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Daily low-intensity pulsed ultrasound-mediated osteogenic differentiation in rat osteoblasts

Akito Suzuki[1,2], Tadahiro Takayama[1]*, Naoto Suzuki[3,4], Michitomo Sato[1], Takeshi Fukuda[1], and Koichi Ito[1,5]

[1]Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan
[2]Division of Applied Oral Sciences, Nihon University Graduate School of Dentistry, Tokyo, Japan
[3]Department of Biochemistry, Nihon University School of Dentistry, Tokyo, Japan
[4]Division of Functional Morphology, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan
[5]Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan
*Correspondence address. Tel: +81-3-32198107; Fax: +81-3-32198349; E-mail: takayama@dent.nihon-u.ac.jp

There were few studies investigating the effects of the mechanical stimulation provided by daily low-intensity pulsed ultrasound (LIPUS) treatment. LIPUS is known to accelerate bone mineralization and regeneration; however, the precise cellular mechanism is unclear. Our purpose was to determine how daily LIPUS treatment affected cell viability, alkaline phosphatase activity, osteogenesis-related gene expression, and mineralized nodule formation in osteoblasts. The typical osteoblastic cell line ROS 17/2.8 cells were cultured in the absence or presence of LIPUS stimulation. Daily LIPUS treatments (1.5 MHz; 20 min) were administered at an intensity of 30 mW/cm^2 for 14 days. Expression of osteogenesis-related genes was examined at mRNA levels using real-time polymerase chain reaction and at protein levels using western blotting analysis. LIPUS stimulation did not affect the rate of cell viability. Alkaline phosphatase activity was increased after 10 days of culture with daily LIPUS stimulation. LIPUS significantly increased the expression of mRNAs encoding Runx2, Msx2, Dlx5, osterix, bone sialoprotein, and bone morphogenetic protein-2, whereas it significantly reduced the expression of mRNA encoding the transcription factor AJ18. Mineralized nodule formation was markedly increased on Day 14 of LIPUS stimulation. LIPUS stimulation directly affected osteogenic cells, leading to mineralized nodule formation. LIPUS is likely to have a fundamental influence on key functional activities of osteoblasts in alveolar bone.

Keywords low-intensity pulsed ultrasound; osteogenesis; osteoblasts; nodule formation

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Introduction

Low-intensity pulsed ultrasound (LIPUS) stimulates fracture healing in animal models [1,2] and in clinical settings [3,4], and has also been reported to accelerate bone maturation in distraction osteogenesis [5,6]. Of all the means to influence bone repair, LIPUS is distinguished by being non-invasive and easy to apply, and the LIPUS signal is of low enough intensity to be considered neither thermal nor destructive. Previous reports have suggested that LIPUS is beneficial for treating non-union of the extremities in humans [7]. Moreover, when applied clinically to treat delayed union and non-union, it stimulates union in a relatively short period of time [8]. However, the underlying mechanism responsible for the pronounced effects of LIPUS on osteogenesis remains unclear.

Bone fracture healing and distraction osteogenesis are complex biological processes that involve the spatial and temporal orchestration of various cell types, many genes, and the extracellular matrix [9]. The process of fracture healing includes cell viability and differentiation, chemotaxis, and synthesis of extracellular matrix. In previous *in vitro* studies, we found that transient LIPUS stimulation converts the differentiation pathway of pluripotent mesenchymal cells into the osteoblast and chondroblast lineages [10], and that it directly affects osteogenic cells, leading to mineralized nodule formation [11]. Moreover, LIPUS treatment elevated Runx2 mRNA expression and progressively promoted osteocalcin mRNA expression in human osteoblasts [12]. In mouse bone marrow-derived stromal cells, Naruse *et al.* [13] found that LIPUS elevated mRNA levels for insulin-like growth factor-I,

osteocalcin, and bone sialoprotein (BSP), which is consistent with a bone-forming response. LIPUS has also been shown to stimulate mRNA expression of osteocalcin and another bone matrix protein, alkaline phosphatase (ALPase), in UMR-106 cells [14]. Although the above-described studies examined the effects of transient LIPUS stimulation on the expression of osteogenesis- and chondrogenesis-related transcription factors in several different cell types, few studies have investigated osteogenesis-related gene and protein expression in detail.

