Low frequency-pulsed electromagnetic fields promote osteogenic differentiation of bone marrow-derived mesenchymal stem cells by regulating connexin 43 expression

ZHI-JUN LU, HOU-YUN GU, ZHI-QIANG LI and FEI-XIANG LIN

Department of Spine Surgery, Ganzhou People's Hospital (The Affiliated Ganzhou Hospital of Jiangxi Medical College of
Nanchang University, Ganzhou Hospital-Nanfang Hospital of Southern Medical University), Ganzhou, Jiangxi 341000, P.R. China

Abstract. The present study investigated the effect of connexin 43 (Cx43) on the regulation of osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (BMSCs) using low-frequency-pulsed electromagnetic fields (LPEMF). The BMSCs were isolated and cultured *in vitro* using adherent whole-bone marrow cultures. CCK-8 assay was used to detect the effects of LPEMF on the proliferation ability of BMSCs and alkaline phosphatase (ALP) activity and the levels of osteogenic marker genes were detected to evaluate the osteogenic ability change following LPEMF treatment. Lentiviral vector-mediated RNA interference was transfected into BMSCs to inhibit the expression of Cx43 and western blotting was used to detect Cx43 expression. The BMSCs showed the highest proliferation following LPEMF treatment at 80 Hz for 1 h. The results of ALP activity, osteogenic marker genes and Alizarin Red S staining showed that the osteogenic ability was notably increased following LPEMF treatment at 80 Hz for 1 h. Cx43 expression increased during the osteogenic differentiation of BMSCs following LPEMF treatment at 80 Hz. The enhanced osteogenic differentiation of the LPEMF-treated BMSCs were partially reversed when Cx43 expression was inhibited. LPEMF may promote the osteogenic differentiation of BMSCs by regulating Cx43 expression and enhancing osteogenic ability.

Correspondence to: Dr Fei-Xiang Lin, Department of Spine Surgery, Ganzhou People's Hospital (The Affiliated Ganzhou Hospital of Jiangxi Medical College of Nanchang University, Ganzhou Hospital-Nanfang Hospital of Southern Medical University), 16 Meiguan Avenue, Ganzhou, Jiangxi 341000, P.R. China
E-mail: feixianglin0@sina.com

Introduction

Bone defects are major problems in trauma orthopedics, leading to non-union and limb disability, making the prognosis of the patient unsatisfactory. It causes severe damage to the physical and mental health of the patient, with a huge economic burden on both patients and society (1). Fracture healing is divided into four major overlapping phases: Inflammation, bridging osseous callus formation, callus mineralization and bone remodeling (2). Functional reduction of osteoblasts is an important cause of bone defects. Therefore, pharmacological or physical interventions that induce osteoblast differentiation and proliferation, thereby promoting bone formation, are potential strategies for treating bone defects.

Low-frequency-pulsed electromagnetic field (LPEMF) is a noninvasive and inexpensive method that has shown efficacy against a wide range of diseases of the skeletal muscle system (3,4). LPEMF may inhibit osteoclast differentiation *in vitro* (5,6), accelerate bone defect repair and shorten the healing time of bone defects by promoting osteogenic differentiation of bone marrow stromal cells (BMSCs) (2). BMSCs have multidirectional differentiation potential and are the main source of osteoblasts (7). Osteogenic differentiation of BMSCs is the physiological basis of bone defect repair and promoting osteogenic differentiation of BMSCs can substantially shorten the time required for bone defect repair (8). Therefore, investigating the underlying mechanisms of LPEMF in osteoblasts is essential for understanding its efficacy in treating osteoblast-related diseases.

Connexin 43 (Cx43) is the most abundant gap junction protein in bone (9). Cx43 is the most widely expressed connexin among bone cell types, encoded by *Gja1* gene (10) and expressed at the cell surface, where it mediates cell communication and allows small molecules and ions to pass between cells (9,11). Cx43 plays important roles in bone formation and homeostasis. Osteoblasts regulate cell proliferation, differentiation and metabolic functions via intercellular communication (9,10,12-14). Cx43 is also expressed in cartilage and is necessary for mineralization and osteoclast formation, suggesting that Cx43 may play an important role in multiple stages of fracture healing (15-17). In three-dimensional culture, overexpression of Cx43 in BMSCs enhances the

size and spatial distribution of gap junctions, intercellular communication and expression of osteogenic markers (18).

LPEMF has the ability to promote the osteogenic differentiation of BMSCs, accelerate the process of bone defect repair and shorten the healing time of bone defects. However, the mechanism of LPEMF on Cx43 expression in promoting the osteogenic differentiation of BMSCs remains to be elucidated. The present study provided a comprehensive and systematic annotation of the role of Cx43 in the regulation of the osteogenic differentiation of BMSCs by LPEMF through *in vitro* experiments at the molecular and cellular levels to explore the mediation of Cx43 and provide a target for the treatment of bone defects.

Materials and methods

Isolation and culture of BMSCs. Primary rat BMSCs were isolated from specific pathogen free (SPF) four to six-week-old rats weighing 100-150 g Sprague-Dawley, which were provided by the SPF grade animal house of Shantou University Medical College (Guangdong, China). A total of 30 rats, 50% male and 50% female, were libitum fed in the same environment were maintained at an indoor temperature of 22°C, relative humidity of 18-22%, and a 12-h light/dark cycle. All animal experimental procedures were approved by the Institutional Ethics Committee for Animal Experimentation of Ganzhou People's Hospital (Ganzhou, China). Rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg) and euthanized by cervical dislocation. The two lower limbs were rapidly dissociated and disinfected by immersion in 75% ethanol for 5-10 min, under aseptic conditions. Subsequently, the femoral bone marrow was lavaged and the lavage fluid was inoculated into a 10-cm dish using the whole bone marrow adherent culture method (19). Briefly, the cells were seeded onto flasks for culturing in a CO_2 cell incubator. The culture medium was maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 0.5 mg/ml penicillin/streptomycin. Cell morphology and growth were observed daily using an inverted microscope. When the cell confluence reached 70-90%, a subculture was carried out and the cells were cultured to the third generation for subsequent experiments.

