Original

Gene Expression in Response to Low-Intensity Pulsed Ultrasound Treatment of Bone Marrow Cells under Osteogenic Conditions In Vitro

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Abstract: This study was conducted to examine the gene-expression changes that might contribute to enhanced osteogenesis after low-intensity pulsed ultrasound (LIPUS) exposure. Bone marrow cells were obtained from the femora of rats and were suspended in an osteogenic medium to prepare cell cultures. After the cultures were established, test cultures were exposed to LIPUS through the base of the cell-culture plates for 15 min/day on Days 3-9 (LIPUS group). Control cultures were not exposed to LIPUS, but were otherwise treated identically to the LIPUS group. On Day 10, total RNA was extracted from both sets of cultures and hybridized to microarray slides, and the obtained datasets were analyzed. Real-time PCR was used to confirm the microarray analysis results. Cell-proliferation assays and Sirius Red staining were performed on Days 4, 7 and 10, and Alizarin Red S staining was performed on Days 10, 14 and 21. Markers for differentiated osteoblasts and osteocytes and genes encoding collagen-related molecules and cell-adhesion factors were upregulated in the LIPUS group on Day 10. Cell proliferation was lower in the LIPUS group than in the controls on Day 7. Sirius Red staining in the LIPUS group was significantly higher than in the controls on Day 10, and the cell areas stained with Alizarin Red S were significantly larger in the LIPUS group than in the controls on each day of the experiment. Thus, LIPUS exposure increased the gene expression of extracellular matrix factors and promoted the differentiation of osteoblast-like cells into osteocytes in an in vitro cell culture model.

Key words: Bone marrow cells, Enhanced osteogenesis, Gene expression, LIPUS, Microarray analysis, Mineralization

Introduction

In the field of orthopedics, low-intensity pulsed ultrasound (LIPUS) has been widely used to promote tissue healing. In early studies, Duarte and demonstrated that ultrasound treatment could stimulate bone growth. Thereafter, randomized clinical trials conducted by Heckman et al., Kristiansen et al. and Mayr et al. have demonstrated the efficacy of low-intensity ultrasound stimulation in accelerating the normal fracture-repair process. These reports provide evidence that LIPUS treatment might substantially reduce the time required for bone fracture healing.

The bone-repair process is also critical in the context of dental treatment procedures such as dental implant therapy. Dental implants can effectively replace missing teeth, although a healing period is required to allow osseointegration of the implants. This healing period for osseointegration has been shortened through the use of technologies of surface modification of fixtures, as well as modified clinical procedures such as immediate-loading protocols.

Recently, attention has been devoted to the use of LIPUS in dental implant therapy. Tanzer et al. used an in vivo animal model and demonstrated that LIPUS treatment markedly stimulated bone ingrowth into titanium porous implants. Fujii et al. reported that LIPUS treatment accelerated bone formation around titanium implants in dog mandibles. Li reported that in an in vivo model, LIPUS treatment accelerated bone-titanium integration at early stages of healing after titanium implantation. However, the biological mechanisms underlying the process remain unclear. In in vitro studies, Kokubu et al. demonstrated that LIPUS treatment increases prostaglandin E2 (PGE2) production in a mouse osteoblastic cell line (MC3T3-E1) through the induction of cyclooxygenase-2 (COX-2). Subsequently, Sena et al. demonstrated that LIPUS stimulates a transient increase in the expression of early response genes, including those encoding c-Jun, c-Myc, COX-2, Egr-1, and TSC-22, in bone marrow-derived osteoblastic cells. Kidokoro et al. reported that LIPUS enhanced the differentiation and mineralization of osteoblasts derived from rat bone marrow stromal cells.

Although LIPUS treatment has been widely demonstrated to
promote bone healing, few studies have addressed the molecular mechanisms involved, such as the underlying changes in gene expression. Therefore, in this study, we used cDNA microarray analysis to comprehensively examine gene-expression changes following LIPUS treatment of cultured bone marrow cells under bone-formation conditions. We also confirmed the stage of differentiation of the cell cultures by performing cell-proliferation, collagen-synthesis, and calcification assays.