The hypothesis of the present study was that daily LIPUS treatment would accelerate the process of bone mineralization during bone healing. To evaluate this hypothesis, we investigated the effects of daily LIPUS treatment on cell viability, ALPase activity, osteogenesis-related gene expression, and mineralized nodule formation in a rat osteosarcoma cell line.

Materials and Methods

Cell culture

Experiments were performed using the rat clonal cell line ROS 17/2.8 (ROS cells) [15]. Although ROS cells originated from rat osteosarcoma, these cells have been treated in numerous studies as typical rat osteoblasts or osteoblast-like cells [16–18]. The cells were maintained in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, USA) and 1% (v/v) penicillin–streptomycin solution (Sigma Chemical, St Louis, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Ultrasound treatment

ROS cells at fifth or sixth passage were seeded into 6- and 96-well plates at a density of 2.0×10^4 cells/cm^2. The cells were subjected to LIPUS with modifications, as reported previously [19,20]. Briefly, a sterilized transducer (Asahi Irika Co, Ltd, Saitama, Japan) generating 1.5-MHz LIPUS in a pulsed-wave mode at 30 mW/cm^2 was placed horizontally into each culture well so that it was barely touching the surface of the medium. The distance between the transducer and cells was 3–4 mm (**Fig. 1**). LIPUS stimulation was performed for 20 min daily for up to 14 days, when the cells reached confluence. The culture medium was replaced with fresh medium once every 3 days. Control samples were treated in the same manner, without exposure to LIPUS.

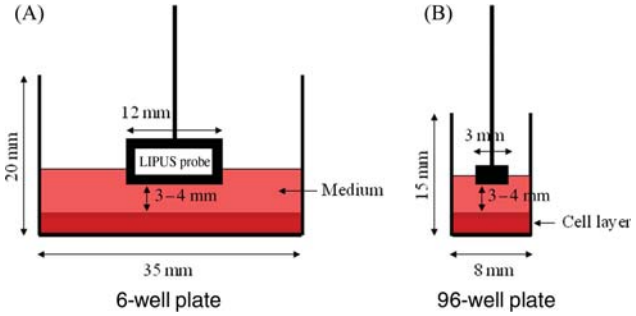

Fig. 1 Diagrammatic representation of LIPUS application The LIPUS transducer probe for 6-well plate (**A**) and 96-well plate (**B**) were suspended above the culture medium (using a clamp stand) so that the probe was about 3–4 mm above the cell layer, partially immersed in the culture medium.

Determination of cell viability

ROS cells were seeded in 96-well microplates at a density of 2.0×10^4 cells/cm^2 and cultured in the presence and absence of daily LIPUS stimulation for up to 14 days. At various time points, the medium was replaced with fresh medium containing 10% (v/v) colorimetric cell-counting reagent (Wako Fine Chemicals, Osaka, Japan), and incubation was continued for 1 h. The intensity of the colored reaction product was measured at 450 nm with a microtiter plate reader (Titertek Multiskan Plus; Flow Laboratories, McLean, USA). The relative cell concentrations were calculated from the relative absorbance values based on a standard curve.

Determination of ALPase activity

ROS cells were seeded in 96-well microplates at a density of 2.0×10^4 cells/cm^2 and cultured in the presence and absence of daily LIPUS stimulation for up to 14 days. A 200 μl aliquot of ALPase enzyme reaction solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ in 0.1 M glycine–NaOH buffer, pH 10.5) was added to each well, and the plates were incubated for 20 min at 37°C. The enzyme reaction was terminated by the addition of 50 μl of 0.5 M NaOH. The amount of *p*-nitrophenol released by ALPase was determined by measuring the absorbance at 405 nm using a microtiter plate reader. One unit of ALPase activity was defined as the amount of enzyme required to liberate 1.0 μmol of *p*-nitrophenol per minute. Enzyme activity was calculated as mU/10^4 cells.

Real-time polymerase chain reaction

ROS cells were plated in 6-well microplates at a density of 2.0×10^4 cells/cm^2 and cultured for up to 14 days in

the presence and absence of daily LIPUS stimulation. At Days 0, 1, 3, 5, 7, 10, and 14 of LIPUS exposure, total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Aliquots containing equal amounts of mRNA were subjected to real-time polymerase chain reaction (PCR) analysis.

