

Research Article



Static magnetic fields promote osteoblastic/cementoblastic differentiation in osteoblasts, cementoblasts, and periodontal ligament cells

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ABSTRACT

Purpose: Although static magnetic fields (SMFs) have been used in dental prostheses and osseointegrated implants, their biological effects on osteoblastic and cementoblastic differentiation in cells involved in periodontal regeneration remain unknown. This study was undertaken to investigate the effects of SMFs (15 mT) on the osteoblastic and cementoblastic differentiation of human osteoblasts, periodontal ligament cells (PDLs), and cementoblasts, and to explore the possible mechanisms underlying these effects.
Methods: Differentiation was evaluated by measuring alkaline phosphatase (ALP) activity, mineralized nodule formation based on Alizarin red staining, calcium content, and the expression of marker mRNAs assessed by reverse transcription polymerase chain reaction (RT-PCR). Signaling pathways were analyzed by western blotting and immunocytochemistry.
Results: The activities of the early marker ALP and the late markers matrix mineralization and calcium content, as well as osteoblast- and cementoblast-specific gene expression in osteoblasts, PDLs, and cementoblasts were enhanced. SMFs upregulated the expression of Wnt proteins, and increased the phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) and total β -catenin protein expression. Furthermore, p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK), and nuclear factor- κ B (NF- κ B) pathways were activated.
Conclusions: SMF treatment enhanced osteoblastic and/or cementoblastic differentiation in osteoblasts, cementoblasts, and PDLs. These findings provide a molecular basis for the beneficial osteogenic and/or cementogenic effect of SMFs, which could have potential in stimulating bone or cementum formation during bone regeneration and in patients with periodontal disease.

Keywords: Bone regeneration; Periodontal guided tissue regeneration; Relative biological effectiveness; Signal transduction; Wnt proteins

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Author Contributions

Conceptualization: Su-Jin Ahn; Formal analysis: Suk-Won Lee; Investigation: Jaesuh Park; Methodology: Eun-Cheol Kim, Su-Jin Ahn; Project administration: Eun-Cheol Kim, Su-Jin Ahn; Writing - original draft: Jaesuh Park, Suk-Won Lee, Su-Jung Park; Writing - review & editing: Eun-Cheol Kim, Il Keun Kwon, Su-Jin Ahn.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

INTRODUCTION

The ultimate goal of periodontal treatment is to prevent further loss of attachment and to regenerate periodontal supporting tissues including cementum, periodontal ligament (PDL), and bone. To achieve periodontal regeneration, several therapeutic approaches have been examined in animal models and clinical studies, including bone grafting, guided tissue regeneration, and the implantation of biologic mediators [1]. However, inconsistent beneficial effects of these treatments have been observed. The application of magnetic fields has been used as an alternative and/or complementary treatment for fracture healing, spinal fusion, osteoarthritis, and wound healing [2]. Magnetotherapy including static magnetic fields (SMFs), pulsed electromagnetic fields (PEMFs), and alternating electromagnetic fields provides a non-invasive, safe, and easy method to directly treat the site of injury, the source of pain and inflammation, and various types of disease. Although PEMFs have been used extensively in clinical treatments of non-united bone fractures for a long time [3], there is some skepticism concerning the proposed exposure protocols [4]. Furthermore, PEMFs are generated by electrical devices.

SMFs are a type of magnetic field used in clinical practice, especially in the dental field [5], because SMFs do not need power devices, as PEMFs do. In addition, SMFs are convenient and suitable for long-term application [6], and do not provide any heat or electric hazard to surrounding tissue [7]. Rare-earth magnets, which generate SMFs, have been used in magnetic retention appliances in implant- or tooth-retained overdentures [8], maxillofacial prostheses after trauma and cancer surgery [9], as well as in orthodontic treatments such as space closure, molar distalization, intrusion, the traction of impacted teeth, and palatal expansion [5]. SMFs increased implant stability and reduced bone loss during the initial weeks of healing [10]. Recently, we demonstrated that exposing sandblasted, large-grit, acid-etched-treated titanium implants to 15-mT SMFs triggered faster early peri-implant bone formation in rabbits [11].

However, the effect of SMFs on growth and osteoblastic differentiation remains controversial. SMF exposure had no significant effect on the proliferation of rat calvaria cells or osteoblast-like cells [12]. The proliferation of human osteoblast cultures was reduced when cells were exposed to continuous low-intensity SMFs [13]. Moreover, SMFs generated by corrosion currents inhibited human osteoblast differentiation [14]. In contrast, SMFs were found to increase the osteoblastic differentiation of rat osteoblasts [12], human osteoblast-like MG63 cells [18], and dental pulp cells [16]. In addition, we found that 15-mT SMFs, an intensity that is relatively low within the intensity range of moderate SMFs (1 mT to 1 T), enhanced the proliferation and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) *in vitro* [17].

Periodontal regeneration involves the recruitment of locally-derived progenitor cells that can subsequently differentiate into periodontal ligament cells (PDLCS), mineral-forming cementoblasts, or bone-forming osteoblasts [18]. Although SMFs can enhance bone fracture healing and bone formation by osteoblasts both *in vivo* and *in vitro* [19,20], their effects on the differentiation of PDLCS, cementoblasts, and osteoblasts are not fully understood. These cells constitute the periodontium and can be directly influenced when SMFs are applied to teeth, implants, and other intraoral structures. Thus, the aim of this study was to investigate the effects of SMFs (15 mT) on the osteoblastic and cementoblastic differentiation of human PDLCS, cementoblasts, and osteoblasts, and to explore the possible mechanisms underlying these effects.

MATERIALS AND METHODS

Cell culture

Human fetal osteoblasts (hFOB 1.19) were obtained from the American Type Culture Collection (CRL-11372, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium-Ham's F-12 (1:1) basal medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine (Gibco, 15140-122, Invitrogen Corporation, Grand Island, NY, USA) in a standard cell-culture incubator at 37°C and 5% CO₂. Differentiation was induced by transfer to osteogenic medium (OM) containing 100 μM ascorbic acid, 2 mM β-glycerophosphate, and 10⁻⁷ M dexamethasone, as previously described [21].

Human PDLs and cementoblasts [22], immortalized by transfection with the telomerase catalytic subunit of the human telomerase reverse transcriptase, were kindly provided by Professor Takashi Takata (Hiroshima University, Hiroshima, Japan). Cells were cultured in α-minimum essential medium (α-MEM; Invitrogen Corporation) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Primary osteoblasts were enzymatically isolated from the calvaria of 1-day-old ICR mice (Samtako, Osan, Korea). Briefly, the calvaria were aseptically dissected, and the fragments were incubated in phosphate-buffered saline (PBS) solution with 0.2% collagenase-dispase solution (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at 37°C. The cells were dissociated from the bone fragments, counted, and plated at 4×10⁴ cells/cm². The cells were grown in α-MEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

All animal experimental procedures in the current study were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (approval number: KHMC-IACUC 2015-002). Primary human PDLs were cultured using an explant technique, as described previously [23]. Briefly, impacted third molars were obtained from 3 healthy patients of the Department of Oral and Maxillofacial Surgery, Kyung Hee University Dental Hospital, Seoul, Korea. The experiments were performed with approval from the Ethical Committee of Kyung Hee University Dental Hospital, and informed written consent was obtained from all patients.

Freshly extracted teeth were immediately placed in PBS supplemented with antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Fungizone). In order to culture PDLs, the PDL was carefully removed from the middle third of the root by a scalpel. Tissues were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 minutes, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

The SMF exposure system

A neodymium (Nd₂Fe₁₄B) disc magnet (1 mm thick and 15.0 mm in diameter, Qingdao Qiangsheng Magnets Co., Ltd., Shandong, China) and 4-well plastic culture plates were used to create an SMF exposure system (Figure 1A). A magnet was placed below the wells horizontally, and the north (N) side of the magnet was oriented towards the well. The desired field intensities (3, 15, and 50 mT) were established by controlling the distance between the

magnet and the culture plates, as previously described [17]. The distance was fixed by the space between the magnet and bottom wall of the well. We measured the SMF intensity with a Gauss meter (TM-701, Kanetec, Tokyo, Japan) that could detect either axial or transverse fields. To eliminate the interference of adjacent SMFs, we attached only 1 magnet disc on 1 culture plate and provided adequate space between adjacent culture plates (at least 30 mm).

