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# Low-intensity pulsed ultrasound-induced ATP increases bone formation via the P2X7 receptor in osteoblast-like MC3T3-E1 cells

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ABSTRACT

**Low-intensity pulsed ultrasound (LIPUS) is used for bone healing in orthopedics and dentistry. It has been shown that LIPUS induces the secretion of extracellular adenosine triphosphate (ATP), a key mediator of osteoblast response to mechanical stimuli. However, the detailed mechanism of LIPUS-induced osteogenesis has been elusive. In this study, we investigated the role of the P2X7 receptor in LIPUS-induced osteogenesis. LIPUS induced the release of extracellular ATP, differentiation of osteoblasts and osteogenesis via the P2X7 receptor, without affecting the activity of alkaline phosphatase (ALPase). These results suggest that LIPUS-induced extracellular ATP promotes bone formation via the osteoblast P2X7 receptor independently of ALPase.**


## 1. Introduction

Bone formation and homeostasis is regulated by hormones, cytokines, and external factors such as mechanical stimuli. In adults, bone mass is maintained via bone remodeling induced by mechanical stress, which is a one of the most important factors affecting the amount and strength of the bone tissue [1].

Nucleotides released by mechanical stimulation or inflammation act as autocrine/paracrine mediators of osteoclasts and osteoblasts that participate in bone remodeling [2]. Adenosine triphosphate (ATP) is a key regulator of osteoblast response to mechanical stimuli [3,4]. Mechanosensitive ATP production is induced in various cell types by such activities as pressure, stretch, and flow shear, as well as osmotic stress [2,5]. It has also been demonstrated that the release of extracellular ATP by mechanically stimulated osteoblasts is calcium-dependent [6]. Extracellular ATP induces a variety of physiological responses through activation of ATP-binding purinergic (P2) receptors. These receptors, expressed on both osteoblasts and osteoclasts, can be divided into the P2Y family of G protein-coupled receptors and the P2X family of ligand-gated cation channels [7]. MC3T3-E1 cells expressed P2X3, P2X4, P2X5 and P2X7 [8]. In previous study, P2X4 is not mediated bone formation by extracellular nucleotides [9]. In particular, the P2X7 receptor has been shown to mediate pore formation in response to fluid shearing stress in mouse calvarial cells. $P2X7^{-/-}$ mice demonstrated reduced periosteal bone formation in long bones and decreased osteogenesis in response to mechanical loading; however, P2X7 deficiency did not affect the length of mouse long bones [10,11]. These reports indicate that the P2X7 receptor may play a critical role in osteogenesis induced by mechanical stimuli.

Mechanical stimulation was applied by low-intensity pulsed ultrasound (LIPUS) treatment accelerates healing and regeneration of bone fractures and is used in clinics [12]. LIPUS exerts a variety of direct and indirect effects such as acoustic radiation force,

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acoustic streaming, and propagation of surface waves, thus promoting fluid flow-induced circulation and redistribution of nutrients, oxygen, and signaling molecules. In addition, the transformation of acoustic wave energy into heat can be ignored, and cavitation at the pressure levels delivered by LIPUS normally does not occur. Previous studies have reported that LIPUS promotes osteoblast differentiation and osteogenesis in vitro [13–16]. Thus, LIPUS induces osteoblast proliferation and expression of receptor activator of nuclear factor-kappa B ligand (RANKL) via ATP release in SaOS-2 cells [17], suggesting that the secretion of extracellular ATP may underlie the osteogenic effects of LIPUS. However, the involvement of the ATP-gated P2X7 receptor in LIPUS-induced bone regeneration is poorly understood. In this study, our main purpose was to elucidate the involvement of the P2X7 receptor in the LIPUS-induced osteogenic effects in calvarial osteoblast-like MC3T3-E1 cells. For this, we assessed the expression of transcription factors RUNX2 and Osterix, which regulate osteoblast differentiation, and measured accumulation of inorganic phosphate and calcium in the extracellular matrix, and the expression of bone ECM proteins.

## 2. Materials and methods

### 2.1. Cell culture

The mouse calvarial cell line MC3T3-E1, obtained from Riken Bio Resource Center (Tsukuba, Japan), was used as a model for osteoblasts. Cells were maintained in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin–streptomycin solution (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. The medium was changed every 3 days.