LPEMF exposure. To evaluate the proliferation and osteogenic differentiation behavior of BSMCs under different intensity gradients of LPEMF, the cells were continuously exposed to sinusoidal LPEMF (20, 40, 60, 80 and 100 Hz) of five different gradient signals with a density of 1 mT in the incubator. A pulsed electromagnetic field *in vitro* cell intervention system (DG1022U; Rigol Technologies, Inc.) generated electromagnetic field signals at a predetermined frequency. The magnetic field was generated using a pair of 60-turn Helmholtz coils. The coil diameter was 300 mm and the spacing was 122.5 mm. A Petri dish was placed at the center of the coil. The control cells were placed in another incubator under the same conditions, but without LPEMF.

BMSCs osteogenic differentiation culture. When P3 cells were cell confluence reached 70-90%, they were subcultured and inoculated at 5,000-10,000 cells/cm². The cells were incubated overnight in a humidified incubator at 37°C, with 5% CO_2 and 95% air and then replaced with an osteogenic induction medium (alpha-MEM; (Gibco; Thermo Fisher Scientific, Inc.; cat. no. C12571500BT) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 12664025), 50 μM vitamin C (MilliporeSigma; cat. no. A4544), 10 mM beta-sodium glycerophosphate (MilliporeSigma; cat. no. G9422) and 100 nM dexamethasone (MilliporeSigma; cat. no. D1756) for further culture. The osteogenic induction medium was replaced once every 2-3 days and cell growth was observed under a light microscope every day. The cells were cultured until they were terminated at 9 days and tested for ALP activity. After 14 days of cell culture, total RNA and total protein were extracted for PCR and Western blotting experiments. Cell cultures were terminated at 14 days and Alizarin red S staining was performed.

Cell Counting Kit-8 (CCK-8) assay. To evaluate cell proliferation, BMSCs were inoculated into 96-well plates at a density of $1x10^4$ cells per well and incubated in a CO_2 cell incubator (37°C, 5% CO_2) for 4 h. Cells were cultured 24 h after electromagnetic field treatment for 5 days and 10 μl CCK8 (Dojindo Laboratories, Inc.) dye solution was added to each well. After 2 h, the absorbance of each well was measured at 450 nm according to the manufacturer's instructions. Each data point was obtained as the mean of five wells.

ALP activity detection. ALP activity is an early marker of bone formation (20). After cells were incubated for the indicated times, cells were washed once with PBS at 4°C, lysed with 1% Triton X-100 and the lysates collected. After centrifugation for 5 min at 4°C and 1,174 x g, the supernatant was collected. Color substrate (50 μl) and 50 μl of the group sample or sample buffer (blank control) was added to the 96-well plate, then placed on a shaker for mixing. After incubation at 37°C for 10 min, 100 μl of the reaction stop solution was added to each well. The absorbance of each well was measured at 405 nm by using a microplate reader.

Alizarin red S staining and quantification. After 14 days of cell culture under different conditions, the medium was removed and 1 ml PBS was added to each well. The cells were gently washed once, fixed with 2 ml anhydrous ethanol for 30 min and cleaned once with ultra-pure water. Alizarin red S solution (1 ml, 0.1%) was added after the cell surface dried. The bed was shaken at 22°C room temperature for 15 min, the Alizarin red S solution was removed, the cells were rinsed again with ultra-pure water three times and the staining of calcium nodules was observed under a light microscope after the cells were dried naturally. After Alizarin Red S staining was completed, 2 ml of 10% CPC sodium phosphate buffer solution was added to each well and incubated at 22°C room temperature for 15 min to elute Alizarin Red S. The Alizarin red S eluent was diluted 10 times with ultra-pure water and 100 μl diluent was absorbed into 96-well plates with five wells per group. The absorbance of each well was measured at 540 nm using a microplate analyzer.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. After treatment, total RNA was extracted from each group of cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.)

Table I. Primers used for quantitative PCR.

Target gene	Oligonucleotide sequence
Runx2	5'-GGGACCGTCCACTGTCACTTTAA-3' (Forward)
	5'-TACAAGTGGCCAGGTTCAACGA-3' (Reverse)
BSP	5'-GCTATGAAGGCTACGAGGGTCAGGATTAT-3' (Forward)
	5'-GGGTATGTTAGGGTGGTTAGCAATGGTGT-3' (Reverse)
Bglap	5'-AGGTGGTGAATAGACTCCG-3' (Forward)
	5'-GGCTGTGCCGTCCATACTT-3' (Reverse)
Osx	5'-GGAGGCACAAAGAAGCCATA-3' (Forward)
	5'-GGGAAAGGGTGGGTAGTCAT-3' (Reverse)
GAPDH	5'-TCCTGCACCACCAACTGCTTAG-3' (Forward)
	5'-AGTGGCAGTGATGGCATGGACT-3' (Reverse)

and the RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Next, mRNA was reverse-transcribed into cDNA using a reverse transcription system (Toyobo Life Science) according to the manufacturer's protocol. Then, cDNA was used as a template to perform qPCR (BeyoFast SYBR Green qPCR Mix; Beyotime Institute of Biotechnology) under the following thermal cycler conditions: 95°C for 5 sec, followed by 45 cycles, including denaturation at 95°C for 30 sec in an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.); further it was annealed at 60°C for 30 sec and extended at 72°C for 30 sec. GAPDH was used as an internal control, and the relative expression level of mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (21). Primers were designed and synthesized by BGI Biological Co. and the sequences are listed in Table I.