**Materials and Methods**

The experimental protocol was approved by the Institutional Committee for Animal Care, Aichi Gakuin University (AGUD 151). The experimental design and analytical time points are shown in Fig. 1.

**Cell culture**

Bone marrow cells were obtained from the femora of 5 rats (8-week-old, male, Sprague-Dawley rats; Japan SLC, Japan), and were suspended in α-MEM (Life Technologies, USA) supplemented with 15% fetal bovine serum (Equitech-Bio, USA), 10 nM dexamethasone (Sigma, USA), 50 mg/mL L-ascorbic acid 2-phosphate (Sigma), 10 mM Na-β-glycerophosphate (Sigma), and an antibiotic-antimycotic solution (Life Technologies, USA). Cell suspensions (5 × 10⁶ cells/ml) were seeded into 12-well cell-culture plates (Corning, USA) and cultured at 37 °C in a 5 % CO₂ atmosphere, and the culture medium was changed once every 3 days.

**Application of LIPUS**

LIPUS exposure was performed for 15 min/day on Days 3-9 after the establishment of cell cultures (LIPUS group). LIPUS signals were transmitted at a frequency of 3 MHz, with a spatial-average intensity of 40 mW/cm² and 2-ms-burst sine waves at 100 kHz, using a clinical LIPUS device (BR-Sonic Pro, ITO, Japan). LIPUS was applied to cells through the bottom of the cell-culture plates via an ultrasound coupling gel (ITO ULTRASOUND GEL, ITO) applied to the LIPUS probes (ERA: 4.5 cm²) (Fig. 2). Control cultures were not exposed to LIPUS, but were otherwise treated identically to the test group.

**Cell-proliferation assay**

Cell proliferation was quantified using the WST-8 method (Cell Counting Kit-8 (CCK-8), Dojindo, Japan) on Days 4, 7, and 10. Cultured cells were rinsed with PBS (phosphate-buffered saline) and then 900 μl of culture medium was added. Subsequently, 100 μl of CCK-8 solution was added to each well of the plate, and after incubation for 20 min in a CO₂ incubator, 100-μl samples of each test solution were transferred into 96-well plates. Absorbance was measured at 450 nm on a microplate reader (Microplate Reader 680, Bio-Rad, USA).

**Collagen synthesis**

Sirius Red staining was performed on Days 4, 7 and 10 to quantify collagen deposition in cell cultures. The stain binds specifically to type I and type III collagen and enables quantitative morphometric measurement of collagen in tissues⁴⁹. Sirius Red (Polysciences, USA) was dissolved in saturated aqueous picric acid (Kanto Chemical, Japan). Cultured cells were washed with PBS, fixed with Bouin’s fluid (Polysciences) for 1 h, and then air-dried. Subsequently, cell cultures were stained with Sirius Red for 1 h, after which they were washed with 0.01 M HCl and then with 0.1 M NaOH to extract the bound dye. Absorbance was measured using a spectrophotometer (Microplate Reader 680) equipped with a 550-nm filter.

**Mineralization assay**

Alizarin Red S staining was performed on Days 10, 14 and 21 to detect calcification in cultured cell samples. Alizarin Red S is a negatively charged dye that specifically binds metal ions, and it is used to analyze calcium deposits. Alizarin Red S (Thermo Fisher Scientific, USA) was dissolved in 100 ml of distilled water and the pH was adjusted to 4.1-4.3 with 0.1% NH₄OH. Cultures were washed with PBS, fixed with 10 % neutral buffered formalin (Wako Pure Chemical Industries, Japan) for 10 min, stained for 10 min, and then washed 4 times with distilled water. The stained areas...
were detected using an imaging scanner and quantified using ImageJ imaging software (NIH, USA).