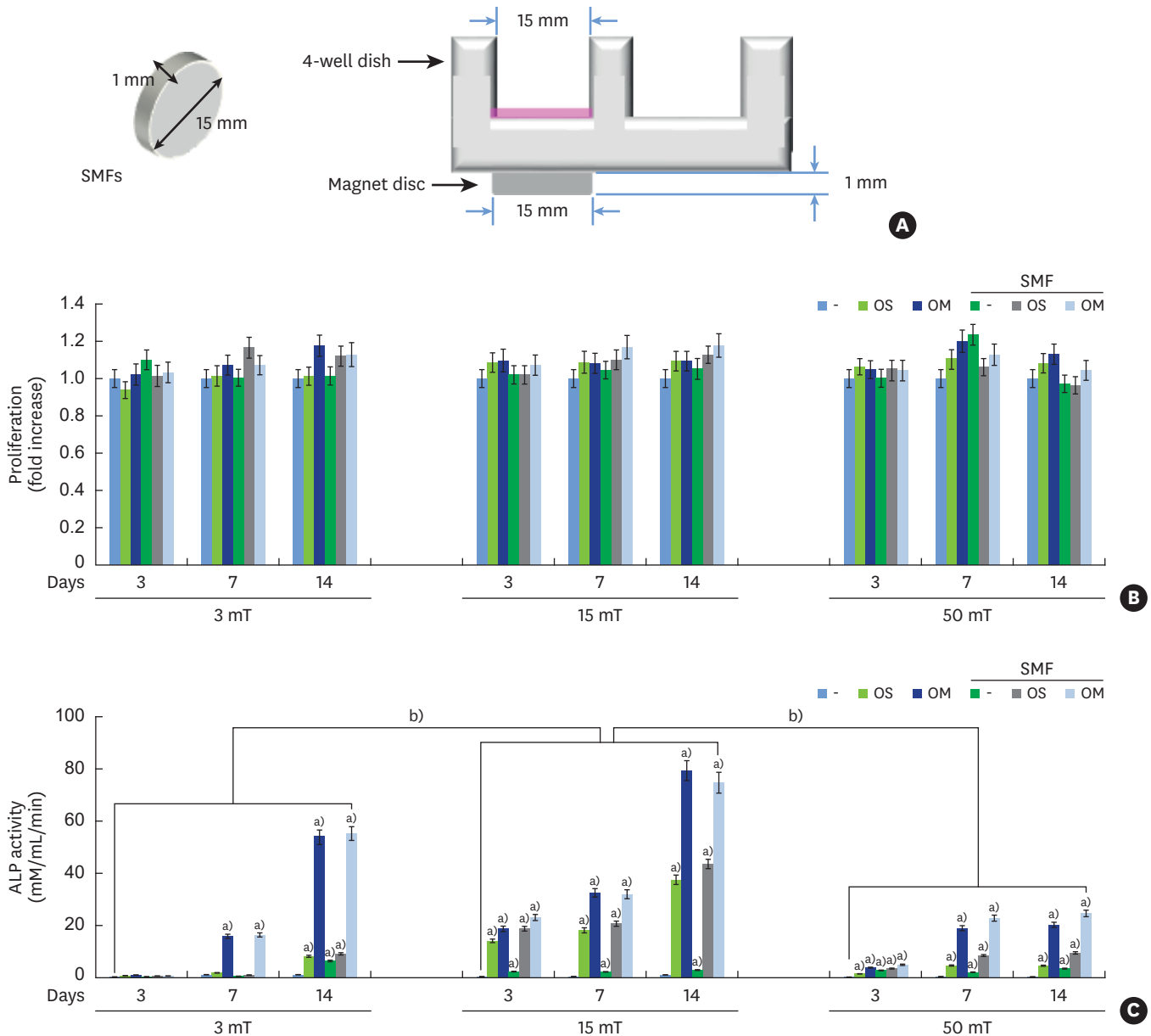


Figure 1. Effects of SMFs on osteoblastic differentiation in human osteoblasts. (A) Schematic diagram of the SMF exposure system. (B) Cell proliferation was examined by an MTT assay at 3, 7, and 14 days. (C) Differentiation was assessed based on ALP activity, (D) Alizarin red staining, (E) calcium content, and (F) expression of the mRNA of bone matrix proteins. The intensity of Alizarin red staining was determined by optical density. Cells were treated with OS medium containing 50 $\mu\text{g}/\text{mL}$ of L-ascorbic acid and 10 mM β -glycerophosphate along with 3-, 15-, and 50-mT SMFs for 7 days and 14 days. OM contained OS and 10^{-7} M dexamethasone. The results are representative of 5 independent experiments that were performed.

SMF: static magnetic field, MTT: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, ALP: alkaline phosphatase, OS: osteogenic supplement, OM: osteogenic medium, Runx2: runt-related transcription factor 2, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

^{a)}Significant difference compared to control ($P < 0.05$); ^{b)}Significant difference between each group ($P < 0.05$).

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SMFs promote differentiation in periodontium cells

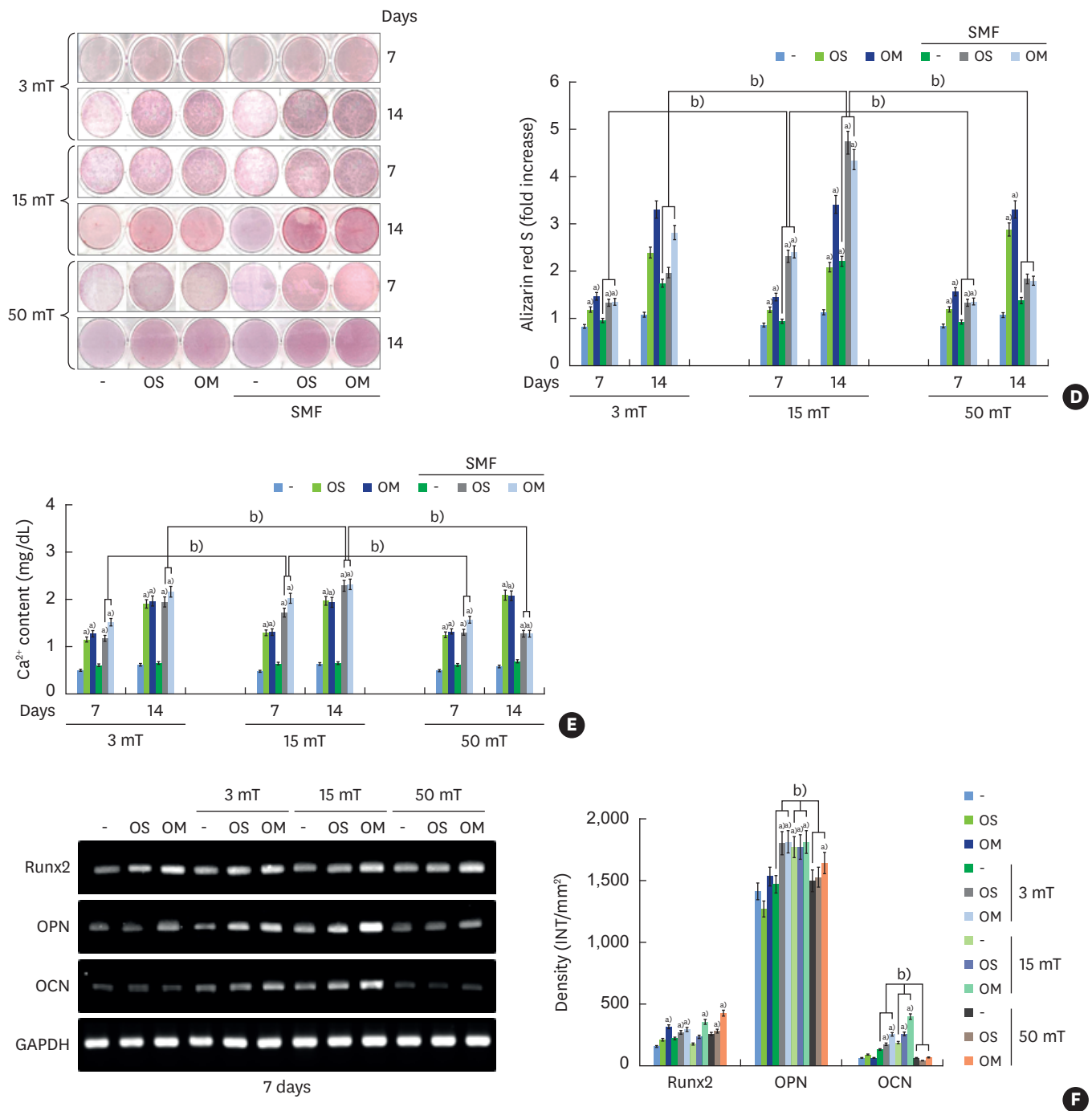


Figure 1. (Continued) Effects of SMFs on osteoblastic differentiation in human osteoblasts. (A) Schematic diagram of the SMF exposure system. (B) Cell proliferation was examined by an MTT assay at 3, 7, and 14 days. (C) Differentiation was assessed based on ALP activity, (D) Alizarin red staining, (E) calcium content, and (F) expression of the mRNA of bone matrix proteins. The intensity of Alizarin red staining was determined by optical density. Cells were treated with OS medium containing 50 µg/mL of L-ascorbic acid and 10 mM β-glycerophosphate along with 3-, 15-, and 50-mT SMFs for 7 days and 14 days. OM contained OS and 10⁻⁷ M dexamethasone. The results are representative of 5 independent experiments that were performed.

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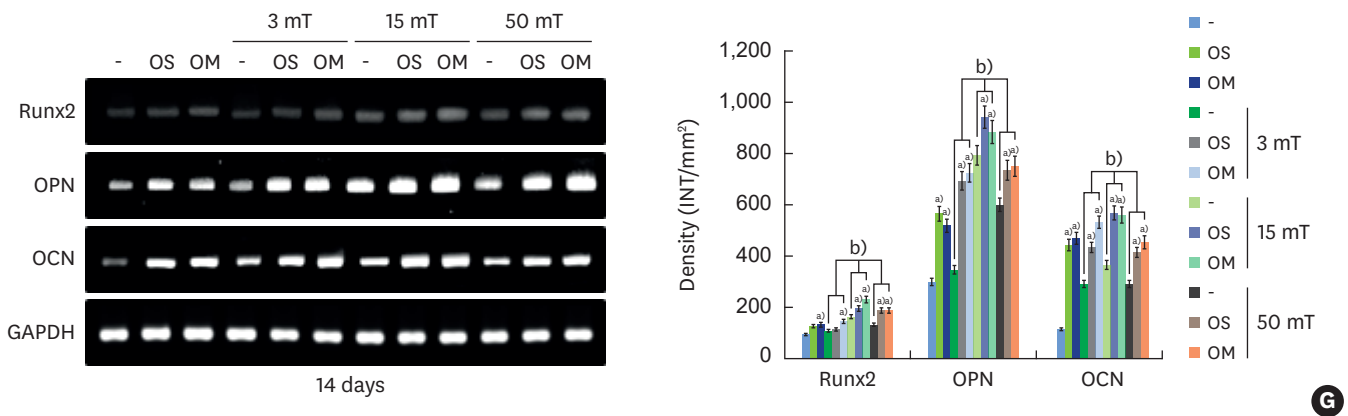


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^{a)}Significant difference compared to control ($P<0.05$); ^{b)}Significant difference between each group ($P<0.05$).