### 2.2. Application of low-intensity pulsed ultrasound (LIPUS)

Cells were seeded on 6-well culture plates (IWAKI, Tokyo, Japan) at the density of $2 \times 10^4$ cells/cm$^2$ for 24 h prior to stimulation with LIPUS (1.5 MHz, pulsed-wave mode intensity of 30 mW/cm$^2$) for a maximum of 24 h [13]. LIPUS was applied using a sterilized transducer (OSTEOTRON D$^2$: Ito Co. Ltd., Tokyo, Japan) placed on the surface of culture medium so that the distance between the transducer and the cells was approximately 3–4 mm. Control cells were seeded at the same density but were not stimulated with LIPUS.

### 2.3. Luciferin–luciferase bioluminescence assay

Cells were seeded at a density of $2 \times 10^4$ cells/cm$^2$ onto 6-well plates 24 h prior to stimulation. The medium was then replaced with 3 ml of fresh culture medium containing ARL67156 (Sigma–Aldrich), an ATPase inhibitor, and the cells were incubated for 30 min and then stimulated with LIPUS for a maximum of 30 min. The concentration of extracellular ATP was determined using the Kinsiro ATP Luminescence kit (Toyo Ink SC Holdings Co. Tokyo, Japan). For this, 50 μl of the luciferin–luciferase assay mix dissolved in culture medium was added to a 50 μl sample and bioluminescence was immediately measured using a Luminescencer Octa luminometer (ATTO Co. LTD., Tokyo, Japan). A standard curve was generated for each luciferase assay using serial dilutions of an ATP standard.

### 2.4. Real-time polymerase chain reaction

Cells were seeded on 6-well plates and cultured for up to 14 days. Total RNA was isolated on days 3, 7, and 14 of culture using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA); RNA concentration was measured by NanoDrop 1000 (ND-1000; Thermo Fisher Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 0.5 μg of DNase-treated total RNA using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan), and the resultant cDNA was analyzed by real-time PCR using the SYBR Green kit (Takara Bio). The reactions were performed in a total volume of 25 μl containing 12.5 μl of SYBR premixed Ex *Taq*, 0.5 μl (20 mM) of each primer (Table 1), 9.5 μl of $dH_2O$, and 2 μl (0.5 μg) of cDNA. The PCR assays were performed in a Smart Cycler II instrument (Cepheid, Sunnyvale, CA, USA) and analyzed using Smart Cycler software. The cycling conditions included 35 cycles at 95 °C for 5 s and 60 °C for 20 s. All real-time PCR experiments were performed in triplicate, and the specificity of the amplified products was verified by melting curve analysis. The calculated values of target gene expression were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control.

### 2.5. SDS–polyacrylamide gel electrophoresis and Western blotting

After LIPUS stimulation, cells were cultured in serum-free medium for additional 24 h, and then collected and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% polyacrylamide gels (8.3 cm × 6.5 cm × 0.75 mm)