**Total RNA extraction**

Total RNA was extracted from LIPUS-treated and control cultures on Day 7 after LIPUS exposure by using the guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol, Invitrogen, USA) and purification columns (RNeasy, QIAGEN, USA), in accordance with the manufacturers’ instructions. The obtained samples were treated with DNase I (TURBO DNA-free, Life Technologies) to remove contaminating DNA. The concentration of the extracted RNA was measured using a spectrophotometer (ND-1000, Thermo Fisher Scientific).

**Microarray analysis**

Total RNA (100 ng/sample) was converted to cDNA, amplified, and labeled with Cy3-labeled CTP (Low Input Quick Amp Labeling kit, Agilent Technologies, USA). The amplified RNA was quantified using a spectrophotometer (ND-1000) and then hybridized to whole rat genome 4 × 44 K oligo-DNA microarray slides (Agilent Technologies); after hybridization, the slides were washed using the provided washing solution (Gene Expression Wash Pack, Agilent Technologies). Fluorescence images of the hybridized arrays were scanned using a microarray scanner (GenePix, Molecular Devices, USA), and the data were extracted using the adjunctive software (GenePix Pro software, Molecular Devices). Result files were imported into Agilent GeneSpring GX software (Version 11.0) for statistical analysis. Genes exhibiting a fold-change (FC) of ≥2 and a p-value of <0.05 (LIPUS-exposed vs. control) were identified as differentially expressed genes. Functional annotation of differentially expressed genes was based on gene-ontology (GO) analysis.

**Real-time PCR analysis**

To confirm the results of the microarray analysis, real-time PCR was performed using a thermal cycler (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, USA). A reverse-transcription reaction system (1st Strand cDNA Synthesis Kit, Takara, Japan) was used to generate cDNA from 500 ng of total RNA by using oligo(dT) primers. The reaction mixture contained 2 × SYBR Green FAST qPCR master mix (Kapa Biosystems, USA), 20 pmol of forward and reverse primers, and 5 μl of cDNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The PCR program included an enzyme activation step (95 °C, 3 min) and 40 cycles of denaturation/annealing-extension (95 °C, 3 s; 60 °C, 31 s). Primers were designed and selected using the Perfect Real-time Primer Support System (Takara). Gene-expression levels were first normalized using the internal *GAPDH* control, and then compared to the control (ΔΔCT method); expression levels are presented as the relative fold-increase compared with the control samples.

**Statistical analysis**

Differences in cell proliferation, collagen synthesis, and calcification were statistically evaluated by means of two-way analysis of variance (ANOVA) performed using the factors “culture day” and “with or without LIPUS.” When two-way ANOVA showed a significant interaction between the 2 factors, a one-way ANOVA was performed for each level of the factor “culture day.” The cDNA microarray data were filtered using 2 methods to identify differentially expressed genes. We performed Student’s *t* tests followed by Benjamini-Hochberg corrections.

**Results**
Figure 5. Mineralized nodule formation.
A-F: Representative images of Alizarin Red S staining in cultured cells.
A, C, and E: Control cultures on Days 10, 14, and 21; B, D, and F: LIPUS-exposed cultures on Days 10, 14, and 21.

**Cell proliferation**

Fig. 3 shows changes in optical density at Days 4, 7 and 10, which serves as a readout of cell proliferation. Cell proliferation was lower in the LIPUS group than in the control group on Day 7 ($p < 0.01$), but proliferation levels did not differ significantly between these groups on Days 4 and 10.

**Collagen synthesis**

Fig. 4 shows the levels of collagen synthesis on Days 4, 7 and 10, quantified using Sirius Red staining. Optical density readings were higher on Day 10 in the LIPUS group than in the control group ($p < 0.01$), but were not significantly different between these groups on Days 4 and 7.

**Mineralized nodule formation**

Fig. 5 shows mineralized nodule formation on Days 10, 14 and 21, as detected using Alizarin Red S staining. One-way ANOVA results showed that the stained areas were significantly larger in the LIPUS group than in the controls on each day of the experiment ($p < 0.01$) (Fig. 6).