Cell viability

Cell viability was confirmed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay kit (CellTiter 96 Aqueous One Solution, Promega, Madison, WI, USA) following the protocol suggested by the manufacturer.

Alkaline phosphatase (ALP) activity

Intracellular and soluble ALP, a marker of osteoblast activity, was assayed by the release of p-nitrophenol from p-nitrophenylphosphate. Cells were washed with PBS and sonicated with a cell disruptor. ALP activity was measured using p-nitrophenyl phosphate (final concentration, 3 mM) in 0.7 M 2-aminomethyl-1-propanol (pH 10.3) and 6.7 mM MgCl₂. Absorbance was measured at 415 nm using an enzyme-linked immunosorbent assay reader (Beckman Coulter, Fullerton, CA, USA).

Alizarin red staining

Alizarin red staining was used to detect mineralization. Cells were fixed in 70% ice-cold ethanol for 1 hour and rinsed with distilled water. Fixed cells were stained with 40 mM Alizarin red (pH 4.2) for 10 minutes with gentle agitation. All Alizarin red staining images were photographed.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA). Then, 1 mg of RNA was reverse transcribed using oligo (dT) 15 primers (Roche Diagnostics, Mannheim, Germany) and AccuPower RT PreMix (Bioneer Corporation, Daejeon, Korea). Thereafter, the reverse transcriptase-generated DNA (2–5 mL) was amplified. Primer sequences are detailed in Table 1. The polymerase chain reaction (PCR) products were subjected to electrophoresis on 1.5% agarose gels, and stained with ethidium bromide.

Table 1. RT-PCR primers and conditions

Genes	Primer sequence (5'-3')	Annealing temperature (°C)	Cycle number	Product size (bp)
OPN	F: 5'-CCAAGTAAGTCCAACGAAAG-3' R: 5'-GGTGATGTCCTCGTCTGTA-3'	55	28	347
OCN	F: 5'-GTGCAGCCTTTGTGTCCAAGCAGGA-3' R: 5'-CCGTAGAAGCGCCGATAGGCC-3'	62	30	196
Runx2	F: 5'-AACCACFAATFCACTATCCA-3' R: 5'-CGGACATACCGAGGGACCTG-3'	63	30	76
CEMP-1	F: 5'-CAGGATCCACATCCGTC-3' R: 5'-CTGAACAGCTTCGAGGC-3'	55	30	434
CAP	F: 5'-GGGTGGTTGGTTCTAGCTATTG-3' R: 5'-CGGCAAGGCAGCGTATATT-3'	58	32	345
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' R: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	62	30	306

RT-PCR: reverse transcription polymerase chain reaction, OPN: osteopontin, OCN: osteocalcin, Runx2: runt-related transcription factor 2, CEMP-1: cementum protein 1, CAP: cementum-derived attachment protein, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Western blot analysis

Aliquots of 1×10^6 cells were solubilized in ice-cold 1% Triton X-100 lysis buffer (Boston BioProducts, Inc., Ashland, MA, USA). After 30 minutes on ice, the lysates were clarified by centrifugation. Proteins (20 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide), transferred to nitrocellulose membranes, and probed with specific antibodies (diluted to 1:1,000), followed by incubation with secondary horseradish peroxidase-conjugated antibody (1:5,000). Products were detected using an enhanced chemiluminescence system (Amersham-Pharmacia, Piscataway, NJ, USA).

Ca²⁺ assays

To measure calcium content, cells were harvested by centrifugation at 13,500 rpm for 1 minutes, resuspended in lysis buffer (0.1% Triton X-100, Boston BioProducts, Inc.) and sonicated on ice for 1 minute to destroy the cell membranes. The resulting supernatant was used for calcium measurements made with a QuantiChrom™ Calcium Assay Kit (DICA-500, BioAssay Systems, Hayward, CA, USA). Calcium content was estimated by measuring absorbance at 612 nm in a microplate reader.

Immunocytochemistry

Cells were fixed with freshly prepared 3% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 (Boston BioProducts, Inc.) for 15 minutes. After 1 hours of incubation with 10% normal goat serum/PBS, cells were incubated with anti-p65 antibody (Santa Cruz Laboratories, Santa Cruz, CA, USA) diluted 1:50 in PBS for 2 hours, washed and incubated with fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G, and diluted at 1:100 in PBS for 1 hour. In order to identify the nuclei, the FITC-labeled samples were counterstained with 10 μ g/mL of propidium iodide for 2 minutes. To acquire the immunofluorescence images, a confocal microscope (Cell Voyager, Yokogawa, Japan) was used.

Statistical analysis

Descriptive statistics (mean \pm standard deviation) for each parameter were evaluated in all the groups. The data distributions satisfied normality as tested by the Shapiro-Wilk test, meaning that intergroup comparisons could be made by using 1-way analysis of variance and the Duncan multiple range test. *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

SMFs promote osteoblastic differentiation in human osteoblasts

To determine the cytotoxic potential of SMFs, its effect on viability of the human osteoblasts was evaluated. Up to a magnitude of 50 mT, no cytotoxic effects could be observed using the MTT assay (Figure 1B). To determine the effects of SMFs on osteoblastic differentiation, we evaluated its effects on ALP activity (an early marker of osteoblastic differentiation) and mineralization. ALP activity was significantly higher ($P < 0.05$) and calcified nodules more frequent after SMF treatment than when the cells were simply maintained in normal osteogenic supplement (OS) or OM (Figure 1C). Osteoblasts exposed to an SMF of 15 mT had significantly higher ALP levels than the other groups ($P < 0.05$). Because the endpoint of osteoblast formation is the production of a calcified, mineralized matrix, calcium content was measured as an indicator of osteodifferentiation. The SMF-exposed osteoblasts had significantly larger mineralized nodules (Figure 1D) and extracellular calcium deposits than their control counterparts (Figure 1E) ($P < 0.05$). The largest number of mineralized nodules and the greatest calcium content was observed in the 15 mT group. In order to explore the effects of SMFs on the osteoblastic differentiation of osteoblasts, we examined the expression of specific markers of osteoblastic differentiation. Treating the osteoblasts with SMFs increased the expression of mRNA encoding the OS- and OM-induced differentiation markers runt-related transcription factor 2 (Runx2), osteopontin (OPN), and osteocalcin (OCN) (Figure 1F and G). Maximal upregulation of the osteoblast marker genes was observed at 15 mT.

SMFs promote osteoblastic/cementoblastic differentiation in PDLCs and cementoblasts

Since SMFs of 15 mT maximally stimulated the osteoblastic differentiation of human osteoblasts, 15 mT was used in further experiments. Cytotoxicity of PDLCs and cementoblasts in response to SMFs was not observed on days 3, 7, or 14 (Figure 2A and B). To investigate whether SMFs stimulated PDLCs and cementoblasts to differentiate toward a cementoblast/osteoblast phenotype, we measured ALP activity and the expression of osteoblast- and cementum-related genes, calcium content, and calcified nodule formation. Treatment with SMFs enhanced ALP activity and mineralized nodule formation in PDLCs and cementoblasts, compared with OS and OM controls (Figure 2C-F). Similar increases in calcium concentration were also observed in SMF-treated PDLCs and cementoblasts (Figure 3A and B). In addition, SMFs upregulated the mRNA expression of osteoblast markers in PDLCs, including Runx2, OPN, and OCN (Figure 3C), as well as the expression of cementoblast markers, such as cementum protein 1 (CEMP-1) and cementum-derived attachment protein (CAP), in cementoblasts (Figure 3D).

SMFs promote osteoblastic differentiation in primary cultured osteoblasts and PDLCs

To further assess the osteoblastic activity of the SMFs, we examined primary cultured osteoblasts and PDLCs. Primary cultured PDLCs and osteoblasts showed no cytotoxicity in response to SMFs, but did show increased ALP activity and mineralization (Figures 4A-F, 5A and B). In addition, the treatment of primary cultured PDLCs and osteoblasts with SMFs increased the expression of mRNA encoding the differentiation markers Runx2, OPN, and OCN (Figure 5C and D).