The amount of RNA was normalized using a human β-actin competitive PCR kit (TaKaRa Shuzo, Shiga, Japan), and the mRNA was converted into cDNA using an RNA PCR kit (GeneAmp; Perkin-Elmer, Branchburg, USA). The resultant cDNA mixtures were diluted 5-fold in sterile distilled water, and 2 ml was subjected to real-time PCR using SYBR Green I dye (BioWhittaker Molecular Applications, Rockland, USA). The reactions were performed in a 25 μl volume of solution containing 1 × R-PCR buffer, 1.5 mM dNTP mixture, 1 × SYBR Green I, 15 mM $MgCl_2$, 0.25 U Ex Taq R-PCR version (TaKaRa Shuzo), and 20 mM primers (sense and antisense; **Table 1**). The primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, USA).

Assays were performed on a Smart Cycler (Cepheid, Sunnyvale, USA) and analyzed using Smart Cycler software (Version 1.2d). The PCR conditions were 95°C for 3 s and 40 cycles at 68°C for 20 s. Measurements were taken at the end of each 68°C annealing step. PCR product specificity was verified by melting curve analysis between 68°C and 94°C. All real-time PCR reactions were performed in triplicates, and the gene expression levels were normalized by dividing the calculated value for the mRNA samples by that for glyceraldehyde-3-phosphate dehydrogenase mRNA at each time point.

SDS–PAGE and Western blotting analysis

ROS cells were plated in 6-well microplates at a density of 2.0×10^4 cells/cm^2 and cultured for up to 14 days in the presence and absence of daily LIPUS stimulation. Aliquots of the conditioned medium and whole-cell extracts containing equal amounts of protein were analyzed by SDS–PAGE on a 5–20%-gradient cross-linked polyacrylamide gel at 150 V for 60 min. The discontinuous Tris–glycine buffer system of Laemmli [21] was used for electrophoresis.

The separated proteins were transferred onto a membrane using a semi-dry transfer unit and a continuous buffer system at 0.8 mA/cm^2 constant amperage for 60–90 min. After the transfer was complete, the transferred membrane was treated with 25% (v/v) blocking reagent at 4°C for 18 h. The membranes were probed with 1:500 dilution of antibodies against Runx2, BSP, and tubulin (all from Santa Cruz Biotechnology, Santa Cruz, USA) and against AJ18 (provided by Dr Jaro Sodek, University of Toronto, Toronto, Canada), followed by the addition of biotin-conjugated secondary antibodies (diluted 1:10,000) [22]. The membranes were then treated with horseradish peroxidase-conjugated streptavidin. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK) with exposure to X-ray film. As a control, the transfer membrane was also exposed to normal rabbit serum at concentrations adjusted to match those of the primary antibodies.

Determination of mineralized nodule formation

Cells were plated into 96-well tissue culture plates at a density of 2.0×10^4 cells/cm^2 and cultured for 14 days in α-MEM containing 50 mM β-glycerophosphate and

Table 1 Primers used in the real-time polymerase chain reaction experiments

Target gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	GenBank accession No.
Runx2	GCGTCAACACCATCATTCTG	CAGACCAGCAGCACTCCATC	NM_004348
Msx2	TCACCACGTCCCAGCTTCTAG	AGCTTTTCCAGTTCCGCCTCC	NM_013601
Dlx5	GCGCTCAACCCATACCAG	ACTCGGGACTCGGTTGTAGG	AB073716
Osterix	AGCTCTTCTGACTGCCTGCCTA	TGGGTGCGCTGATGTTTGCT	AY177399
AJ18	CCCCAAGGAAGTCACCAGT	CTTTCTATGGGATCGGTCTCTT	AF321874
BMP-2	CAACACCGTGCTCAGCTTCC	TTCCCACTCATTTCTGAAAGTTCC	NM_012512
BSP	AAAAATGCTCACCATCACTGC	AATTGCCACACTGACTTCCAC	NM_004967
GAPDH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG	NG_003018

50 μg/ml ascorbic acid, in the presence and absence of daily LIPUS stimulation. The culture medium was replaced by a fresh medium every 2 or 3 days. Cell condition and nodule formation were checked routinely by phase contrast microscopy (Nikon, Tokyo, Japan). The presence of mineralized nodules was determined by staining with alizarin red (Wako), as described by Willams and Rietschel [23].