Figure 6. Pixel-based quantitation of Alizarin Red staining in cells; $**p < 0.01$.

Figure 7. Scatter plot of gene-expression levels in LIPUS-exposed and control cultures.
A: Scatter plot of expression data for 30,331 genes before filtering. B: Scatter plot of gene-expression data for reliable genes after filtering. The scatter plot of the gene-expression data summarizes both $t$-test and fold-change criteria. Differential expression was assessed using $t$-test analysis, followed by fold-change analysis. Multiple-testing correction was performed using the Benjamini-Hochberg False Discovery Rate (FDR) correction. An FDR of $< 0.05$ was considered significant.

**Microarray analysis of gene expression**

The microarray data were normalized using the 75th percentile value for each assay; the scatter plots of gene expression are shown in Fig. 7. We detected 559 differentially expressed genes, of which 45 genes were upregulated and 514 were downregulated. Among the genes upregulated in the LIPUS group, 33 were genes of known function (Table 1). Table 2 shows a list of differentially expressed genes, together with their associated GO annotations. The entire microarray dataset can be accessed from Gene Expression Omnibus (Accession Number GSE 70662).

**Real-time PCR**

Real-time PCR was performed to confirm the results of the microarray analysis. Fig. 8 presents graphs showing the relative quantitation of gene expression. One-way ANOVA revealed that all selected genes were more strongly upregulated in the LIPUS group than in the controls ($p < 0.01$).
Table 1. Genes with a fold change (FC) of ≥ 2 (LIPUS-exposed vs. control)

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**Figure 8.** Real-time PCR analysis of mRNA levels compared to control cultures, LIPUS-exposed cultures exhibited upregulated mRNA expression of collagen triple helix repeat containing 1 (Cthrc1), galanin prepropeptide (Gal), matrix extracellular phosphoglycoprotein (MEPE), bone γ-carboxyglutamate protein (Bglap), secreted phosphoprotein 1 (Sppl), lysyl oxidase (Lox), thrombospondin 1 (Thbs1), dickkopf homolog 1 (Dkk1), carboxypeptidase Z (CPZ), dentin matrix phosphoprotein 1 (Dmp1), collagen type XI a2 (Col11a2), tissue inhibitor of metalloproteinase 1 (Timp1), cell adhesion molecule 1 (Cadm1), and leucine-rich repeat-containing G protein-coupled receptor 4 (Lgr4). The relative expression levels of these genes on Day 10 are shown; **p < 0.01. Values represent means ± SD (n = 3). The Ct value of the control was used as a baseline, with a value of 1. Relative expression levels were calculated using the 2^{-ΔΔCt} model.

**Discussion**

**Cell culture**

In this study, dexamethasone, L-ascobic acid 2-phosphate, and β-glycerophosphate were added to the culture medium to promote the differentiation of bone marrow cells into osteoblast-like cells. Multipotent mesenchymal stem cells (MSCs) are present in the bone marrow, and the addition of the aforementioned supplements to the growth medium can promote the differentiation of MSCs into osteoblast-like cells. MSCs can also differentiate into chondrocytes, myoblasts, and adipocytes. Osteoblast differentiation is associated with the expression of runt-related transcription factor 2 (Runx2), and of osterix, which functions downstream from Runx2. Runx2 also controls the expression of type I collagen (col I), bone γ-carboxyglutamate (gla) protein (osteocalcin, Bglap), secreted phosphoprotein 1 (osteopontin, Sppl), and bone sialoprotein, and thus enables osteoblasts to act as bone-forming cells. We observed increased expression of col I, osteocalcin, osteopontin, and bone sialoprotein, which are all markers for osteoblast-like cell differentiation, in the RNA samples that were collected 10 days after the cultures were established. This suggests that differentiation into osteoblast-like cells was completed after 10 days of culture under the
might be due to a suppression of cell proliferation after the onset was lower in the LIPUS group than in the control group. This suggests that the LIPUS exposure might stimulate osteoblast-like differentiation through interactions with bone marrow components other than MSCs, such as bone cells or blood stem cells. However, this hypothesis could not be confirmed using our microarray analysis. Another possibility is that one or more required growth factors were present at sufficient concentrations to permit differentiation in the bone marrow cultures, but not in isolated MSC cultures. We propose that the differentiation of undifferentiated MSCs into osteoblast-like cells is one of the mechanisms contributing to osseointegration. We hypothesized that the mechanical stress induced by LIPUS exposure would promote differentiation into osteoblast-like cells, which would be accompanied by characteristic changes in gene expression. To test this hypothesis, we exposed bone marrow cells to LIPUS under conditions that promote differentiation into osteoblast-like cells, and then used genome-wide microarray analysis to compare gene-expression patterns in LIPUS-exposed and control cells.