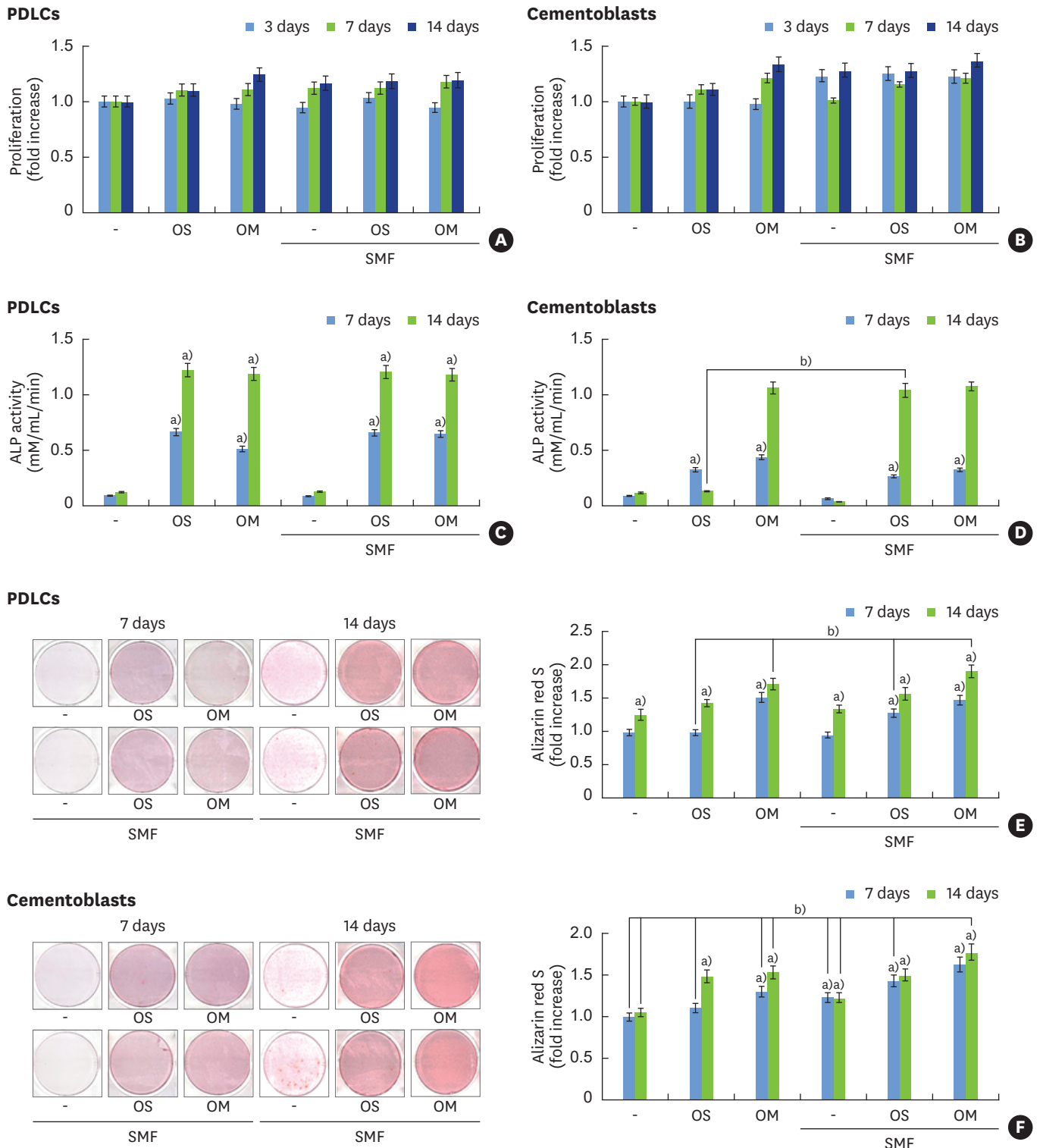


Figure 2. Effect of SMFs on osteoblastic differentiation in a cell line of human PDLs and cementoblasts. (A, B) Cell proliferation was examined by an MTT assay at 3, 7, and 14 days. (C, D) ALP activity was determined and normalized to protein content. (E, F) Matrix mineralization was evaluated by Alizarin red staining. Cells were treated with 15-mT SMFs and OS or OM for 14 days. The results are representative of 5 independent experiments.

SMF: static magnetic field, PDL: periodontal ligament cell, MTT: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, ALP: alkaline phosphatase, OS: osteogenic supplement, OM: osteogenic medium.

^a)Significant difference compared to control ($P < 0.05$); ^b)Significant difference between each group ($P < 0.05$).

SMFs promote differentiation in periodontium cells

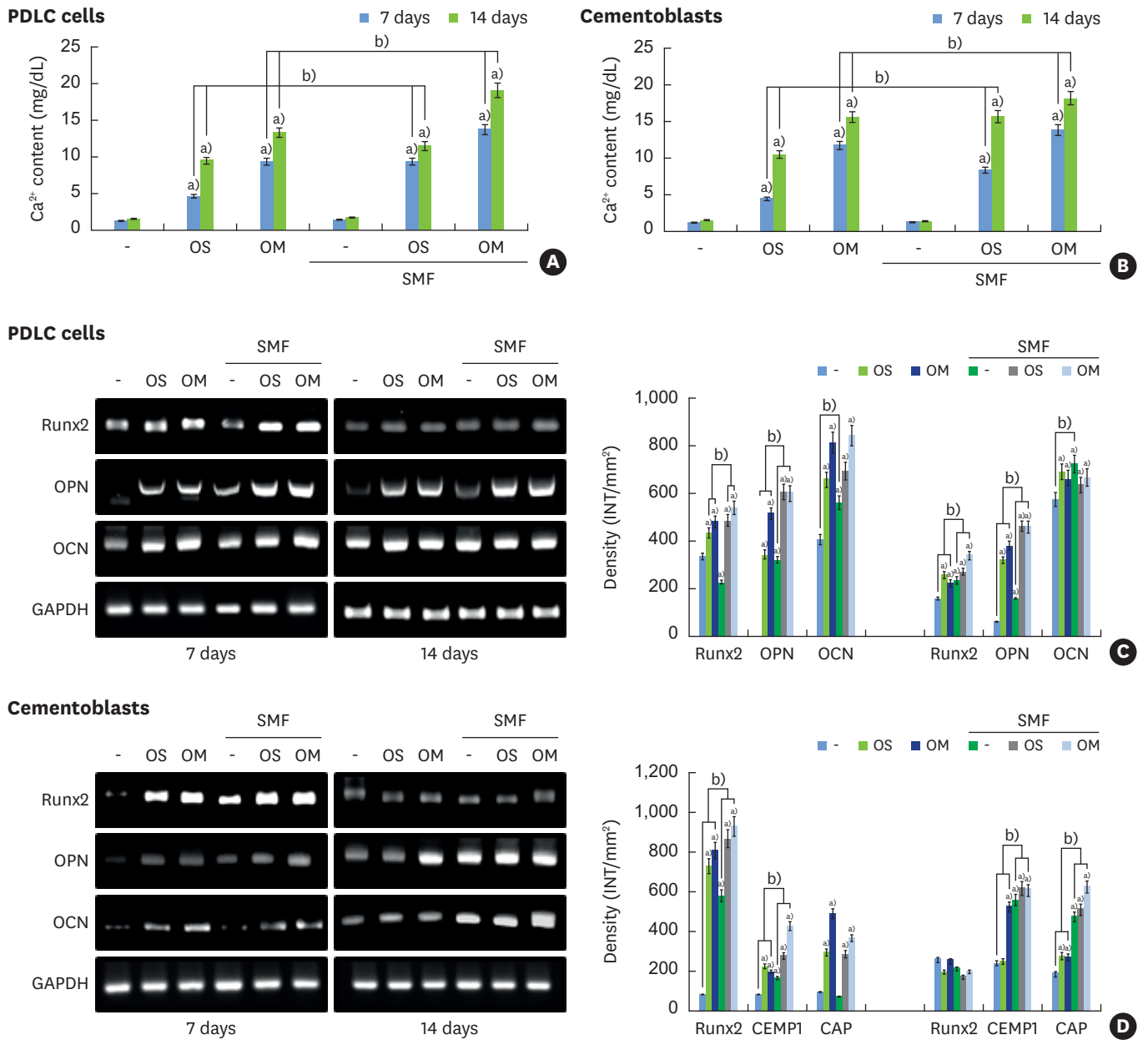


Figure 3. Effects of SMFs on calcium content and osteoblastic or cementoblastic gene expression in a cell line of human PDLCs and cementoblasts. (A, B) Ca²⁺ concentration was measured using a calcium quantification assay kit. (C, D) mRNA was determined by RT-PCR analysis. Cells were treated with a 15-mT SMF and OS or OM for 14 days. The results are representative of 5 independent experiments.

SMF: static magnetic field, PDL: periodontal ligament, PDLC: periodontal ligament cell, RT-PCR: reverse transcription polymerase chain reaction, OS: osteogenic supplement, OM: osteogenic medium, Runx2: runt-related transcription factor 2, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, CEMP-1: cementum protein 1, CAP: cementum-derived attachment protein.

