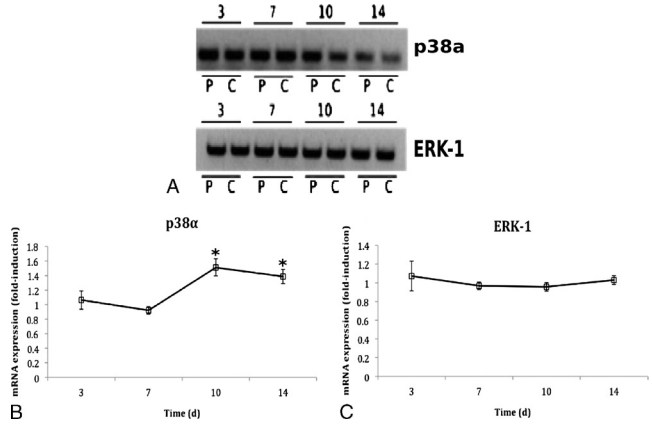

FIGURE 5. High-frequency PEMF stimulation alters MAP kinase signaling, but these changes do not appear to exclusively regulate osteogenic differentiation. A, Endogenous expression of p38α and ERK-1 in mesenchymal progenitor cells. Total RNA was isolated from stimulated and unstimulated C3H10T1/2 cells on days 3, 7, 10, and 14 of PEMF stimulation. Semiquantitative RT-PCR was performed using primer pairs specific for mouse p38α and mouse ERK-1. The PCR products were resolved on 1% agarose gels and visualized under a UV lamp. B and C, Reverse transcriptase–PCR data for p38α and ERK-1 were quantitatively analyzed by densitometry using ImageJ software and normalized to GAPDH signals obtained from the same time point (Fig. 3A). Normalized data for stimulated cells were then converted to fold induction by expressing the densitometric data as a ratio of stimulated cells to unstimulated controls. Numbers in A correspond to days of stimulation. P indicates PEMF stimulated; C, control (unstimulated). Data points represent the mean ± SD. $*P < 0.05$.