**Table 1**
PCR primers used in the experiments.

| Target | Primers | GenBank Acc. |
|---|---|---|
| RUNX2 | 5′-CACTCTGGCTTTGGGAAGAG-3′<br>5′-GCAGTTCCCAAGCATTTCAT-3′ | NM_001145920.1 |
| Osterix | 5′-GGTAGGCGTCCCCCATGGTTT-3′<br>5′-AGACGGGACAGCCAACCCTAG-3′ | NM_130458.3 |
| P2X7 | 5′-TGCAGCTGGAACGATGTCTTG-3′<br>5′-CGCTGGTACAGCTTATCGCTCA-3′ | NM_011027.2 |
| Type I collagen | 5′-TGGGCGCGGCTGGTATGAGTTC-3′<br>5′-ACCCTGCTACGACAACGTGCC-3′ | NM_007743.2 |
| Bone sialoprotein | 5′-AATTCTGACCCTCGTAGCCTTCATA-3′<br>5′-GAGCCTCGTGGCGACACTTA-3′ | NM_008318.3 |
| Osteopontin | 5′-TACGACCATGAGATTGGCAGTGA-3′<br>5′-TATAGGATCTGGGTGCAGGCTGTAA-3′ | NM_009263.3 |
| Osteocalcin | 5′-AAGCAGGAGGGCAATAAGGT-3′<br>5′-ACCCTGCTACGACAACGTGCC-3′ | NM_007541.2 |
| GAPDH | 5′-AAATGGTGAAGGTCGGTGTG-3′<br>5′-TGAAGGGGTCGTTGATGG-3′ | NM_008084.2 |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and a discontinuous Tris–glycine buffer system. The samples containing 10 ng of extracellular protein were dissolved in 10 μl of sample buffer containing 1% SDS, 2 M urea, 15 mg/ml dithiothreitol, and bromophenol blue, and then heated at 95 °C for 5 min before loading onto the gel. Proteins were separated at 150 V for 60 min and transferred onto a PVDF membrane using a semi-dry electrotransfer unit and transfer buffer containing 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 20% (v/v) methanol at the constant current of 0.8 mA/cm$^2$ for 60–90 min. The membrane was then treated with 25% (v/v) blocking reagent in Tris-buffered saline (TBS) (10 mM Tris, 145 mM NaCl, pH 7.4) for 18 h at 4 °C, washed in Tween 20-containing TBS (TBS-Tween), and incubated with rabbit polyclonal IgG antibodies against RUNX2, Osterix, P2X7 and β-tubulin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in 10% (v/v) blocking reagent in water, for 90 min at room temperature; β-tubulin was used as an internal standard. The membranes were washed in TBS-Tween and incubated with biotin-conjugated secondary antibodies diluted 1:10,000 in 10% blocking agent in distilled water for 60 min at room temperature. The membranes were washed in TBS-Tween and phosphate-buffered saline (PBS; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and incubated with horseradish peroxidase-conjugated streptavidin in PBS for 30 min at room temperature. Immunoreactive proteins were visualized using a chemiluminescence kit (Amersham Life Sciences, Buckinghamshire, UK) and autoradiography (X-ray) films (Eastman Kodak, New Haven, CT, USA). Pre-stained molecular weight standards were run on the same gel. The band intensity was quantified using digital image analysis software (GS-800 Calibrated Densitometer and Quantity One Version 4.2.1, Bio-Rad, Hercules, CA, USA).

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

After LIPUS stimulation, cells were cultured in serum-free medium for additional 24 h. The concentrations of type I collagen, bone sialoprotein, osteopontin, and osteocalcin in the culture medium at 3, 7, and 14 days were determined using commercially available ELISA kits (R&D Systems, Cusabio Biotech Co., Uscn Life Science Inc. and Biomedical Technologies Inc.) according to the manufacturers' instructions.

### 2.7. Alkaline phosphatase activity assay

Cells were fixed with 4% neutral buffered formalin overnight at 4 °C. Alkaline phosphatase (ALPase) activity was assessed by treatment with a mixture of 0.25 mM naphthol AS-MX phosphate (Sigma–Aldrich) and 1.25 mM Fast Red B salt (Sigma–Aldrich) in 0.1 M Tris–HCl, pH 8.0, for 1 h in the dark. The cells were observed under a phase-contrast microscope with a Plan ×10 DL objective (Nikon, Tokyo, Japan). The planar area of ALP positive staining was quantified using NIS-Elements D 3.2 software (Nikon).

### 2.8. Phosphate assay and mineral content in ECM

After LIPUS stimulation, the cells were cultured in serum-free medium for an additional 24 h. The concentration of inorganic phosphate (Pi) in the culture medium at 3, 7, and 14 days was determined using the Malachite Green Phosphate Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

To quantify mineral content, the cells were cultured in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate [18] for 14 days; the medium was changed every 2–3 days. At the end of cultivation, 300 μl of 0.5 M HCl was added to each well, and the cells were left overnight for decalcification of mineralized nodules. Calcium content was determined quantitatively using the Calcium E-Test kit (Wako Fine Chemicals, Osaka, Japan) according to the manufacturer's instructions. Protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories) after HCl evaporation.

### 2.9. Cell viability

After LIPUS stimulation, cells were stained with 0.4 w/v% trypan blue solution to determine cell viability. Viable (trypan blue-negative) cells were counted using a DIAPHOT phase-contrast microscope.

### 2.10. Knockdown of P2X7 by short hairpin RNA in MC3T3-E1 cells

To obtain stable P2X7-deficient clones, the cells were transfected with Lentiviral Particles containing the puromycin resistance gene for antibiotic selection and P2X7 short hairpin RNA (shRNA) or non-target (scrambled) control shRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and cultured in the presence of 5 μg/ml puromycin (Enzo Life Sciences Inc., Farmingdale, NY, USA). After stable clones had been selected, real-time PCR and Western blotting were performed to confirm P2X7 knockdown.