Western blotting. Western blotting was performed according to standard procedures. Briefly, proteins were extracted from cells or tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B). Protein concentrations were determined using the BCA Protein Assay Kit (Solarbio, Beijing, China). After denaturation ~20 µg of protein per lane was separated by 10% sodium dodecyl sulfate polyacrylamide gel, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (0.45 µm; MilliporeSigma). The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat milk for 1 h at room temperature. Subsequently, it was incubated with primary antibodies [rabbit anti-Cx43 (1:1,000; Cell Signaling Technology; cat. no. 3512), rabbit anti-GAPDH (1:1,000; Panera; cat. no. SF-PA005)] overnight at 4°C, then washed with TBST (0.1% Tween20) and incubated with the corresponding secondary antibody (Jackson ImmunoResearch; cat. no. 111-035-003) for 2 h. A chemiluminescence system (ECL Kit; Biosharp Life Sciences) was used to observe the protein bands and digital images were captured, which were then analyzed using ImageJ v6.0 (National Institutes of Health).

Statistical analysis. All data were analyzed using SPSS 19.0 software (IBM Corp.) and the results were expressed as mean ± standard deviation. The t-test was used for comparisons between two groups and one-way ANOVA followed by Tukey's post hoc test was used for comparisons between multiple groups. P<0.05 was considered to indicate a statistically significant difference. All experiments were independently repeated at least three times.

Results

Effect of different frequencies of LPEMF treatment on the proliferation ability of BMSCs. Evaluation of the effect of LPEMF on BMSCs proliferation *in vitro*: BMSCs were treated with different intensity gradients (0, 20, 40, 60, 80 and 100 Hz) of LPEMF for 24 h and the viability of BMSCs treated with LPEMF for 24 h was determined using CCK-8. The results showed that the proliferation viability of BMSCs increased with increasing magnetic field strength from 0 to 80 Hz peaking at 80 Hz (P<0.05). However, when the intensity reached 100 Hz, the proliferation viability of BMSCs was significantly inhibited and exhibited toxic side effects (Fig. 1). Therefore, the 100 Hz magnetic field intensity was removed in subsequent experiments as the research condition and 80 Hz magnetic field stimulation was used as the optimal stimulation intensity for the LPEMF treatment of BMSCs.

Effect of LPEMF treatment on ALP activity during osteogenic differentiation of BMSCs. To test whether LPEMF can promote the osteogenic differentiation of BMSCs, the ALP activity of each group was measured. The results showed that, compared with the control group, the ALP activity of BMSCs pretreated with LPEMF at different frequencies was enhanced on day 9 after osteogenic induction culture and the intensity showed a dose-dependent trend, with significant differences among all groups (Fig. 2).

Effect of LPEMF treatment on the expression of osteogenic marker genes during osteogenic differentiation of BMSCs. The present study examined the effect of LPEMF treatment on the expression of osteogenic marker genes during osteogenic differentiation of BMSCs. Following LPEMF treatment at different frequencies, BMSC osteogenic differentiation was induced and cultured for 7 and 14 days and the transcription levels of the osteogenic marker genes *Runx2*, *BSP*, *Bglap* and *Osx* in each group were measured. The results showed that
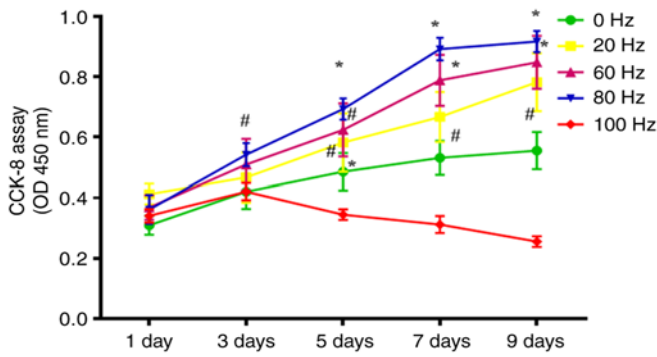
Figure 1. Effect of different frequencies of LPEMF treatment on rat BMSCs. The proliferation activity of BMSCs increased with the increase of magnetic field intensity at 0-80Hz and reached the peak value at 80 Hz. When the intensity reached 100 Hz, the proliferation activity of BMSC was significantly inhibited. Data is expressed as mean ± standard deviation and the experiments were performed in quadruplicate (n=4). *P<0.05 vs. controls at the same time point; #P<0.01 vs. controls at the same time point, controls were anterior frequency gradient at the same time point-. LPEMF, low-frequency-pulsed electromagnetic field; BMSC, bone marrow-derived mesenchymal stem cell.
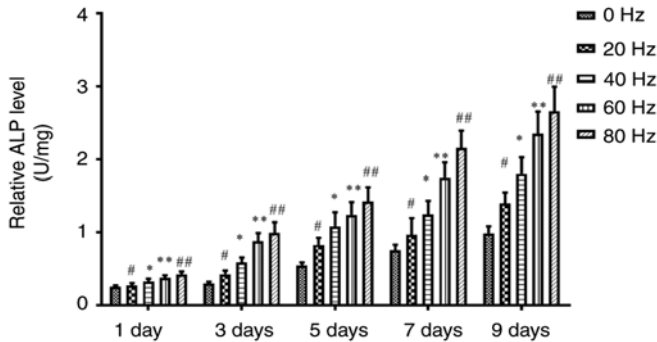

Figure 2. Effect of different frequency LPEMF treatment on the ALP activities of BMSCs. ALP activities were measured by conventional methods and the data represent the mean ± standard deviation of quadruplicate samples (n=4). #P<0.05 vs. the 0 Hz group; *P<0.01 vs. the 20 Hz group; **P<0.01 vs. the 40 Hz group; ##P<0.05 vs. the 60 Hz group. LPEMF, low-frequency-pulsed electromagnetic field; ALP, alkaline phosphatase; BMSC, bone marrow-derived mesenchymal stem cell.

the transcription levels of osteogenic marker genes on day 7 (Fig. 3A) and 14 (Fig. 3B) were enhanced with an increase in the LPEMF frequency and there was a significant difference between the groups with different frequencies (P<0.05). After cells were treated with LPEMF at the same frequency, the transcription levels of osteogenic marker genes on day 14 were higher than those on day 7 (P<0.05).