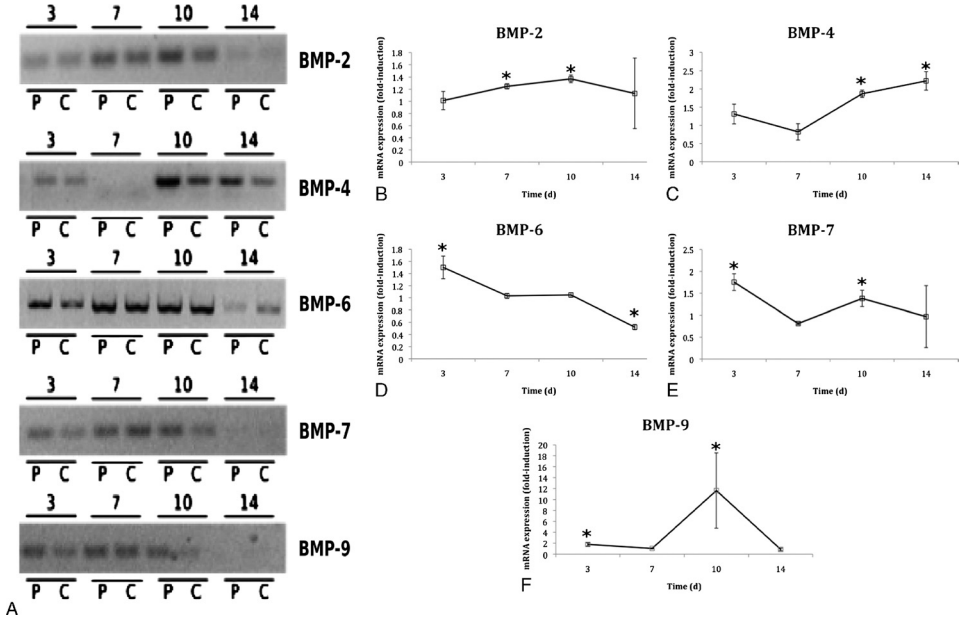

FIGURE 6. High-frequency PEMF stimulation may enhance osteogenic differentiation by inducing increased BMP expression. A, Endogenous expression of BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 in mesenchymal progenitor cells. Total RNA was isolated from stimulated and unstimulated C3H10T1/2 cells on days 3, 7, 10, and 14 of PEMF stimulation. Semiquantitative RT-PCR was performed using primer pairs specific for mouse BMP-2, mouse BMP-4, mouse BMP-6, mouse BMP-7, and mouse BMP-9. The PCR products were resolved on 1% agarose gels and visualized under a UV lamp. B–F, Reverse transcriptase–PCR data for BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 were quantitatively analyzed by densitometry using ImageJ software and normalized to GAPDH signals obtained from the same time point (Fig. 3A). Normalized data for stimulated cells were then converted to fold induction by expressing the densitometric data as a ratio of stimulated cells to unstimulated controls. Numbers in A correspond to days of stimulation. P indicates PEMF stimulated; C, control (unstimulated). Data points represent the mean ± SD. $*P < 0.05$.

disadvantages.[48–50] Thus, continued investigation into alternative forms of bone regeneration and repair is necessary.

Tissue engineering strategies, which combine engineering technology with the principles of biology to regenerate lost or damaged tissue, have been the subject of much investigation in recent years.[51] Strategies include cell- and/or growth factor–based approaches. One modality combining these 2 approaches without altering the cellular genome is biophysical stimulation. Biophysical stimulation consisting of PEMFs to stimulate bone growth, which was approved by the FDA in 1979, is still widely used for this purpose.

The effects of PEMFs on cells of the osteogenic lineage are complex and pleiotropic and have yet to be fully described. It is known that PEMF stimulation is associated with increased expression of osteogenic cytokines[15] and receptors for osteogenic transcription factors,[18] as well as enhanced proliferation in osteoblasts.[17,39] However, there is great variability within the literature with respect to the effects PEMF stimulation, often due to varying methodologies used. In general, however, authors have studied only very low- or low-frequency PEMF stimulation (ie, 5–30 Hz). The current study is unique in its use of a PEMF delivery device that emits PEMFs at a frequency of 27.1 MHz.

Interestingly, previous studies have often reported that, for osteogenic differentiation to occur in the presence of low-frequency PEMFs, an additional costimulant is required. For example, Schwartz et al[16] found that PEMF stimulation (15 Hz) enhances the osteogenic effects of BMP-2 on MSCs when cultured on calcium phosphate substrates. Also, culture medium is often enhanced with pro-osteogenic factors such as ascorbic acid, β-gylcerophosphate, and/or dexamethasone.[17] In contrast, the current study demonstrates an augmented osteogenic response to high-frequency PEMF stimulation without the aid of additional costimulants or osteogenic media. Specifically, high-frequency PEMF stimulation enhanced ALP activity as well as the expression of OCN and OPN both at the transcriptional level as well as protein level. Matrix mineralization was also enhanced in the presence of high-frequency PEMFs. In summary, markers of early, late, and terminal osteogenic differentiation were up-regulated by high-frequency PEMF stimulation in vitro. Of note, cellular proliferation was not significantly altered by this form of biophysical stimulation. This is in contrast to low-frequency PEMF exposure, which has been shown to induce overexpression of proliferation markers such as c-myc and c-fos.[18]

This study is also unique in its use of osteoprogenitor cells rather than terminally differentiated cells of the osteogenic lineage. Whereas most authors have studied PEMF stimulation of osteoblasts, we elected to study osteoprogenitor cells as they have become important targets for bone tissue engineering because of their large quantity within humans and their ease of isolation.[52] C3H10T1/2 cells, which are considered to retain the ability to differentiate into bone, cartilage, and fat,[20] displayed enhanced early, middle, and late osteogenic differentiation in the setting of high-frequency PEMF stimulation. Similarly, immortalized juvenile calvarial cells (iCALs) yielded a similar osteogenic response when stimulated. Unpublished data from our laboratory support that iCALs are in fact progenitor cells as they bear surface antigens similar to MSCs and have the ability to differentiate into multiple tissues of mesenchymal origin.