### 2.11. Statistical analysis

All data present the results of three independent experiments performed in triplicate or quintuplicate. Each value represents the mean ± standard error (S.E.M.). The significance of differences was determined using Student's *t*-test, one-way analysis of variance followed by Tukey's multiple comparisons test or two-way analysis of variance by Bonferroni's multiple comparisons test. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. LIPUS effects on the release of extracellular ATP

We examined the effect of LIPUS on the release of extracellular ATP by MC3T3-E1 osteoblasts. LIPUS significantly stimulated extracellular ATP release at 1 and 3 min compared to the untreated control cells, which did not secrete ATP (Fig. 1).

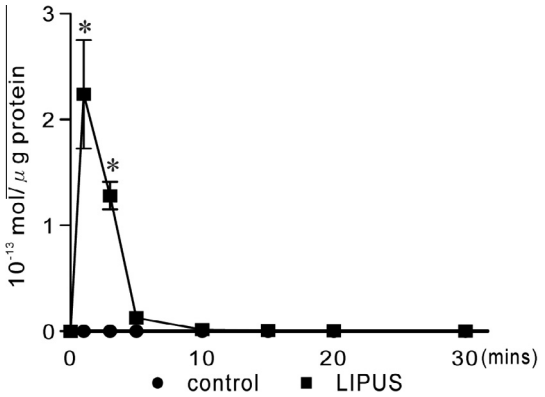


**Fig. 1.** Time course of ATP release by LIPUS. MC3T3-E1 cells were cultured with the ATPase inhibitor ARL67156 (50 μM) for 30 min before LIPUS stimulation for 0, 1, 5, 10, 15, 20, or 30 min. ATP release was quantified by the luciferin–luciferase bioluminescence assay. Data are expressed as the mean ± standard error (S.E.M.) of three independent experiments performed in triplicate; $^*P < 0.05$, LIPUS vs. 0 min (control).

### 3.2. LIPUS effect on the expression of RUNX2, Osterix, and the P2X7 receptor

LIPUS gradually increased mRNA expression of RUNX2 in osteoblasts through day 3 of culture; the expression subsequently decreased by day 5. In osteoblasts stimulated by LIPUS for 5, 10, 20, and 30 min, RUNX2 expression was significantly increased, by 1.7-, 1.7-, 1.8-, and 2.8-fold, respectively, at day 3 of culture compared to the untreated cells (Fig. 2a).

LIPUS also significantly increased Osterix mRNA in osteoblasts, especially at day 1 of culture. Osterix expression in osteoblasts stimulated by LIPUS for 5, 10, 20, and 30 min gradually increased, by 1.6-, 1.9-, 2.2-, and 2.9-fold, respectively, compared to the untreated control, at days 1, 3, and/or 5 of culture (Fig. 2b).

A similar effect was observed on the expression of the P2X7 receptor in osteoblasts, where LIPUS stimulation for 5, 10, 20, and 30 min upregulated P2X7 by 2.1-, 2.0-, 2.5- and 4.1-fold, respectively, at day 3 compared to control (Fig. 2c).

These results indicate that LIPUS upregulated mRNA expression of RUNX2, Osterix, and P2X7 in a time-dependent manner and the treatment for 30-min had the maximum effect.

### 3.3. Effects of P2X7 receptor antagonist A438079 on RUNX2 and Osterix expression

We investigated the effect of P2X7 on the LIPUS-induced expression of RUNX2 and Osterix by treating LIPUS-stimulated cells with A438079, a selective antagonist of the P2X7 receptor [19,20]. We chose to use the 30 min/day stimulation with LIPUS because it had the strongest effect compared to other time points according to Fig. 2. LIPUS increased mRNA levels of RUNX2 and Osterix in osteoblasts by day 3 of culture (by 1.7- and 1.9-fold, respectively), but the effect gradually decreased by day 14 (Fig. 3a and b). However, the LIPUS-elicited upregulation of mRNA and protein expression of RUNX2 and Osterix on day 3 was inhibited by A438079 (Fig. 3c).