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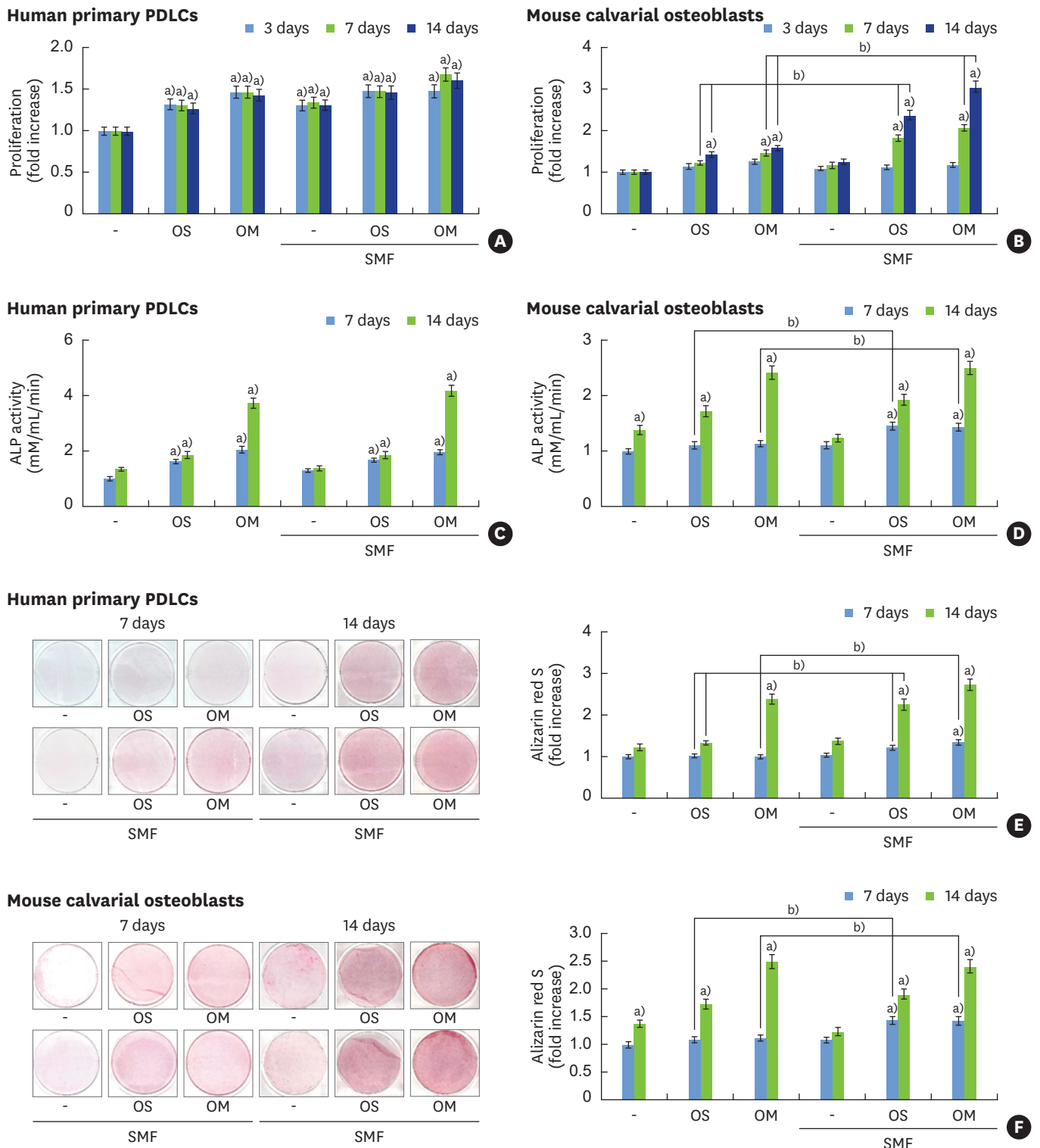


Figure 4. Effect of SMFs on proliferation, ALP activity, and mineralized nodule formation in primary cultured human PDLCs and osteoblasts. (A, B) Cell proliferation was examined by an MTT assay at 3, 7, and 14 days. (C, D) ALP activity was determined and normalized to protein content. (E, F) Matrix mineralization was evaluated by Alizarin red staining as described in the Materials and Methods. Cells were treated with 15-mT SMFs and OS or OM for 14 days. The results are representative of 5 independent experiments.

SMF: static magnetic field, ALP: alkaline phosphatase, PDLC: periodontal ligament cell, MTT: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, OS: osteogenic supplement, OM: osteogenic medium.

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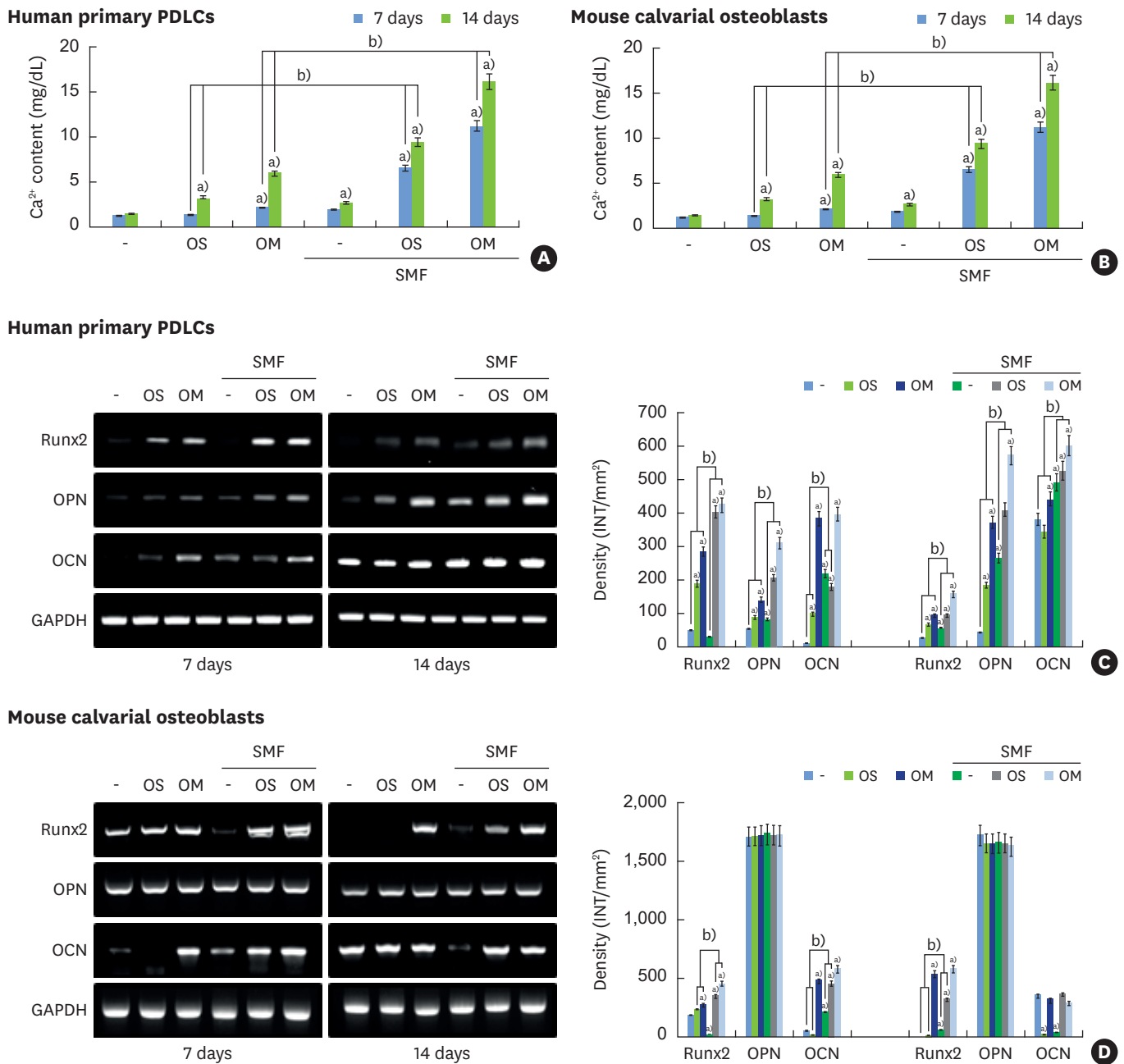


Figure 5. Effect of SMFs on calcium content and osteoblastic gene expression in primary cultured human PDLs and osteoblasts. (A, B) Ca²⁺ concentration was measured using a calcium quantification assay kit. (C, D) mRNA was determined by RT-PCR analysis. The results are representative of 5 independent experiments.

SMF: static magnetic field, PDL: periodontal ligament cell, RT-PCR: reverse transcription polymerase chain reaction, Runx2: runt-related transcription factor 2, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

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SMFs activate Wnt/ β -catenin, p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs), and nuclear factor- κ B (NF- κ B) signaling, but not extracellular signal-regulated kinase (ERK) signaling

To determine the basis of the effects of SMFs on osteoblast differentiation, their effects on Wnt/ β -catenin, MAPK, and NF- κ B signaling were examined. As shown in Figure 6A and

B, SMFs increased *Wnt1* and *Wnt3a* expression, the phosphorylation of glycogen synthase kinase-3 β (GSK-3 β), and total β -catenin protein levels in osteoblasts. Moreover, treatment with SMFs increased OM-induced p38 and JNK phosphorylation, but not that of ERK (Figure 6C). In addition, it enhanced the OM-induced nuclear translocation of p65 (Figure 6D). Immunocytochemical observations showed that the nuclear content of NF- κ B p65 increased markedly in SMF-treated osteoblasts compared with the OM control (Figure 6E).

To confirm the involvement of Wnt/ β -catenin, MAPKs, and NF- κ B pathways in SMF-induced osteoblastic differentiation, inhibitors of Wnt (DKK1), ERK (PD98059), JNK (SP6100126), p38 (SB203580), MAPK, and NF- κ B (PDTC) signaling were used. SMF-induced ALP activity, mineralization nodule formation, calcium concentration, and the mRNAs of osteoblast markers were effectively inhibited by DKK1, SP600126, SB203580, and PDTC, but not by PD98059 (Figure 7A-D).