Although augmented osteogenic differentiation in the setting of high-frequency PEMF stimulation has been demonstrated in the current study, the mechanisms underlying this response remain unclear. The International Commission of Non-ionizing Radiation Protection and others have described potential mechanisms of interaction between cells and surrounding electromagnetic fields.[53,54] These discussions, however, have been largely theoretical. Perhaps more useful from a clinical perspective is the characterization of osteogenic cytokine modulations that occur in the presence of PEMF stimulation. The MAP kinase signaling family, to which both p38α and ERK-1 belong, has been implicated in osteoblastic differentiation and function,[40–42,55] as well as the cellular stress-response pathway.[43,44] We therefore evaluated whether

high-frequency PEMF stimulation alters the expression level of these proteins. We found that p38α transcript levels were modestly elevated in stimulated cells, but that this occurred well after osteogenic differentiation had commenced. Also, we found that ERK-1 transcript levels were unchanged compared with unstimulated cells. Interestingly, the activation (phosphorylation) of p38α has been shown to be a downstream event of BMP-mediated osteogenic induction. Noth and colleagues[56] reported that BMP-2 in particular must activate p38α to mediate osteogenic differentiation. Data in the current study show that BMP-2 gene up-regulation is late and slightly elevated in stimulated cells, which may explain the delay and modest increase in p38α gene up-regulation. However, it is also plausible that space limitations within the culture plates (due to morphologic changes of differentiating osteoprogenitor cells) stimulated a stress response in PEMF-treated cells, thus accounting for the rise in the level of p38α mRNA at days 10 and 14 in these cells. Consequently, PEMF induction of osteoprogenitor cell differentiation may proceed through MAP kinase–dependent and –independent pathways. Further studies are required to more fully determine the role of MAP kinase–related factors in the cellular response to PEMF stimulation.

Bone morphogenetic proteins, which are members of the transforming growth factor β superfamily, also play an important role in osteoblast differentiation and subsequent bone formation.[26] Approximately 20 BMP isoforms, with varying osteoinductive potentials, have been described. Studies from our laboratory have previously demonstrated that BMP-2, BMP-6, BMP-9, and, to a lesser extent, BMP-4 and BMP-7 are the most osteogenic.[20,25,26,46] Therefore, we evaluated whether high-frequency PEMF stimulation corresponded to modulation of these osteogenic BMPs during differentiation. We found that mRNA transcript levels of BMP-6, BMP-7, and BMP-9 were elevated in stimulated cells during the early phase of differentiation at day 3. Interestingly, mRNA transcript levels of these proteins were similar in stimulated and unstimulated cells at day 7, but again were elevated in PEMF-treated cells at day 10 (BMP-7 and BMP-9). These findings are not surprising given the role of BMPs in osteogenic differentiation. This is especially true for BMP-6, which is known to be expressed to an elevated degree early in the course of osteogenic differentiation.[46] In contrast, BMP-2 and BMP-4 mRNA expression levels were first elevated in stimulated cells later during differentiation, at days 7 and 10 (BMP-2) and days 10 and 14 (BMP-4) of stimulation. It is therefore plausible that high-frequency PEMF stimulation facilitates enhanced osteogenic differentiation of osteoprogenitor cells by inducing up-regulation of certain BMPs (eg, BMP-6, BMP-7, and BMP-9) but not others. However, further studies are necessary to evaluate if a cause-and-effect relationship between PEMF stimulation, BMP up-regulation, and enhanced osteogenesis exists.

The current study is the first, to our knowledge, to demonstrate that high-frequency PEMFs delivered by the novel device used here are capable of inducing osteogenic differentiation of murine osteoprogenitor cells. Although we found high-frequency PEMF stimulation to be associated with enhanced BMP expression, it remains unclear whether the rise in BMP mRNA levels in stimulated cells is indeed due to PEMF stimulation or due to other factors. Bone morphogenetic protein induction of cells requires phosphorylation of Smad proteins. Thus, to more definitively examine the relationship between high-frequency PEMF stimulation and BMP modulation, it would be useful to assess whether high-frequency PEMF stimulation enhances BMP receptor–Smad reporter activity and the nuclear translocation of Smad1/5/8. Similarly, whether high-frequency PEMF stimulation induces differentiation via an MAP kinase–dependent and/or –independent pathway remains in question. It is probably the case that the mechanism of PEMF induction of osteoprogenitor cell differentiation is multifactorial. Moreover, further experiments to address the potential role of this form of biophysical stimulation in in vivo models of bone formation are necessary. Finally, these findings suggest that high-frequency PEMF stimulation of osteoprogenitor cells may be further investigated as an effective bone regeneration option to treat critical-size osseous defects of the craniofacial and axial skeleton.

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