**LIPUS conditions**

LIPUS is widely used in the treatment of bone fractures, and its clinical benefits have been demonstrated. Although LIPUS is used therapeutically at a constant frequency of 1-5 MHz, a previous study reported that the use of frequencies of <1 MHz might result in a high frequency of cavitation. Furthermore, ultrasound exposure produces not only physical effects, such as cavitation, but also hyperthermic effects. Optimized conditions for LIPUS treatment have been established, which involve the use of pulsed waves of extremely low intensity. This promotes tissue regeneration by inducing minimal mechanical stress in the absence of damage to the regenerating tissues or excessive temperature elevation. In this study, LIPUS was used with the following parameter settings: a frequency of 3 MHz, with a spatial-average intensity of 40 mW/cm² and 2-ms-burst sine waves at 100 kHz. These parameters are identical to those used by Li in their study of bone formation around implants. Moreover, Kidokoro et al. reported that these conditions are applicable to cell cultures.

**Cell proliferation**

The cell-proliferation level measured after 7 days of culture was lower in the LIPUS group than in the control group. This might be due to a suppression of cell proliferation after the onset of osteoblast-like differentiation. Preosteoblasts present in early stages of culture exhibit high proliferative capacity, but this decreases as cell differentiation progresses and is accompanied by the production of extracellular matrix (ECM) proteins such as collagens.

**Collagen synthesis**

After 10 days of culture, collagen production in the LIPUS group was significantly higher than that in the control cultures. Sirius Red staining enables sensitive and quantitative detection of type I and type III collagen. Type III collagen is detected in preosteoblasts, whereas type I collagen accounts for approximately 90% of the ECM in bone. Therefore, our results indicate that LIPUS exposure promotes the differentiation of bone marrow-derived cells into osteoblast-like cells.

**Calcified nodule formation**

After 10, 14 and 21 days of culture, calcified nodule areas (i.e., the areas stained with Alizarin Red S) were significantly larger in the LIPUS group than in the control group. The difference in these areas in the stained cultures in the 2 groups on each sampling day was clearly evident to the naked eye (Fig. 5). Increases in collagen production and calcified nodule formation can be explained based on the following evidence. During early stages of calcification, osteoblast-like cells secrete matrix vesicles that deposit ECM proteins, such as collagens, around the cells. In parallel, hydroxyapatite crystals develop within the matrix vesicles. When these crystals are released from the matrix vesicles, they infiltrate the intermolecular spaces of the surrounding collagens and become crystalline nuclei. This results in the calcification of collagen fibers. Our results indicate that these calcification processes were enhanced by LIPUS exposure.

**Analysis of the gene-expression profile following LIPUS exposure**

Gene-expression analysis: Rat bone marrow cells were cultured under conditions that promote osteoblast differentiation, and LIPUS exposure-induced changes in gene expression were analyzed using a cDNA microarray. GO analysis was used for functional annotation of the gene list obtained, and functional categories associated with the genes of interest were extracted. The expression of these genes was quantified using real-time PCR, which confirmed the results of the microarray analysis.