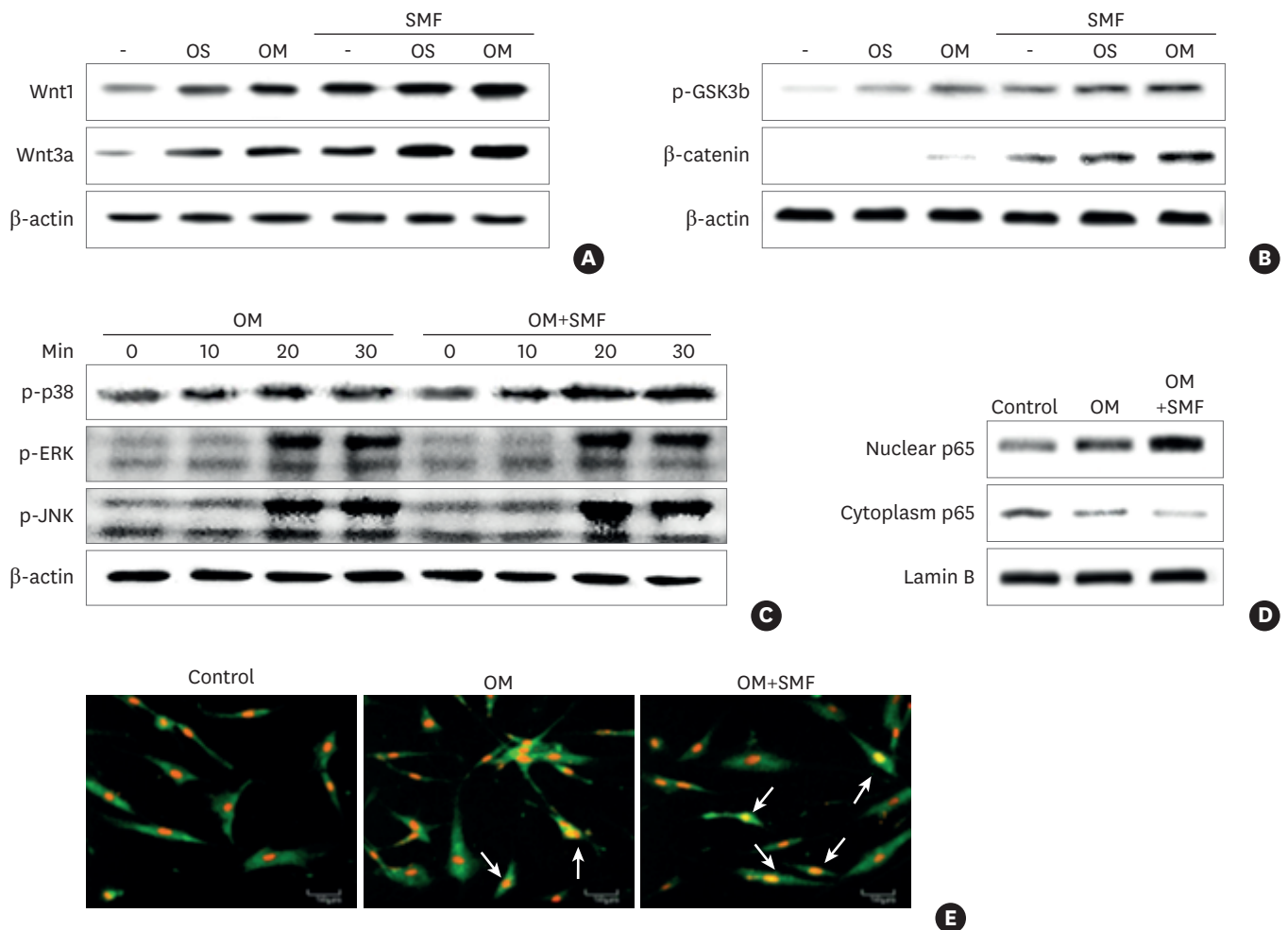


Figure 6. Effects of SMFs on the Wnt/ β -catenin, MAPK, and NF- κ B signaling pathways in human osteoblasts. (A, B) Cells were treated with SMFs and OS or OM for 2 days, (C) 30 minutes, and (D, E) 45 minutes. (A-D) Protein levels were assessed by western blot analysis and (E) immunofluorescence staining. Arrows (yellow) indicate the nuclear translocation of NF- κ B p65. The data presented are representative of five independent experiments.

SMF: static magnetic field, MAPK: mitogen-activated protein kinase, NF- κ B: nuclear factor- κ B, OS: osteogenic supplement, OM: osteogenic medium, GSK-3 β : glycogen synthase kinase-3 β , ERK: extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase.

SMFs promote differentiation in periodontium cells

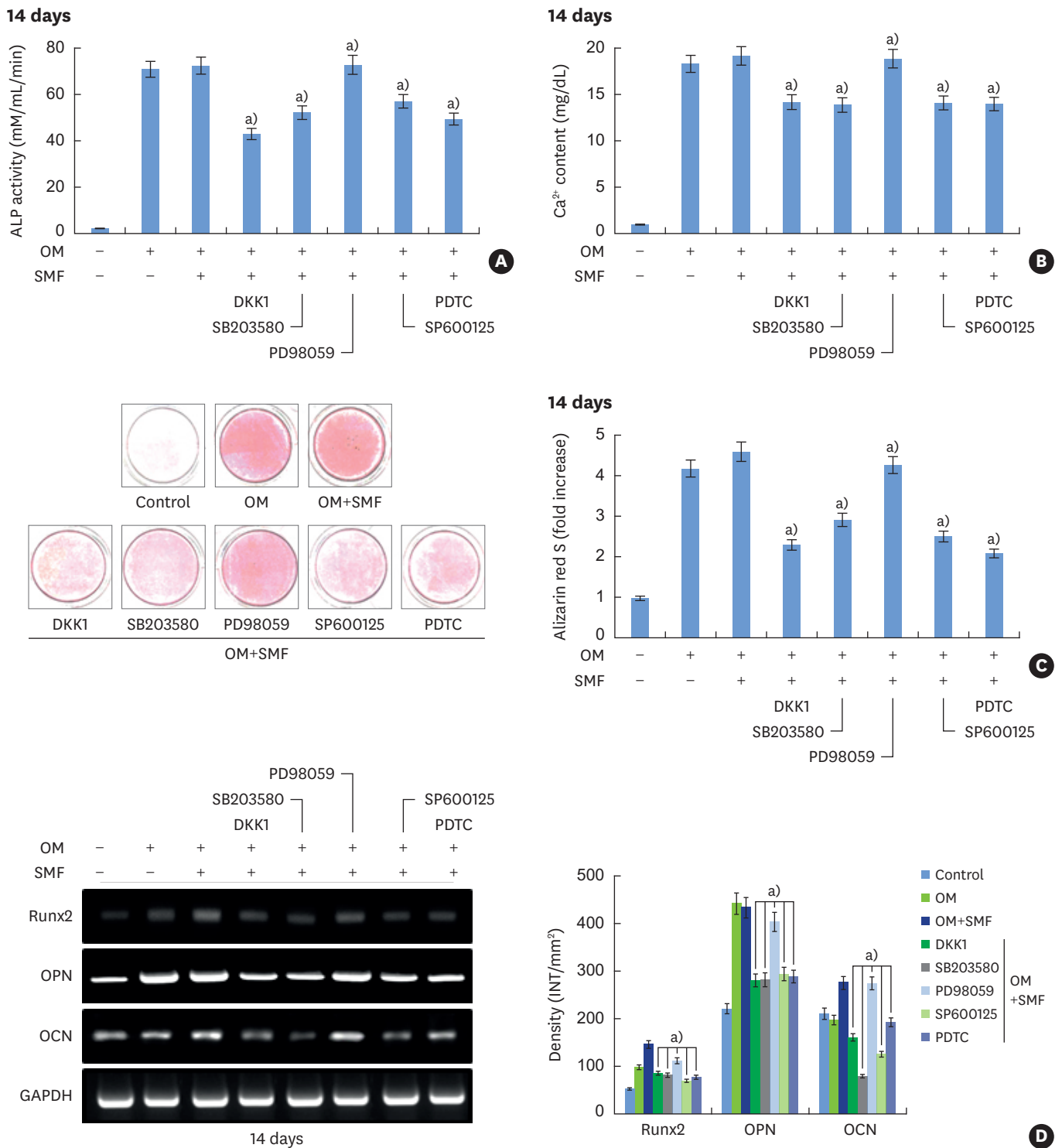


Figure 7. Effects of various inhibitors of signal transduction on SMF-induced osteoblastic differentiation in human osteoblasts. (A-D) Cells were pretreated for 2 hours with DKK1 (0.5 µg/mL), SB203580 (20 µM), PD98059 (20 µM), SP600125 (10 µM), and PDTC (10 µM), and then cultured in OS with 15-mT SMFs for 14 days. The results are representative of 5 independent experiments.

SMF: static magnetic field, OS: osteogenic supplement, Runx2: runt-related transcription factor 2, OPN: osteopontin, OCN: osteocalcin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

^{a)}Significant difference between each group ($P < 0.05$).