### 3.4. Effects of LIPUS and A438079 on the expression of ECM proteins

To clarify the role of LIPUS-induced ATP on osteogenesis, we determined the expression of ECM proteins at the mRNA and protein levels in MC3T3-E1 cells stimulated with LIPUS and treated with A438079.

In LIPUS-stimulated osteoblasts, mRNA expression of type I collagen was upregulated by 2.0-fold at day 3, and that of bone sialoprotein, osteopontin, and osteocalcin was increased by 1.4-, 3.75-, and 2.1-fold, respectively, at day 14 of culture, compared to the untreated control (Fig. 4). The increase in protein expression was consistent with that in mRNA (Fig. 5). However, P2X7 antagonist A438079 blocked LIPUS stimulatory effect on the expression of these ECM components both at mRNA and protein levels (Figs. 4 and 5).

### 3.5. Effects of LIPUS and A4348079 on ALPase activity, cell viability, phosphate concentration, and calcium content in the ECM

To investigate the role of LIPUS-induced ATP in the formation of mineralized nodules, we determined ALPase activity and calcium content in the ECM produced by MC3T3-E1 cells after stimulation with LIPUS and treatment with A438079. ALPase activity and cell viability were not affected by LIPUS (Fig. 6a–d). LIPUS significantly increased phosphate concentration (by 1.3-fold) and calcium content (by 1.8-fold) in the ECM on 14 day of culture compared to the untreated cells (Fig. 6e and f). However, A438079 reduced the stimulatory effects of LIPUS on the deposition of phosphate and calcium in the ECM.

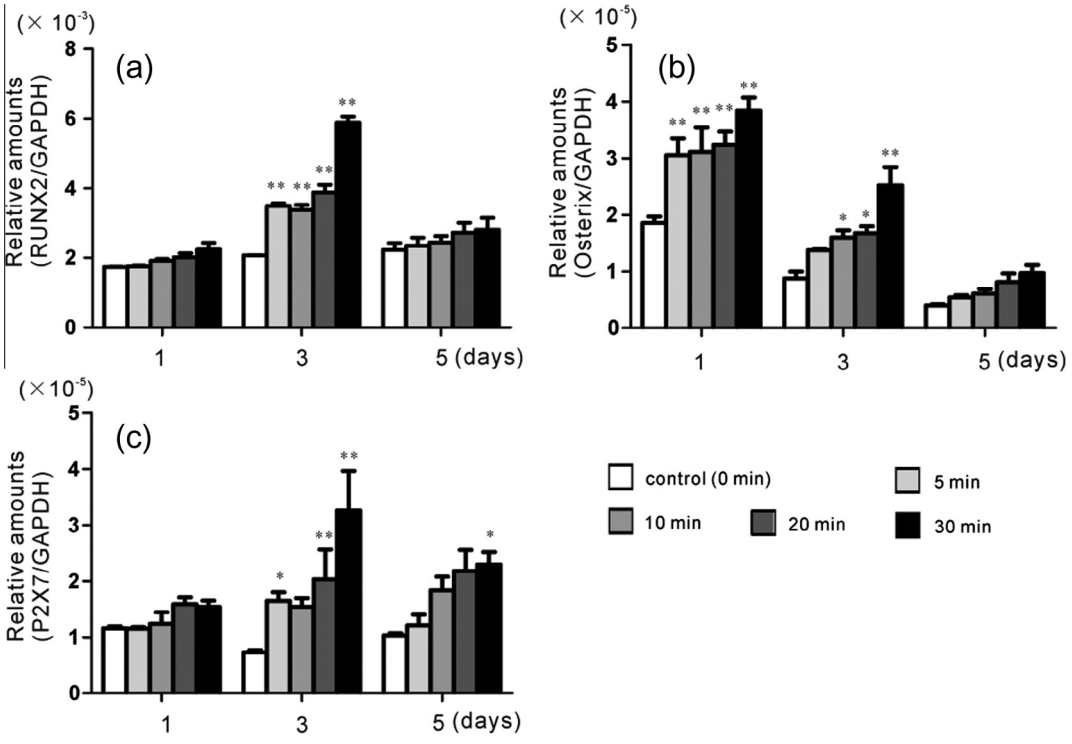


**Fig. 2.** Effects of LIPUS on the expression of RUNX2, Osterix, and the P2X7 receptor. MC3T3-E1 cells were stimulated with LIPUS (5, 10, 20, and 30 min) or left without stimulation (control) and mRNA expression of RUNX2 (a), Osterix (b), and P2X7 (c) was determined on days 1, 3 and 5 of culture using real-time PCR. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, LIPUS vs. control.