Expression of osteoblast-differentiation markers: Non-collagenous proteins account for approximately 10% of total bone-matrix proteins. The majority of these are acidic proteins, and the proteins that interact with hydroxyapatite are considered to be involved in bone calcification. Osteocalcin, which accounts for approximately 20% of the non-collagenous protein content of the bone matrix, is produced by mature osteoblasts and is deposited...
in the ECM of bone tissue, and therefore it is used as a marker for differentiated osteoblasts\textsuperscript{31}. Osteopontin, which contains an “RGD” (Arg-Gly-Asp) sequence, is considered to bind to osteoblasts and osteoclasts localized on bone surfaces and to promote bone resorption by osteoclasts\textsuperscript{21}. In this study, both osteocalcin and osteopontin were upregulated in LIPUS-exposed cells after 10 days of culture. These results indicate that LIPUS exposure induced gene-expression changes that are consistent with the enhancement of osteoblast-like cell differentiation. Naruse et al.\textsuperscript{35} reported that when bone marrow-derived stromal-cell clone ST2 cells were exposed to LIPUS at 1.5 MHz for 20 min, the expression of genes encoding insulin-like growth factor 1 and osteocalcin was increased, and that the expression of these genes was still detectable at 24 h after LIPUS treatment. This increase in osteocalcin gene expression agrees with our results, although the expression was examined using slightly different exposure conditions and analytical time points.

Expression of osteocyte-specific markers: Dentin matrix protein 1 (Dmp1), matrix extracellular phosphoglycoprotein (MEPE), sclerostin (SOST), fibroblast growth factor 23 (FGF23), and phosphate-regulating neutral endopeptidase (PHEX) are considered to be expressed specifically by osteocytes\textsuperscript{36}. Among these genes, Dmp1, MEPE, and PHEX showed upregulated expression following LIPUS exposure. Dmp1, a non-collagenous bone matrix protein expressed by osteocytes but not osteoblasts\textsuperscript{37}, is a strongly acidic and negatively charged protein\textsuperscript{38} that can bind Ca\textsuperscript{2+} and is involved in bone calcification\textsuperscript{39}. Narayanan et al.\textsuperscript{40} proposed that during early stages of osteoblast differentiation, Dmp1 is localized in the nuclear compartment and acts as a transcription factor that specifically activates osteoblast factors such as osteocalcin; however, when Ca\textsuperscript{2+} surges into the nucleus from the cytoplasm, it triggers Dmp1 phosphorylation by a nuclear isoform of casein kinase II, following which phosphorylated Dmp1 is secreted into the ECM, which initiates the calcification process.

MEPE, another non-collagenous bone matrix protein, is synthesized as a precursor protein that is cleaved by cathepsin B, which results in the secretion of the acidic serine-aspartate-rich MEPE-associated motif (ASARM) peptide. The ASARM peptide is then phosphorylated, and this prevents bone calcification\textsuperscript{41}. The upregulation of MEPE observed here might have been the result of negative-feedback mechanisms that antagonize calcification in the cultured tissue.

In the case of PHEX, another gene that was upregulated here, the matrix protein target of this molecule has not been identified, but PHEX was recently shown to act as an inhibitory factor that antagonizes FGF23 expression\textsuperscript{42}. Furthermore, MEPE has been reported to bind to PHEX, which protects MEPE from cleavage by cathepsin B\textsuperscript{43}. Conversely, our study demonstrated no increase in the expression of SOST, a gene that is expressed in osteocytes at a late stage of maturation\textsuperscript{44}. The increased expression of bone matrix-specific markers indicates that LIPUS exposure promoted the differentiation of osteoblast-like cells into osteocytes. It was recently shown that mechanical stress perception can be mediated by integrin adhering to the surface of the bone canaliculus\textsuperscript{45}. In addition, it has been reported that osteocytes have primary cilia that act as mechanosensors\textsuperscript{46}. Physical stimulation by LIPUS is considered to transfer the signal via these mechanosensors on osteocyte membrane, and Li et al.\textsuperscript{47} reported increased secretion of PGE\textsubscript{2}, and nitric oxide in MLO-Y4 cells.