Effect of LPEMF treatment on osteogenic mineralization of BMSCs. The effect of LPEMF treatment on osteogenic mineralization of BMSCs was investigated. BMSCs from each group were terminated after being cultured under osteogenic inductive conditions for 14 days after treatment with LPEMF at different frequencies and mineralization was assessed using Alizarin Red S staining. The gross specimens are shown (Fig. 4A). The formation of calcium nodules was lowest in the control group and numerous calcium nodules were observed in the other groups. The amount of calcium nodule deposition

increased with an increase in LPEMF frequency. Alizarin Red S quantitative results showed that the number of calcium nodules significantly increased with an increase in frequency in each group (P<0.05; Fig. 4B).

Effect of LPEMF treatment on Cx43 expression during osteogenic differentiation of BMSCs. To detect the expression of Cx43 following LPEMF treatment during the process of osteogenic differentiation of BMSCs, Cx43 expression levels in BMSCs treated with LPEMF at 0 and 80 Hz for 14 days after osteogenic induction culture were determined using western blotting. The results showed that Cx43 expression was significantly enhanced in the 80 Hz LPEMF-treated group compared with that of the 0 Hz LPEMF-treated group (P<0.05; Fig. 5).

Inhibition of Cx43 expression attenuates the role of LPEMF in promoting osteogenic differentiation of BMSCs. To verify the role of Cx43 in promoting the osteogenic differentiation of BMSCs by LPEMF, BMSCs were transfected with a CX43-specific lentivirus vector to inhibit the expression of Cx43. Transfection efficiency was detected using western blotting and the results showed that Cx43 expression was significantly inhibited in BMSCs after transfection with the Cx43 specific lentivirus vector. Following Cx43 inhibition, BMSCs were treated with an 80 Hz LPEMF. The indicators of osteogenic differentiation of BMSCs were significantly lower than those of Cx43 without inhibition (P<0.05; Fig. 6).

Discussion

The present study established an *in vitro* osteogenic differentiation model for LPEMF-induced BMSCs. It showed that the proliferation, osteogenic differentiation and mineralization of BMSCs were enhanced in a magnetic field intensity-dependent manner by LPEMF intervention.

BMSCs are the main cell source of osteoblasts in fracture healing. They are adult stem cells with self-renewal, proliferation and multidirectional differentiation potential and can differentiate into osteoblasts, adipocytes, chondrocytes, nerve cells and myoblasts (22,23). The osteogenic differentiation of BMSCs is a continuous and dynamic process. Generally, osteogenic differentiation of BMSCs is divided into three stages: Osteoblast precursor cells, immature osteoblasts and osteoblasts. Massive deposition of mineralized matrix marks the formation of osteoblasts and the main component of the mineralized matrix is calcium salt (24). Therefore, the present study investigated the mechanism of LPEMF healing from a cytological perspective and used BMSCs to study the process of LPEMF-promoting BMSC osteogenic differentiation from multiple aspects and at different stages. LPEMF has been used to promote fracture healing for more than half a century and Wang *et al* (25) showed that it plays a positive role in promoting bone regeneration. However, the mechanism through which LPEMF promotes bone regeneration remains to be elucidated. Currently, there are theories, such as 'non-thermal effect' (26), 'window effect' hypothesis (27), local blood flow increase theory, calcium ion deposition theory (28), mediating mechanism of second messenger molecule (29) and endocrinoid effect of pulsed electromagnetic field (30).

Figure 3. Expression of osteogenic gene expression was measured using RT-qPCR after (A) 7 and (B) 14 days of osteogenic induction and different frequency LPEMF treatment. Expression of *Runx2*, *BSP*, *Bglap* and Osx was measured using real-time RT-qPCR after 7 and 14 days of induction, respectively. LPEMF, low-frequency-pulsed electromagnetic field. Data are expressed as the mean ± standard deviation of the values in 0 Hz. Values represent analysis from three separate experiments. *P<0.05 vs. the 0 Hz group; #P<0.05 vs. the 20 Hz group; **P<0.05 vs. the 40 Hz group; ##P<0.05 vs. the 60 Hz group. RT-qPCR, reverse transcription-quantitative PCR; LPEMF, low-frequency-pulsed electromagnetic field.