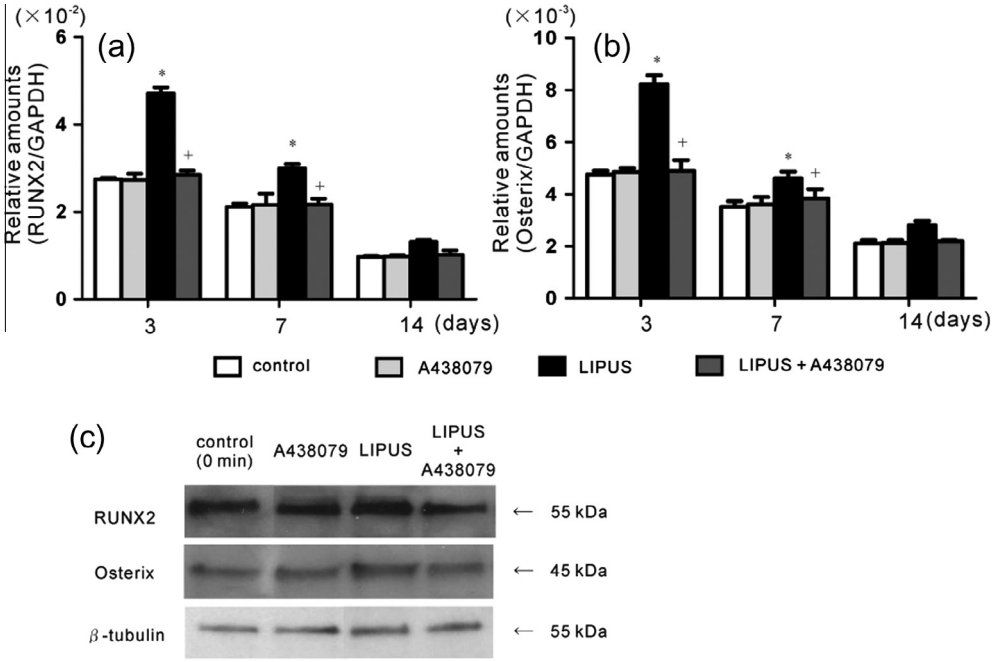


**Fig. 3.** Effects of A438079 on the expression of RUNX2, Osterix. Cells were stimulated or not with LIPUS in the presence or absence of A438079 (10 μM) and mRNA expression of RUNX2 (a) and Osterix (b) was determined by real-time PCR at day 3 of culture. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, LIPUS vs. control; $^{+}P < 0.05$, $^{++}P < 0.01$, LIPUS vs. LIPUS + A438079. The protein expression of RUNX2 and Osterix was determined by Western blotting on day 3 of culture (c).