Expression of collagen-related genes: Collagen cross-linking within the ECM has been reported to be associated with the bone calcification process, and this cross-linking has been suggested to be critical for bone formation. Here, collagen production was increased in the LIPUS group, and this was accompanied by upregulated mRNA expression of lysyl oxidase (Lox), collagen triple helix repeat containing 1 (Cthrc1), and type XI collagen α2 (Col11a2). Whereas Lox is a crucial enzyme involved in collagen cross-linking\textsuperscript{48}, Cthrc1 is a positive regulator of bone formation by osteoblasts\textsuperscript{49}, and Col11a2 has been reported to be associated with epithyseal cartilage growth\textsuperscript{50}. These results indicate that LIPUS exposure induces the expression of proteins involved in collagen cross-linking, a process that enhances bone strength.

Expression of cell adhesion-related genes: Various adhesion factors, including thrombospondin 1 (Thbs1) and cell adhesion molecule 1 (Cadm1), were also identified to be upregulated. Thbs1 is an ECM protein that acts as an adhesion factor; it binds to cell membrane receptors such as integrins through distinct protein domains\textsuperscript{51}. Although we did not observe an increase in integrin levels, these molecules are recognized to play roles in the cellular signal transduction elicited by mechanical stimulation, such as that induced by LIPUS exposure\textsuperscript{52}. Cadm1 is an intercellular adhesion molecule that belongs to the immunoglobulin superfamily of cell-adhesion molecules (IgCAMs), and its expression is strongly correlated with the bone-forming capacity of human MSCs\textsuperscript{53}. The upregulation of these genes observed here suggests that LIPUS stimulation promotes cell adhesion.

Expression of other ECM-related genes: Carboxypeptidase Z (CPZ) is an ECM-localized enzyme, and CPZ enzyme activity has been observed in both cultured cells and human tissues\textsuperscript{40}. Wang et al.\textsuperscript{55} reported that in growth-plate chondrocytes, CPZ binds to and activates components of the Wnt signal transduction pathway. Another ECM-related molecule, leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4), was shown to be involved in R-spondin-mediated signal transduction in the context of Wnt/β-catenin and Wnt/PCP signaling\textsuperscript{50}. Thus, the upregulation of CPZ and LGR4 suggests that LIPUS exposure activates one or more Wnt signal transduction pathways. We also observed an increase in the expression of dickkopf homolog 1 (Dkk1)\textsuperscript{57}, which is an inhibitory molecule in the Wnt/β-catenin signaling pathway, although the underlying reason is unknown.
Matrix metalloproteinases, which are involved in ECM remodeling, are known to be controlled by the common endogenous protease inhibitor Timp1 (tissue inhibitor of metalloproteinase 1); Timp1 belongs to a family of 4 protease inhibitors: Timp1, Timp2, Timp3, and Timp4. We observed the upregulation of Timp1 gene expression, which agrees with the observations of Ito et al., who reported increased Timp1 gene expression in chondrocytes exposed to LIPUS at 1.0 MHz.

Expression of neurotransmitter genes: Intriguingly, we also observed increased expression of a gene encoding a neurotransmitter, galanin (Gal). Gal is a 29-amino-acid neuropeptide that has been reported to control feeding and inhibit the secretion of growth hormone in the hypothalamus. Previously, McDonald et al. reported increased Gal expression in osteoblast-like cells after bone fractures, and noted its effect on promoting bone formation through the inhibition of TNFα and IL-1β production. These observations suggest a novel mechanism for the stimulation of osteoblast differentiation. In the context of our experiments, LIPUS exposure-induced upregulation of Gal gene expression could have enhanced osteoblast differentiation by inhibiting the production of inflammatory cytokines.

Competing Interests
The authors have declared that no COI exists.

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