ALP is an enzyme secreted by BMSCs during the early stages of osteogenic differentiation and its activity reaches its peak before the mineralization stage. Through the hydrolysis of pyrophosphate, ALP provides phosphate ions and hydroxyl radicals for the deposition of hydroxyapatite crystals, thus forming a mineralized bone matrix (31). The proliferation of
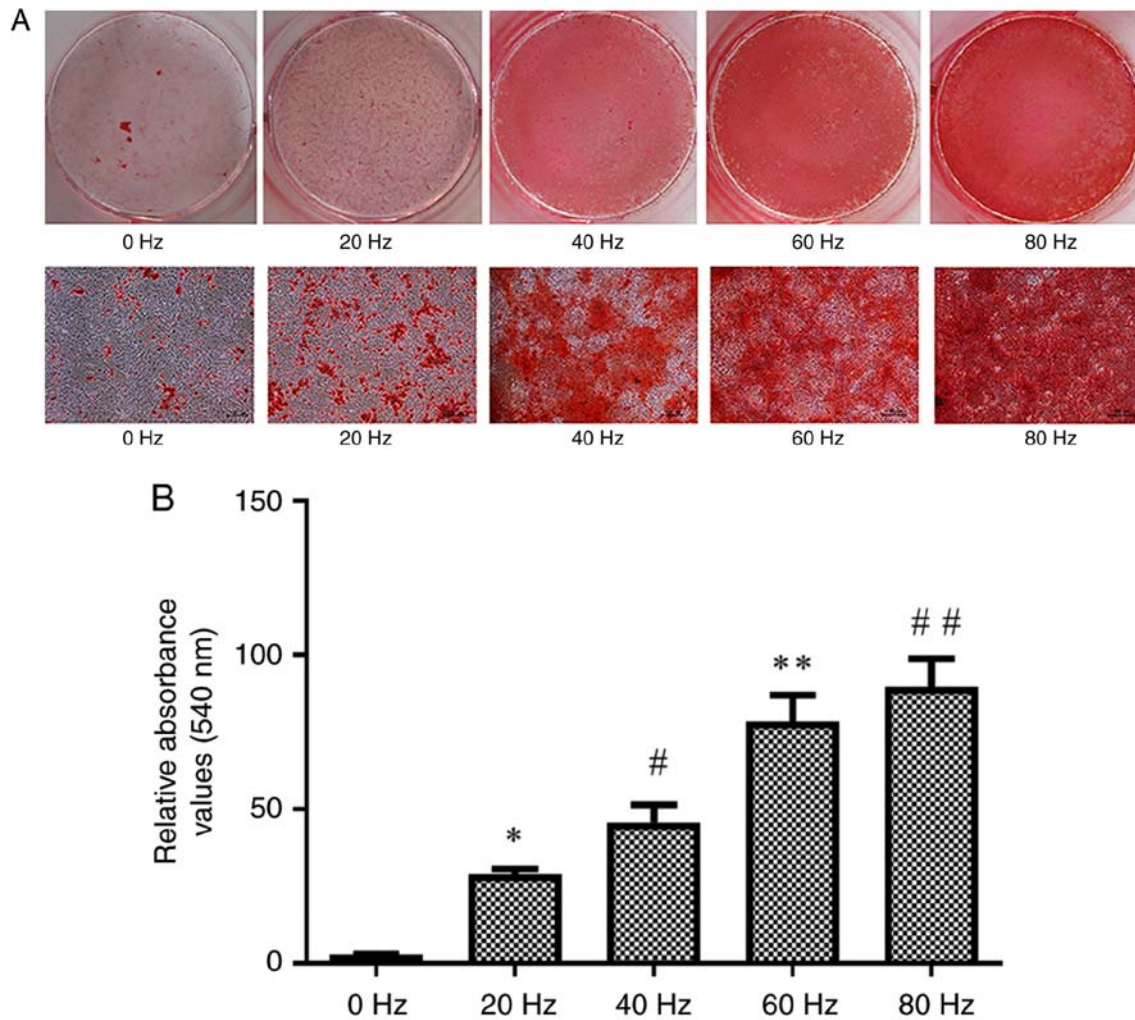
Figure 4. Mineralization level as assessed by alizarin red S staining after 14 days of differentiation. (A) Alizarin red S staining with (B) quantitative results of calcium deposition after 14 days of osteogenic induction and different frequency LPEMF treatment. Data represent the mean ± standard deviation of five independent experiments (n=5). *P<0.05 vs. the 0 Hz group; #P<0.05 vs. the 20 Hz group; **P<0.05 vs. the 40 Hz group; ##P<0.05 vs. the 60 Hz group. LPEMF, low-frequency-pulsed electromagnetic field.
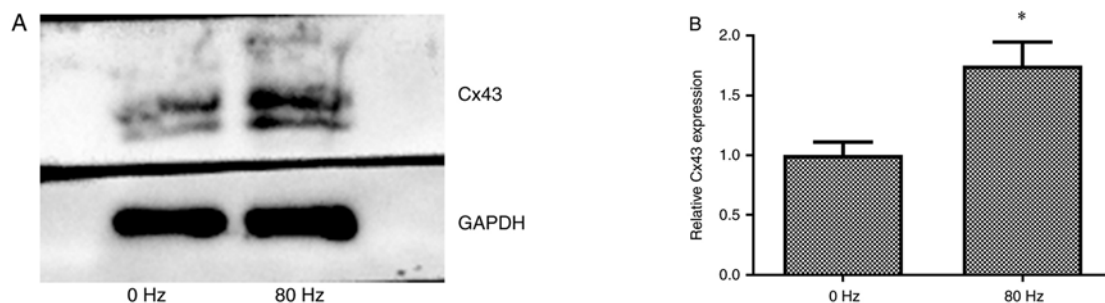
Figure 5. Cx43 expression after 14 days of osteogenic induction and 80 Hz LPEMF treatment. (A and B) Cx43 expression after 14 days of osteogenic induction and 80 Hz LPEMF treatment. Data represent the mean ± standard deviation of five independent experiments (n=5). *P<0.05 vs. the 0 Hz group. Cx43, connexin 43; LPEMF, low-frequency-pulsed electromagnetic field.