Statistical analysis

All data are presented as the mean ± standard deviation (SD). The statistical significance was determined using Bonferroni's modification of Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Cell viability

When ROS cell viability in the presence and absence of daily LIPUS stimulation was measured, LIPUS stimulation for up to 14 days did not affect the rate of cell viability (**Fig. 2**).

ALPase activity

ALPase activity was measured for up to 14 days in culture (**Fig. 3**). Regardless of LIPUS stimulation, ALPase activity increased gradually through Day 7 of culture and decreased at Day 10. At Days 5, 7, 10, and 14 of culture, ALPase activity was significantly higher in the LIPUS-treated cells than that in the non-treated control cells.

Effect of LIPUS on expression of osteogenesis-related genes

The effect of LIPUS stimulation on the expression of mRNAs encoding Runx2 [**Fig. 4(A)**], Msx2 [**Fig. 4(B)**], Dlx5 [**Fig. 4(C)**], osterix [**Fig. 4(D)**], AJ18 [**Fig. 4(E)**], bone morphogenetic protein (BMP)-2 [**Fig. 4(F)**], and BSP [**Fig. 4(G)**] in ROS cells was determined by real-time PCR. Compared with control cells, LIPUS-treated cells expressed significantly more Runx2 and Msx2 mRNA on Days 5–14 of culture; the levels peaked at Day 5 and remained nearly the same through Day 14. The LIPUS-treated cells also expressed more Dlx5 and BMP-2 mRNA on Days 5–14, with peak expression at Day 7. LIPUS stimulation also markedly increased the expression of osterix and BSP mRNA on Days 10–14, and it slightly decreased expression of AJ18 mRNA on Days 3–14.

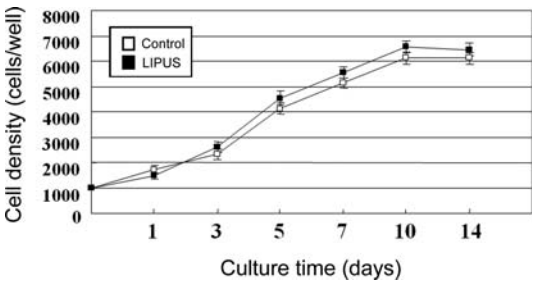

Fig. 2 Effect of LIPUS stimulation on cell viability ROS cells were cultured in the presence and absence of daily LIPUS stimulation and the cell numbers were determined at Days 1, 3, 5, 7, 10, and 14 of culture. The data are shown as the mean ± SD of three separate experiments.

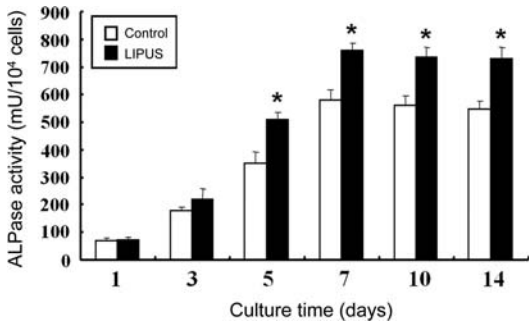

Fig. 3 Effect of LIPUS stimulation on ALPase activity The cells were cultured in the presence and absence of daily LIPUS stimulation and ALPase activity were determined at Days 1, 3, 5, 7, 10, and 14 of culture. The data are shown as the mean ± SD of three separate experiments. *$P < 0.05$ for LIPUS-treated versus control.

Effect of LIPUS on expression of osteogenesis-related proteins

The expression of Runx2 and AJ18 proteins in ROS whole-cell exacts and BSP protein in the conditioned medium of cultured ROS cells treated with LIPUS was examined using western blotting analysis. At Day 7 of LIPUS stimulation, the amount of Runx2 [**Fig. 5(A)**] was substantially increased and that of AJ18 [**Fig. 5(B)**] was markedly decreased. At Day 14, BSP expression [**Fig. 5(C)**] was significantly increased.