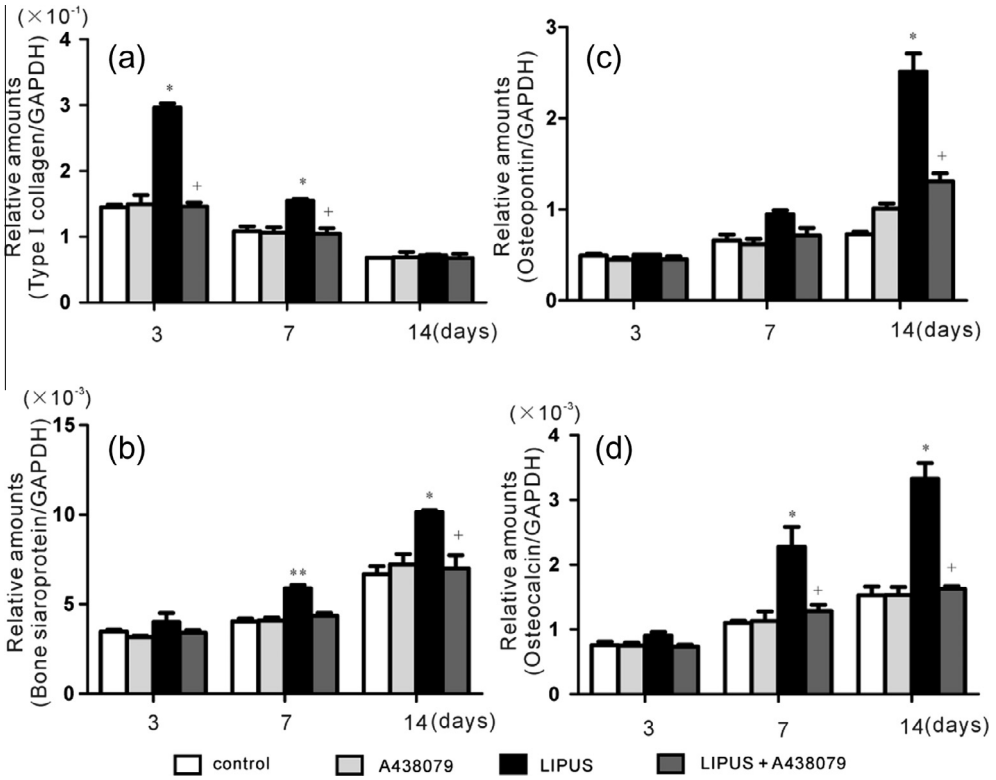


**Fig. 4.** Effects of LIPUS and A438079 on the mRNA expression of ECM proteins. MC3T3-E1 cells stimulated or not with LIPUS for 30 min per day during 14 days in the presence or absence of A438079 (10 μM) and mRNA expression of type I collagen (a), bone sialoprotein (b), osteopontin (c), and osteocalcin (d) was determined by real-time PCR on days 3, 7, and 14 of culture. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, LIPUS stimulation vs. control, $^{+}P < 0.05$, $^{++}P < 0.01$, LIPUS vs. LIPUS + A438079.

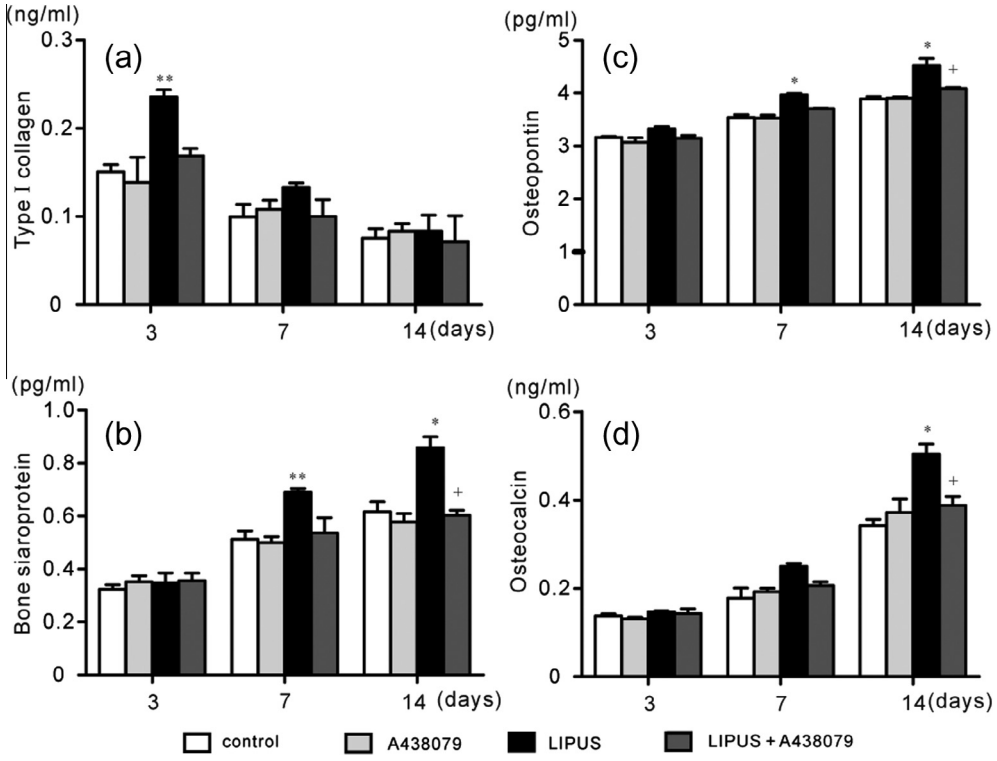


**Fig. 5.** Effects of LIPUS and A438079 on the protein expression of ECM proteins. Protein expression of type I collagen (a), bone sialoprotein (b), osteopontin (c), and osteocalcin (d) was determined by ELISA on days 3, 7, and 14 of culture. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, LIPUS stimulation vs. control, $^{+}P < 0.05$, $^{++}P < 0.01$, LIPUS vs. LIPUS + A438079.