osteoblasts isolated from ALP knockout mice was normal, but there was no normal mineral salt deposition following culture. The quantitative measurement of ALP activity is often used as an indicator of changes in the strength of early osteogenic differentiation (20). Runx2 activation controls the initiation of the osteogenic differentiation switch in BMSCs and promotes osteoblast maturation (32). Runx2 gene defects cause BMSCs to

have no osteogenic differentiation ability and endometrial and endochondral osteogenic functions are lost in Runx2 knockout mice, suggesting osteoblast maturation disorder (33). Runx2 binds to osteoblastic heterozygous elements to regulate the expression of osteoblastic extracellular matrix protein genes Bglap, osteopontin, BSP and Col1α1 and participate in the synthesis of extracellular matrix (34). Osx is a key gene that
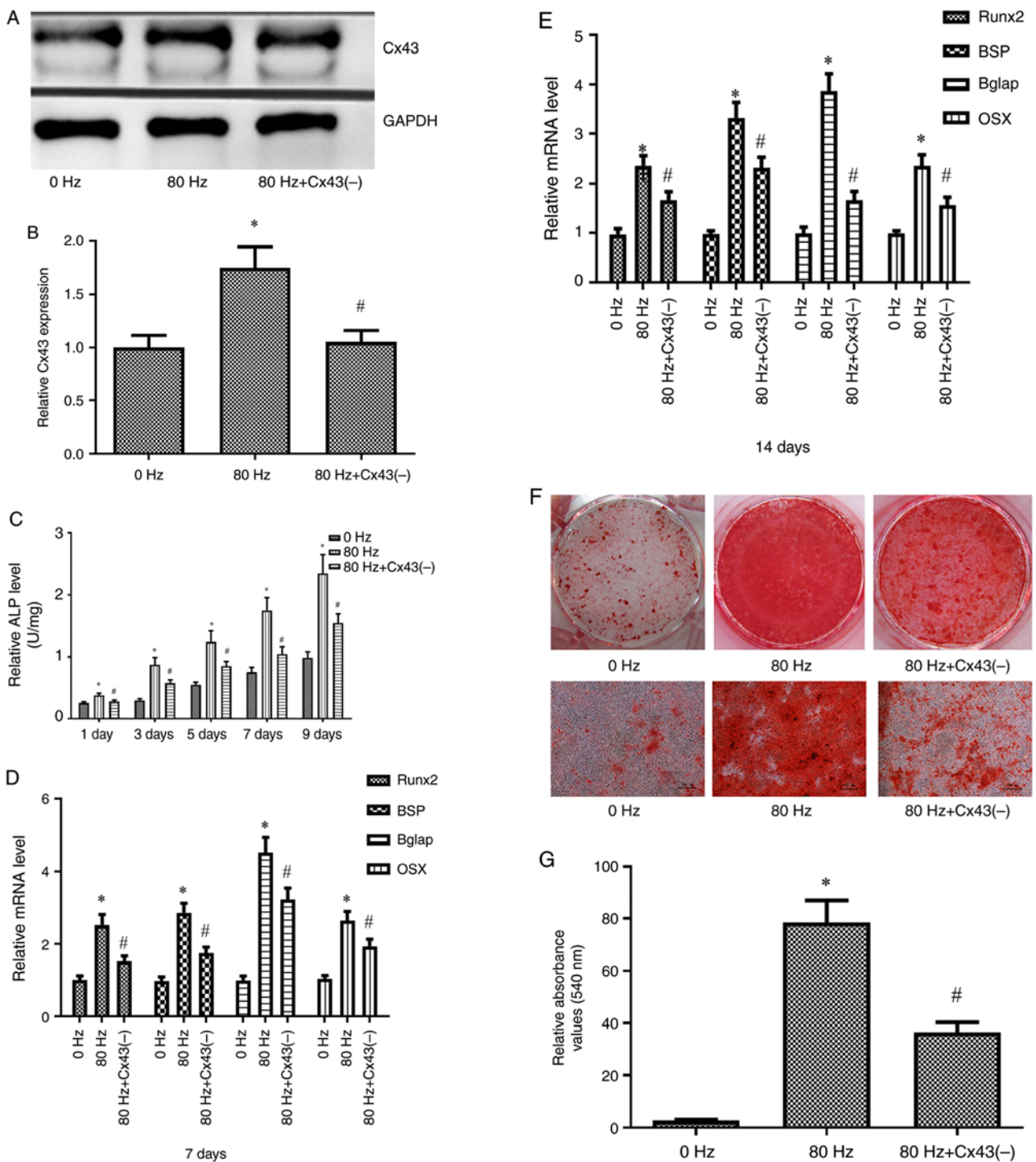
Figure 6. Osteogenic differentiation level of BMSCs while inhibiting Cx43 expression. (A and B) Cx43 expression after 14 days of osteogenic induction, 80 Hz LPEMF treatment and Cx43 inhibition. (C) Effect of different LPEMF treatment frequencies on the ALP activities of BMSC expression of osteogenic gene expression was measured using reverse transcription-quantitative PCR after (D) 7 and (E) 14 days of osteogenic induction. (F) Alizarin red S staining and (G) quantitative results of calcium deposition after 14 days of osteogenic induction and different LPEMF treatment frequencies. Data represent the mean ± standard deviation of four independent experiments (n=4). *P<0.05 vs. the 0 Hz group; #P<0.05 vs. the 80 Hz group. Cx43, connexin 43; LPEMF, low-frequency-pulsed electromagnetic field; ALP, alkaline phosphatase; BMSC, bone marrow-derived mesenchymal stem cell.

controls BMSCs during osteoblast differentiation. Osx was first discovered during the process of osteogenic differentiation induced by BMP-2 in C2C12 myoblasts and regulates the expression of Bglap, osteopontin, bone salivariin, type I collagen and other genes (35). BSP and Bglap are secreted by mature osteoblasts, which together with osteopontin and type I collagen participate in the synthesis of osteoids and deposition of calcium

salts and other minerals (36). The increased secretion of BSP and osteocalcin signals the transformation of immature osteoblasts into mature osteoblasts and can be used as indicators of middle and late osteogenic differentiation of BMSCs (36-38). Alizarin red S forms a complex with calcium ion chelation and is an orange-red deposition. Alizarin red S staining is commonly used to detect the maturity of BMSCs during the late stage of

osteogenic differentiation (39). In the present study, ALP activity and Runx2, Osx, BSP and Bglap were used as markers of osteogenic differentiation of BMSCs. Alizarin Red S staining was used to detect calcium nodules and reflect the level of osteogenic differentiation in BMSCs. The results of the present study showed that LPEMF stimulated the proliferation of BMSCs in a frequency-dependent manner in the range of 0-80 Hz, whereas at 100 Hz, LPEMF inhibited the growth of BMSCs, presenting a toxic side effect. Therefore, it can be concluded that a magnetic field with a frequency <80 Hz is safe for stimulating BMSCs. Following LPEMF treatment, the activity of ALP and expression of the osteogenic marker genes Runx2, BSP, Bglap and Osx notably increased during BMSCs osteogenic differentiation. The mineralization level of BMSCs was also notably increased, suggesting that LPEMFs can promote bone formation.