DISCUSSION

Successful periodontal reconstruction requires the regeneration of multiple tissues, including cementum, PDL, and bone. Osteoblasts are specialized mesenchymal-derived cells capable of forming new bone. PDLCs retain the capability to differentiate into the osteoblast lineage, and are considered to play a key role in the regeneration of periodontal tissues [24]. In addition, cementoblasts may play a key role in the formation of the PDL-cementum apparatus during periodontal regeneration [25]. Thus, complete periodontal repair entails *de novo* cementogenesis, osteogenesis, and the formation of PDL fibers, and we focused here on the effects of SMFs on the osteoblastic/cementoblastic responsiveness of cells associated with the periodontium such as osteoblasts, cementoblasts, and human PDLCs, to explore the possibility of using SMFs as a therapeutic agent in periodontal or bone regeneration.

The present study showed that SMF exposure did not affect or impair cell proliferation, even over the course of 14 days of culture. Therefore, the present results agreed with previous findings that demonstrated that SMF exposure did not affect cell proliferation in rat calvaria cells or osteoblast-like cells [12]. However, SMFs significantly inhibited the proliferation of human osteoblasts [13], but conversely stimulated the proliferation of MSCs *in vitro* [17]. These discrepancies may stem from differences in culture conditions, cell type, and the application methods or type of magnetic fields.

ALP activity is an early maker of osteoblasts, whereas mineralization of the extracellular matrix detected by Alizarin red staining is a marker of mature osteoblasts [26]. OCN and OPN are non-collagenous proteins found in the extracellular matrix produced by osteoblasts, and are late osteoblast differentiation markers [27]. Runx2 is an osteoblast-specific-transcription factor essential for early osteoblastic differentiation [28]. hFOB 1.19 cells have the matrix-synthesizing properties of differentiated osteoblasts, and are an excellent model for the study of osteoblast biology *in vitro* [29]. We reported above that SMFs increased ALP activity, upregulated OCN, OPN, and Runx2 mRNA, and promoted the formation of mineralized nodules, as well as increasing calcium content in osteoblasts. Evidently, SMFs stimulated both early and late osteoblastic differentiation, as well as mineralization in the osteoblasts, which is consistent with previous data obtained using rat osteoblasts [12], human osteoblast-like MG63 cells [15], dental pulp cells [16], and MSCs [17].

Cementoblasts share many characteristics with osteoblast-like cells, including the ability to form a mineralized matrix, and express genes associated with osteogenic differentiation, including OPN, OCN, CEMP-1, and CAP [22]. PDLCs have the capacity to differentiate *in vitro* into osteoblasts and cementoblasts, as they have high ALP activity and produce considerable OPN, bone sialoprotein, and OCN [30]. Thus, the regulation of osteogenic/cementoblastic differentiation in PDLCs and cementoblasts has important implications for the development of new therapeutic strategies for periodontitis. We observed that SMFs stimulated PDLCs and cementoblasts to acquire cementoblast/osteoblast characteristics as measured by increased ALP activity, osteogenesis/cementogenesis-related gene expression, enhanced calcium content, and mineral nodule formation. These results support existing data suggesting that SMFs enhance osteoblastic/cementoblastic differentiation and the mineralization of osteoblasts, PDLCs, and cementoblasts.

Wnt/ β -catenin signaling plays an important role in skeletal development and osteoblast differentiation [31]. Binding of Wnt to its receptors leads to the inhibition of GSK-3 β and the

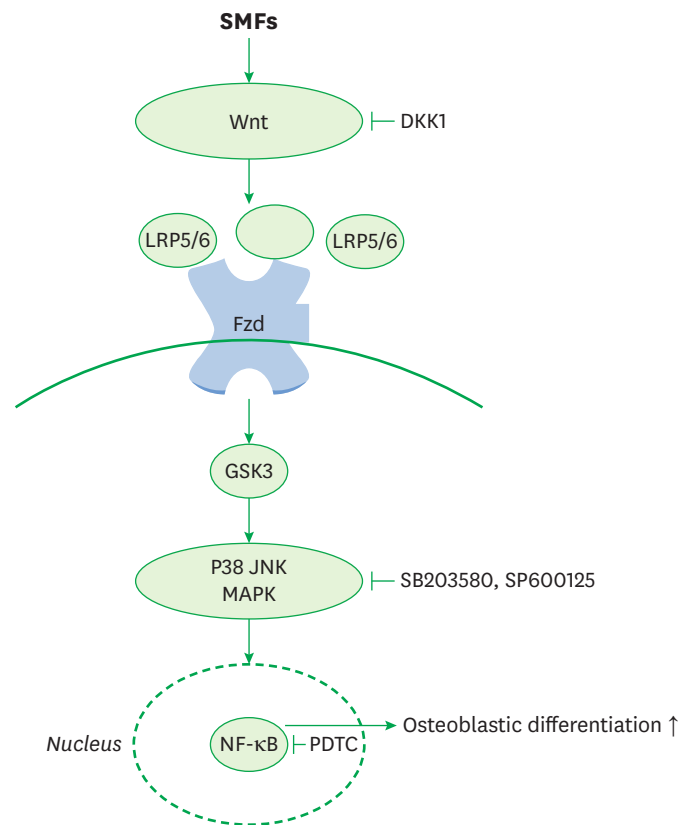


Figure 8. Schematic diagram illustrating the Wnt, Akt, MAPK, and NF- κ B signaling pathways triggered by exposure to SMFs, which ultimately stimulate the osteoblastic differentiation of human osteoblasts. MAPK: mitogen-activated protein kinase, NF- κ B: nuclear factor- κ B, SMF: static magnetic field, Fzd: Frizzled, GSK-3 β : glycogen synthase kinase-3 β , JNK: c-Jun N-terminal kinase.

stabilization and accumulation of β -catenin, which then translocates to the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor family of transcription factors, resulting in the activation of Wnt downstream target genes [32]. A recent study showed that PEMFs can prevent ovariectomy-induced bone loss and the deterioration of bone microarchitecture and strength by activating this Wnt/ β -catenin signaling pathway [33]. In the present study, SMFs significantly upregulated canonical Wnt ligands (*Wnt1* and *Wnt3a*), and stimulated phosphorylation of GSK-3 β and total β -catenin expression in osteoblasts. These results suggest that SMFs stimulate osteoblastic differentiation by activating the Wnt/ β -catenin signaling pathway.

The MAPK and NF- κ B pathways are known to play essential roles in cell differentiation [22]. Recently it was reported that extremely low-frequency magnetic fields inhibited the adipogenic differentiation of human MSCs via the JNK-dependent Wnt signaling pathway [34]. However, electromagnetic fields activated the ERK/MAPK pathway in fibroblast-like cells [35] and hippocampal neurons [36], as well as p38 signaling in a Chinese hamster lung cell line [37]. In addition, treatment with SB203580, an inhibitor of p38 MAPK, suppressed extremely low-frequency electromagnetic field-induced collagen production in mouse osteoblast-like MC3T3-E1 cells [38]. PEMF exposure significantly increased the anti-inflammatory effect of the A_{2A} and A₃ adenosine receptors by inhibiting NF- κ B pathways in chondrocytes and osteoblasts [39]. In contrast, strong SMFs (6 T) induced the

secretion of prostaglandin E2 and the expression of cyclooxygenase 2 in MC3T3-E1 cells, via the translocation of NF- κ B [40]. Our results confirm that the p38, JNK/MAPK, and NF- κ B signaling pathways are activated in SMF-treated osteoblasts. Furthermore, inhibitors of Wnt, p38, JNK MAPK, and NF- κ B significantly reduced osteoblastic differentiation in response to SMFs. Hence, SMF may promote osteoblast differentiation by activating the Wnt, p38, JNK/MAPK, and NF- κ B signaling pathways.

In summary, this is the first study to report that SMFs promoted osteoblastic/cementoblastic differentiation by activating the Wnt/ β -catenin, p38, JNK/MAPK, and NF- κ B signaling pathways in osteoblasts, cementoblasts, and PDLcs. Figure 8 is a schematic representation of the signaling pathway involved in the effects of SMFs on the osteoblastic differentiation in osteoblasts. Wnt binds to the transmembrane receptor Frizzled (Fzd) and co-receptor LRP5/6. SMF-activated Wnt-Fzd-LRP5/6 binding events lead to the intracellular accumulation of β -catenin and phosphorylation of GSK3, which activates MAPK and NF- κ B for osteoblastogenic gene and Runx2 transcription. Our results also show that SMFs have beneficial effects on bone healing and periodontal regeneration.