Mineralized nodule formation

Mineralized nodule formation was determined at Day 14 of culture in the presence and absence of daily LIPUS stimulation. Alizarin red staining of mineralized nodules was markedly more intense in ROS cells daily stimulated with LIPUS than in control cells (**Fig. 6**).

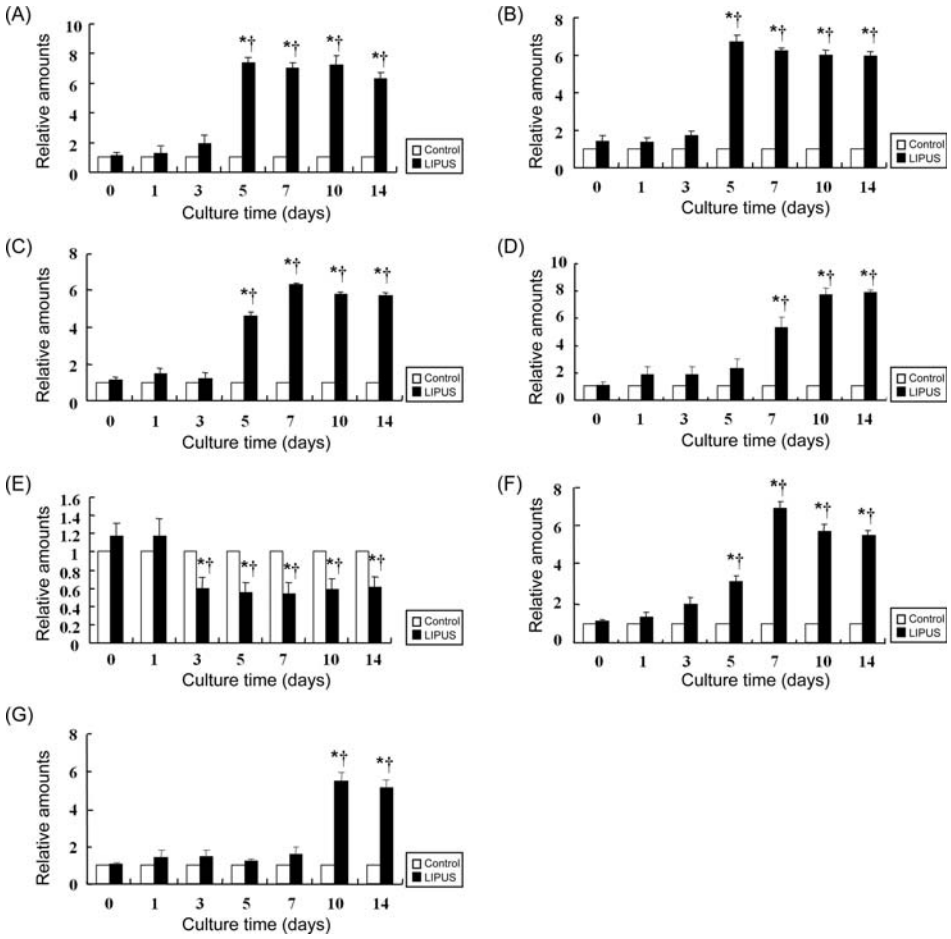

Fig. 4 Effect of LIPUS stimulation on the gene expression of tissue-specific differentiation markers The cells were cultured in the presence and absence of daily LIPUS stimulation. The expressions of the mRNA for (**A**) Runx2, (**B**) Msx2, (**C**) Dlx5, (**D**) osterix, (**E**) AJ18, (**F**) BMP-2, and (**G**) BSP were determined using real-time PCR. The data are shown as the mean ± SD of three separate experiments. $^{*}P < 0.05$ for LIPUS-treated versus control. $^{\dagger}P < 0.05$ for 1, 3, 5, 7, 10, and 14 days versus 0 day after LIPUS treatment.

Discussion

The central findings of this study are that daily LIPUS stimulates the temporal expression of continuous-response and osteogenesis-related genes in osteoblasts, and that this stimulation promotes specific actions related to osteogenesis in osteoblasts, leading to mineralized nodule formation.