Cx43 is a connexin encoded by Gja1 that is widely expressed in bone tissue cells (40). Cx43 is an essential gene for the survival, proliferation, differentiation and other physiological processes of osteoblasts and interference with Cx43 expression can significantly inhibit the differentiation of osteoblasts (40-42). In a three-dimensional osteogenic induction culture of BMSCs, the overexpression of Cx43 increased the number and spatial distribution of GJIC and enhanced the transcription level of osteogenic marker genes (43). Osteoblast differentiation of BMSCs is regulated by various signaling pathways. The present study hypothesized that LPEMF may promote BMSC osteoblast differentiation by regulating Cx43 gene expression. The results showed that Cx43 expression was significantly increased in BMSCs treated with 80 Hz LPEMF for 14 days after osteogenic induction. According to the present study, LPEMF may promote osteogenic differentiation of BMSCs by upregulating ALP activity, expression of osteogenic marker genes and calcium salt formation and Cx43 upregulation may be involved in LPEMF promoting the osteogenic differentiation of BMSCs; however, the specific mechanism is still not completely clear and needs to be further verified by more cell and animal experiments. In tissue engineering, BMSCs have the characteristic of directional differentiation into osteoblasts and are often used as the seed cells of bone cells. Using a pulsed electromagnetic field to stimulate osteogenesis has the advantages of being non-invasive, having no complications and suitable for popularization and application. The application of LPEMF to promote the osteogenic differentiation of BMSCs will provide a new treatment for clinical bone nonunion and bone defects.

In conclusion, the present study concluded that when the LPEMF frequency was <80 Hz, the proliferation, osteogenic differentiation and mineralization of BMSCs were enhanced in a magnetic field intensity-dependent manner. LPEMF enhanced the osteogenic differentiation of BMSCs by regulating ALP activity and osteogenic marker gene expression. The upregulation of Cx43 expression may be involved in the process by which LPEMF promotes the osteogenic differentiation of BMSCs. This provided an experimental basis for the treatment of non-unions and osteoporosis caused by bone defects. These findings suggested that LPEMF may be an effective strategy for the treatment of osteoblast-related diseases.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FL, HG and ZLu conceived the study. FL performed the experiments. ZLu, HG and ZLi analyzed the data. FL wrote the manuscript. FL, HG and ZLu confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ganzhou People's Hospital Animal Care and Use Committee (Ganzhou, China; approval no. 20211210038).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. El-Rashidy AA, Roether JA, Harhaus L, Kneser U and Boccaccini AR: Regenerating bone with bioactive glass scaffolds: A review of in vivo studies in bone defect models. Acta Biomater 62: 1-28, 2017.
2. Schindeler A, McDonald MM, Bokko P and Little DG: Bone remodeling during fracture repair: The cellular picture. Semin Cell Dev Biol 19: 459-466, 2008.
3. Jing D, Li F, Jiang M, Cai J, Wu Y, Xie K, Wu X, Tang C, Liu J, Guo W, *et al*: Pulsed electromagnetic fields improve bone microstructure and strength in ovariectomized rats through a Wnt/Lrp5/β-catenin signaling-associated mechanism. PLoS One 8: e79377, 2013.
4. Hannemann PFW, Mommers EHH, Schots JPM, Brink PRG and Poeze M: The effects of low-intensity pulsed ultrasound and pulsed electromagnetic fields bone growth stimulation in acute fractures: A systematic review and meta-analysis of randomized controlled trials. Arch Orthop Trauma Surg 134: 1093-1106, 2014.
5. Chang K, Chang WHS, Tsai MT and Shih C: Pulsed electromagnetic fields accelerate apoptotic rate in osteoclasts. Connect Tissue Res 47: 222-228, 2006.
6. He Z, Selvamurugan N, Warshaw J and Partridge NC: Pulsed electromagnetic fields inhibit human osteoclast formation and gene expression via osteoblasts. Bone 106: 194-203, 2018.
7. Huo SC and Yue B: Approaches to promoting bone marrow mesenchymal stem cell osteogenesis on orthopedic implant surface. World J Stem Cells 12: 545-561, 2020.