REFERENCES

1. Shimono M, Ishikawa T, Ishikawa H, Matsuzaki H, Hashimoto S, Muramatsu T, et al. Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech* 2003;60:491-502.
[PUBMED](#) | [CROSSREF](#)
2. Cook JJ, Summers NJ, Cook EA. Healing in the new millennium: bone stimulators: an overview of where we've been and where we may be heading. *Clin Podiatr Med Surg* 2015;32:45-59.
[PUBMED](#) | [CROSSREF](#)
3. Funk RH, Monsees T, Ozkucur N. Electromagnetic effects - from cell biology to medicine. *Prog Histochem Cytochem* 2009;43:177-264.
[PUBMED](#) | [CROSSREF](#)
4. Gaetani R, Ledda M, Barile L, Chimenti I, De Carlo F, Forte E, et al. Differentiation of human adult cardiac stem cells exposed to extremely low-frequency electromagnetic fields. *Cardiovasc Res* 2009;82:411-20.
[PUBMED](#) | [CROSSREF](#)
5. Sakata M, Yamamoto Y, Imamura N, Nakata S, Nakasima A. The effects of a static magnetic field on orthodontic tooth movement. *J Orthod* 2008;35:249-54.
[PUBMED](#) | [CROSSREF](#)
6. Zhang J, Ding C, Ren L, Zhou Y, Shang P. The effects of static magnetic fields on bone. *Prog Biophys Mol Biol* 2014;114:146-52.
[PUBMED](#) | [CROSSREF](#)
7. Markov MS. Magnetic field therapy: a review. *Electromagn Biol Med* 2007;26:1-23.
[PUBMED](#) | [CROSSREF](#)
8. Yang TC, Maeda Y, Gonda T, Wada M. Magnetic attachment for implant overdentures: influence of contact relationship with the denture base on stability and bending strain. *Int J Prosthodont* 2013;26:563-5.
[PUBMED](#) | [CROSSREF](#)
9. Aksu AE, Dursun E, Calis M, Ersu B, Safak T, Tözüm TF. Intraoral use of extraoral implants for oral rehabilitation of a pediatric patient after resection of ewing sarcoma of the mandible and reconstruction with iliac osteocutaneous free flap. *J Craniofac Surg* 2014;25:930-3.
[PUBMED](#) | [CROSSREF](#)
10. Siadat H, Bassir SH, Alikhasi M, Shayesteh YS, Khojasteh A, Monzavi A. Effect of static magnetic fields on the osseointegration of immediately placed implants: a randomized controlled clinical trial. *Implant Dent* 2012;21:491-5.
[PUBMED](#) | [CROSSREF](#)
11. Leesungbok R, Ahn SJ, Lee SW, Park GH, Kang JS, Choi JJ. The effects of a static magnetic field on bone formation around a sandblasted, large-grit, acid-etched-treated titanium implant. *J Oral Implantol* 2013;39:248-55.
[CROSSREF](#)

12. Yamamoto Y, Ohsaki Y, Goto T, Nakasima A, Iijima T. Effects of static magnetic fields on bone formation in rat osteoblast cultures. *J Dent Res* 2003;82:962-6.
[PUBMED](#) | [CROSSREF](#)
13. Denaro V, Papapietro N, Sgambato A, Barnaba SA, Ruzzini L, Paola BD, et al. Periprosthetic electrochemical corrosion of titanium and titanium-based alloys as a cause of spinal fusion failure. *Spine* 2008;33:8-13.
[PUBMED](#) | [CROSSREF](#)
14. Denaro V, Cittadini A, Barnaba SA, Ruzzini L, Denaro L, Rettino A, et al. Static electromagnetic fields generated by corrosion currents inhibit human osteoblast differentiation. *Spine* 2008;33:955-9.
[PUBMED](#) | [CROSSREF](#)
15. Chiu KH, Ou KL, Lee SY, Lin CT, Chang WJ, Chen CC, et al. Static magnetic fields promote osteoblast-like cells differentiation via increasing the membrane rigidity. *Ann Biomed Eng* 2007;35:1932-9.
[PUBMED](#) | [CROSSREF](#)
16. Hsu SH, Chang JC. The static magnetic field accelerates the osteogenic differentiation and mineralization of dental pulp cells. *Cytotechnology* 2010;62:143-55.
[PUBMED](#) | [CROSSREF](#)
17. Kim EC, Leesungbok R, Lee SW, Lee HW, Park SH, Mah SJ, et al. Effects of moderate intensity static magnetic fields on human bone marrow-derived mesenchymal stem cells. *Bioelectromagnetics* 2015;36:267-76.
[PUBMED](#) | [CROSSREF](#)
18. Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 2006;40:29-49.
[PUBMED](#) | [CROSSREF](#)
19. Miyakoshi J. Effects of static magnetic fields at the cellular level. *Prog Biophys Mol Biol* 2005;87:213-23.
[PUBMED](#) | [CROSSREF](#)
20. Wang Y, Qin QH. A theoretical study of bone remodelling under PEMF at cellular level. *Comput Methods Biomech Biomed Engin* 2012;15:885-97.
[PUBMED](#) | [CROSSREF](#)
21. Chung JH, Kim YS, Noh K, Lee YM, Chang SW, Kim EC. Deferoxamine promotes osteoblastic differentiation in human periodontal ligament cells via the nuclear factor erythroid 2-related factor-mediated antioxidant signaling pathway. *J Periodontol Res* 2014;49:563-73.
[PUBMED](#) | [CROSSREF](#)
22. Kitagawa M, Tahara H, Kitagawa S, Oka H, Kudo Y, Sato S, et al. Characterization of established cementoblast-like cell lines from human cementum-lining cells *in vitro* and *in vivo*. *Bone* 2006;39:1035-42.
[PUBMED](#) | [CROSSREF](#)
23. Pi SH, Lee SK, Hwang YS, Choi MG, Lee SK, Kim EC. Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival fibroblasts and periodontal ligament cells. *J Periodontol Res* 2007;42:104-13.
[PUBMED](#) | [CROSSREF](#)
24. Beertsen W, McCulloch CA, Sodek J. The periodontal ligament: a unique, multifunctional connective tissue. *Periodontol 2000* 1997;13:20-40.
[PUBMED](#) | [CROSSREF](#)
25. Chen FM, Jin Y. Periodontal tissue engineering and regeneration: current approaches and expanding opportunities. *Tissue Eng Part B Rev* 2010;16:219-55.
[PUBMED](#) | [CROSSREF](#)
26. Kim MB, Song Y, Hwang JK. Kirenol stimulates osteoblast differentiation through activation of the BMP and Wnt/ β -catenin signaling pathways in MC3T3-E1 cells. *Fitoterapia* 2014;98:59-65.
[PUBMED](#) | [CROSSREF](#)
27. Nöth U, Tuli R, Seghatoleslami R, Howard M, Shah A, Hall DJ, et al. Activation of p38 and Smads mediates BMP-2 effects on human trabecular bone-derived osteoblasts. *Exp Cell Res* 2003;291:201-11.
[PUBMED](#) | [CROSSREF](#)
28. Franceschi RT, Xiao G. Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. *J Cell Biochem* 2003;88:446-54.
[PUBMED](#) | [CROSSREF](#)
29. Subramaniam M, Jalal SM, Rickard DJ, Harris SA, Bolander ME, Spelsberg TC. Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: bone formation *in vivo* and karyotype analysis using multicolor fluorescent *in situ* hybridization. *J Cell Biochem* 2002;87:9-15.
[PUBMED](#) | [CROSSREF](#)

30. Hayami T, Zhang Q, Kapila Y, Kapila S. Dexamethasone's enhancement of osteoblastic markers in human periodontal ligament cells is associated with inhibition of collagenase expression. *Bone* 2007;40:93-104.
[PUBMED](#) | [CROSSREF](#)
31. Bodine PV, Komm BS. Wnt signaling and osteoblastogenesis. *Rev Endocr Metab Disord* 2006;7:33-9.
[PUBMED](#) | [CROSSREF](#)
32. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature* 2005;434:843-50.
[PUBMED](#) | [CROSSREF](#)
33. Zhou J, He H, Yang L, Chen S, Guo H, Xia L, et al. Effects of pulsed electromagnetic fields on bone mass and Wnt/ β -catenin signaling pathway in ovariectomized rats. *Arch Med Res* 2012;43:274-82.
[PUBMED](#) | [CROSSREF](#)
34. Du L, Fan H, Miao H, Zhao G, Hou Y. Extremely low frequency magnetic fields inhibit adipogenesis of human mesenchymal stem cells. *Bioelectromagnetics* 2014;35:519-30.
[PUBMED](#) | [CROSSREF](#)
35. Wolf-Goldberg T, Barbul A, Ben-Dov N, Korenstein R. Low electric fields induce ligand-independent activation of EGF receptor and ERK via electrochemical elevation of H(+) and ROS concentrations. *Biochim Biophys Acta* 2013;1833:1396-408.
[PUBMED](#) | [CROSSREF](#)
36. Ma J, Zhang Z, Su Y, Kang L, Geng D, Wang Y, et al. Magnetic stimulation modulates structural synaptic plasticity and regulates BDNF-TrkB signal pathway in cultured hippocampal neurons. *Neurochem Int* 2013;62:84-91.
[PUBMED](#) | [CROSSREF](#)
37. Sun W, Yu Y, Chiang H, Fu Y, Lu D. Exposure to power-frequency magnetic fields can induce activation of P38 mitogen-activated protein kinase. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 2002;20:252-5.
[PUBMED](#)
38. Soda A, Ikehara T, Kinouchi Y, Yoshizaki K. Effect of exposure to an extremely low frequency-electromagnetic field on the cellular collagen with respect to signaling pathways in osteoblast-like cells. *J Med Invest* 2008;55:267-78.
[PUBMED](#) | [CROSSREF](#)
39. Vincenzi F, Targa M, Corciulo C, Gessi S, Merighi S, Setti S, et al. Pulsed electromagnetic fields increased the anti-inflammatory effect of A₂A and A₃ adenosine receptors in human T/C-28a2 chondrocytes and hFOB 1.19 osteoblasts. *PLoS One* 2013;8:e65561.
[PUBMED](#) | [CROSSREF](#)
40. Sakurai T, Terashima S, Miyakoshi J. Enhanced secretion of prostaglandin E2 from osteoblasts by exposure to a strong static magnetic field. *Bioelectromagnetics* 2008;29:277-83.
[PUBMED](#) | [CROSSREF](#)