ALPase, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in the calcification of bone. We found that ALPase activity in both the presence and the absence of daily LIPUS stimulation increased gradually through Day 7 of culture, when the deposition of hydroxyapatite crystals began, and slightly decreased at Day 10. However, the ALPase activity level at Days 5, 7, 10, and 14 was significantly higher in the LIPUS-treated cells than in the controls, suggesting that daily LIPUS stimulation may enhance mineralization by increasing ALPase activity.

To confirm the above findings, we performed real-time PCR analysis to assess the expression of osteogenesis-related marker mRNA. In cells cultured in the presence of LIPUS stimulation, we found that the levels of Runx2 and Msx2 mRNA were significantly higher at Days 5–14, peaking at Day 5 and remaining nearly constant through Day 14, providing evidence for the first time that Runx2 acts as a transcription factor in LIPUS-stimulated ROS cells. This conclusion is in agreement with the observations that Runx2 enhances early stage osteoblast differentiation and inhibits the late stage of osteoblast maturation [24,25]. Therefore, Runx2 appears to be a crucial transcriptional factor for osteoblast differentiation.

We also studied four other important osteogenesis-related transcription factors: Msx2, Dlx5, osterix, and

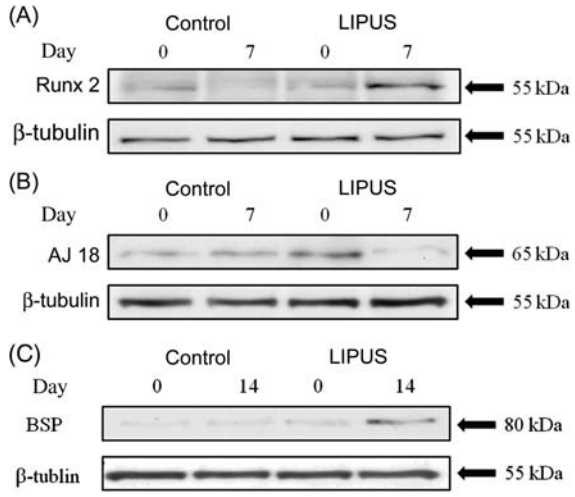

Fig. 5 Effect of LIPUS stimulation on expression levels of osteogenesis-related proteins The protein expression of Runx2 (**A**) and (**B**) AJ18 in ROS cells was determined by western blotting analysis of cells cultured at Day 7 in the presence and absence of daily LIPUS stimulation. The protein expression of BSP (**C**) in ROS cells was determined by western blotting analysis of cells cultured at Day 14 in the presence and absence of daily LIPUS stimulation.

AJ18. The homeobox proteins Dlx5 and Msx2 appear to regulate the development of mineralized tissues, including bone, cartilage, and dentin [26–30]. Dlx5 expression correlates with osteoblast differentiation and is at a maximum in the final stages of osteoblast differentiation *in vitro* when the extracellular matrix mineralizes, suggesting that Dlx5 may be involved in the differentiation of osteogenic cells [31]. In contrast, Msx2 is predominantly expressed by proliferating osteoblasts and pre-osteoblasts, but its expression decreases according to terminal osteoblast differentiation [26,30,31]. Msx2 inhibits the activity of Runx2 by binding to it, and Dlx5 interferes with the activity of Msx2; thus Dlx5 can indirectly activate Runx2 activity by alleviating the inhibitory effect of Msx2 [32].