8. Liang J, Li W, Zhuang N, Wen S, Huang S, Lu W, Zhou Y, Liao G, Zhang B and Liu C: Experimental study on bone defect repair by BMSCs combined with a light-sensitive material: g-C$_3$N$_4$/rGO. J Biomater Sci Polym Ed 32: 248-265, 2021.
9. Donahue HJ: Gap junctions and biophysical regulation of bone cell differentiation. Bone 26: 417-422, 2000.
10. Batra N, Kar R and Jiang JX: Gap junctions and hemichannels in signal transmission, function and development of bone. Biochim Biophys Acta 1818: 1909-1918, 2012.
11. Unger VM, Kumar NM, Gilula NB and Yeager M: Three-dimensional structure of a recombinant gap junction membrane channel. Science 283: 1176-1180, 1999.
12. Saez JC, Berthoud VM, Branes MC, Martinez AD and Beyer EC: Plasma membrane channels formed by connexins: Their regulation and functions. Physiol Rev 83: 1359-1400, 2003.
13. Liao Y, Day KH, Damon DN and Duling BR: Endothelial cell-specific knockout of connexin 43 causes hypotension and bradycardia in mice. Proc Natl Acad Sci USA 98: 9989-9994, 2001.
14. Plotkin LI: Connexin 43 hemichannels and intracellular signaling in bone cells. Front Physiol 5: 131, 2014.
15. Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH and Civitelli R: Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol 151: 931-944, 2000.
16. Tonon R and D'Andrea P: The functional expression of connexin 43 in articular chondrocytes is increased by interleukin 1beta: Evidence for a Ca2+-dependent mechanism. Biorheology 39: 153-160, 2002.
17. Ransjö M, Sahli J and Lie A: Expression of connexin 43 mRNA in microisolated murine osteoclasts and regulation of bone resorption in vitro by gap junction inhibitors. Biochem Biophys Res Commun 303: 1179-1185, 2003.
18. Loiselle AE, Paul EM, Lewis GS and Donahue HJ: Osteoblast and osteocyte-specific loss of Connexin43 results in delayed bone formation and healing during murine fracture healing. J Orthop Res 31: 147-154, 2013.
19. Meirelles Lda S and Nardi NB: Murine marrow-derived mesenchymal stem cell: Isolation, in vitro expansion, and characterization. Br J Haematol 123: 702-711, 2003.
20. Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH and Millán JL: Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15: 1879-1888, 2000.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
22. Phinney DG, Kopen G, Isaacson RL and Prockop DJ: Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: Variations in yield, growth, and differentiation. J Cell Biochem 72: 570-585, 1999.
23. Gurusamy N, Alsayari A, Rajasingh S and Rajasingh J: Adult stem cells for regenerative therapy. Prog Mol Biol Transl Sci 160: 1-22, 2018.
24. Muruganandan S and Sinal CJ: The impact of bone marrow adipocytes on osteoblast and osteoclast differentiation. IUBMB Life 66: 147-155, 2014.
25. Wang T, Yang L, Jiang J, Liu Y, Fan Z, Zhong C and He C: Pulsed electromagnetic fields: Promising treatment for osteoporosis. Osteoporos Int 30: 267-276, 2019.
26. Weber C, Thai V, Neuheuser K, Groover K and Christ O: Efficacy of physical therapy for the treatment of lateral epicondylitis: A meta-analysis. BMC Musculoskelet Disord 16: 223, 2015.
27. Ibiwoye MO, Powell KA, Grabiner MD, Patterson TE, Sakai Y, Zborowski M, Wolfman A and Midura RJ: Bone mass is preserved in a critical-sized osteotomy by low energy pulsed electromagnetic fields as quantitated by in vivo micro-computed tomography. J Orthop Res 22: 1086-1093, 2004.
28. Yildiz M, Cicek E, Cerci SS, Cerci C, Oral B and Koyu A: Influence of electromagnetic fields and protective effect of CAPE on bone mineral density in rats. Arch Med Res 37: 818-821, 2006.
29. Lohmann CH, Schwartz Z, Liu Y, Li Z, Simon BJ, Sylvia VL, Dean DD, Bonewald LF, Donahue HJ and Boyan BD: Pulsed electromagnetic fields affect phenotype and connexin 43 protein expression in MLO-Y4 osteocyte-like cells and ROS 17/2.8 osteoblast-like cells. J Orthop Res 21: 326-334, 2003.
30. Nelson FRT, Brighton CT, Ryaby J, Simon BJ, Nielson JH, Lorich DG, Bolander M and Seelig J: Use of physical forces in bone healing. J Am Acad Orthop Surg 11: 344-354, 2003.
31. Coleman JE: Structure and mechanism of alkaline phosphatase. Annu Rev Biophys Biomol Struct 21: 441-483, 1992.
32. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, et al: Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765-771, 1997.
33. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, et al: Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89: 755-764, 1997.
34. Franceschi RT and Xiao G: Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. J Cell Biochem 88: 446-454, 2003.
35. Zou L, Zou X, Li H, Mygind T, Zeng Y, Lü N and Bünger C: Molecular mechanism of osteochondroprogenitor fate determination during bone formation. Adv Exp Med Biol 585: 431-441, 2006.
36. Gordon JA, Tye CE, Sampaio AV, Underhill TM, Hunter GK and Goldberg HA: Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization in vitro. Bone 41: 462-473, 2007.
37. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR and de Crombrugghe B: The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108: 17-29, 2002.
38. Oldknow KJ, Macrae VE and Farquharson C: Endocrine role of bone: Recent and emerging perspectives beyond osteocalcin. J Endocrinol 225: R1-R19, 2015.
39. Gregory CA, Gunn WG, Peister A and Prockop DJ: An Alizarin red-based assay of mineralization by adherent cells in culture: Comparison with cetylpyridinium chloride extraction. Anal Biochem 329: 77-84, 2004.
40. Gramsch B, Gabriel HD, Wiemann M, Grümmer R, Winterhager E, Bingmann D and Schirrmacher K: Enhancement of connexin 43 expression increases proliferation and differentiation of an osteoblast-like cell line. Exp Cell Res 264: 397-407, 2001.
41. Merrifield PA and Laird DW: Connexins in skeletal muscle development and disease. Semin Cell Dev Biol 50: 67-73, 2016.
42. Plotkin LI, Laird DW and Amedee J: Role of connexins and pannexins during ontogeny, regeneration, and pathologies of bone. BMC Cell Biol 17 (Suppl 1): S19, 2016.
43. Rosselló RA, Wang Z, Kizana E, Krebsbach PH and Kohn DH: Connexin 43 as a signaling platform for increasing the volume and spatial distribution of regenerated tissue. Proc Natl Acad Sci USA 106: 13219-13224, 2009.