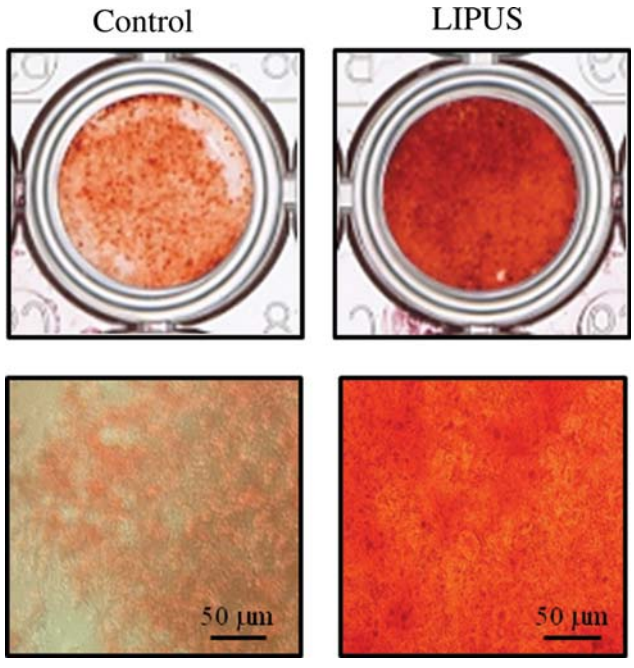

Fig. 6 Effect of LIPUS-treated conditioned medium on mineralized nodule formation Cells were cultured with or without daily LIPUS stimulation and the formation of mineralized nodules were determined by staining with alzarin red at day 14 of culture after daily LIPUS stimulation. Bar = 50 μm.

Recently, the zinc-finger-containing transcription factor osterix was also demonstrated to be critical for osteoblast differentiation and bone formation [33]. Osterix acts downstream of Runx2 to induce the differentiation of pre-osteoblasts into fully functional osteoblasts [33], and the over-expression of osterix has been shown to be sufficient to guide the differentiation of embryonic stem cells toward the osteoblastic lineage *in vitro* [34]. AJ18 was identified based on the differential display of genes that are up-regulated in cultured fetal rat calvarial cells by treatment with BMP-7 [35]. It binds to osteoblast-specific element 2 and modulates transactivation by Runx2 [35]. In addition, decreased expression of AJ18 is essential for increased osteoblastic differentiation [36].

In the present study, when ROS cells were cultured with daily LIPUS stimulation, Dlx5 mRNA expression was significantly elevated on Days 5–14, with a peak at Day 7, and osterix mRNA expression was markedly increased on Days 7–14. AJ18 mRNA expression, however, decreased slightly between Day 3 and Day 14 of culture with LIPUS stimulation. Our findings demonstrate that ROS cells respond to LIPUS treatment by increasing Runx2, Msx2, Dlx5, and osterix mRNA expression and decreasing AJ18 mRNA expression. Western blotting confirmed that LIPUS treatment increased expression of Runx2 protein and decreased AJ18 protein. Our results provide a molecular explanation for LIPUS-promoted osteogenesis of ROS cells and show that daily LIPUS stimulation promotes the differentiation of osteoblasts.

Our results are the first to show that daily LIPUS stimulation increases BMP-2 gene expression in ROS cells. Several studies have demonstrated that loading of mechanical stress onto bone causes osteoblasts to respond in various ways [37], such as by promoting BMP production [38], but no studies have previously investigated the effect of daily LIPUS on the expression of BMPs. We found that LIPUS treatment significantly

increased BMP-2 mRNA expression at Days 5–14, with a peak at Day 7. Unfortunately, the precise cellular mechanism by which this occurs is unclear. Therefore, further experiments are needed to clarify this phenomenon.

The major non-collagenous proteins of bone have been characterized. Because BSP is thought to play a specific role in bone mineralization and might be a useful marker of bone formation, we focused on the alteration of BSP expression by LIPUS [39]. The expression of BSP mRNA and protein was markedly higher in LIPUS-stimulated cells than in control cells, indicating that LIPUS stimulates mineralization by increasing the expression of BSP, which acts to create a nucleus for calcification.

We also examined the formation of mineralized nodules in the presence and absence of daily LIPUS stimulation. We found that the LIPUS stimulation significantly increased the formation of mineralized nodules and resulted in nodules with markedly more intense alizarin red staining. Considering these findings, we hypothesized that the conditioned medium from LIPUS-stimulated cells would increase calcium deposition in mineralized nodules produced by ROS cells. This result shows that LIPUS stimulation promotes the formation of bone by osteoblasts. In addition, BSP was expressed in the late stage of osteoblast differentiation. Thus, LIPUS may stimulate not only the differentiation but also the maturation of osteoblasts.

The results of this study indicated that daily LIPUS stimulation directly affected osteogenic cells, leading to mineralized nodule formation.

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