### *3.6. Effects of P2X7 gene silencing on the expression of ECM proteins*

To clarify the role of LIPUS-induced ATP on osteogenesis, we determined the expression of the ECM proteins at the mRNA level in the P2X7-deficient cells stimulated with LIPUS. For this, MC3T3-E1 osteoblasts were transfected with P2X7-specific shRNA (sh P2X7), which decreased the expression of P2X7 mRNA by more than 2-fold compared to the cells transfected with scrambled shRNA (sh control) (Fig. 7a). Consistently, sh P2X7 also reduced P2X7 protein expression by 2-fold compared to sh control (Fig. 7b and c).

In the control shRNA-transfected cells stimulated with LIPUS, mRNA expression of type I collagen was increased by 1.4-fold on day 3, and that of bone sialoprotein, osteopontin, and osteocalcin was induced by 1.3-, 2.1-, and 2.0-fold, respectively, on day 14 of culture, compared to the untreated cells (Fig. 8a–d). However, this stimulatory effect was blocked in the sh P2X7-transfected cells (Fig. 8a–d).

## 4. Discussion

LIPUS is a medical technology based on the application of mechanical stress, which has been proven to have clinical effects on bone fracture healing [21]. A previous *in vitro* study has shown that LIPUS induces osteogenesis and suppresses adipogenesis in mouse bone marrow cells [22].

Mechanical stress such as pressure, stretch, and fluid shear, as well as osmotic stress induce the release of extracellular ATP in various cell types [2,5], including osteoblasts where extracellular ATP release is stimulated via both constitutive and inductive mechanisms [23]. It has been demonstrated that the secretion of extracellular ATP promotes osteogenesis in rat calvarial cells *in vitro* [24].

The P2X7 receptor is a non-selective cation channel permeable to $Na^+$, $K^+$, and $Ca^{2+}$. ATP and 2′(3)-O-(4-benzoylbenzoyl) ATP (BzATP) are agonists of P2X7 that induce the opening of P2X7 channels causing the elevation in intracellular calcium and depolarization of the plasma membrane [25]. Osteoblasts express functional P2X7 receptors both *in situ* and *in vitro* [26]. *In vivo* studies have shown that $P2X7^{-/-}$ mice have reduced osteogenesis in load-bearing bones, suggesting the role of P2X7 in the skeletal bone response to mechanical stress [10,11].

In this study, we tested the hypothesis that LIPUS promotes osteogenesis through the ATP-P2X7 pathway by examining the secretion of extracellular ATP after LIPUS stimulation of osteoblast-like MC3T3-E1 cells. Previous findings indicate that a P2X7 receptor agonist BzATP promotes the production of lysophosphatidic acid (LPA) which is involved in P2X7-induced osteogenesis via activation of phospholipases D and $A_2$, and induction of the cyclooxygenase (COX) pathway, acting in concert with LPA receptor signaling in osteoblasts [26]. COX enzymes mediate the conversion of arachidonic acid into prostaglandin (PG) $E_2$ [26], shown to be induced by ATP release in response to mechanical stimulation in MC3T3-E1 cells [2]. We observed that LIPUS induced COX-2 expression via P2X7 (data not shown); therefore, the released ATP could activate P2X7 and upregulate LPA or $PGE_2$ via COX-2 activity. We hypothesized that LIPUS-induced ATP may stimulate these physiological responses and promote osteogenesis. Our results indicate that LIPUS stimulation for 1 and 3 min induced extracellular ATP release, which was reversed after 10 min. The half-life of osteoblast-released extracellular ATP has been reported to be 10 min [27]; however, the downstream events caused by LIPUS-induced ATP are likely to last longer [7]. Indeed, we showed that LIPUS stimulation for 30 min had maximum effect on the increase of RUNX2 and Osterix mRNA, the key transcriptional regulators of osteoblast differentiation, as evidenced by the study of

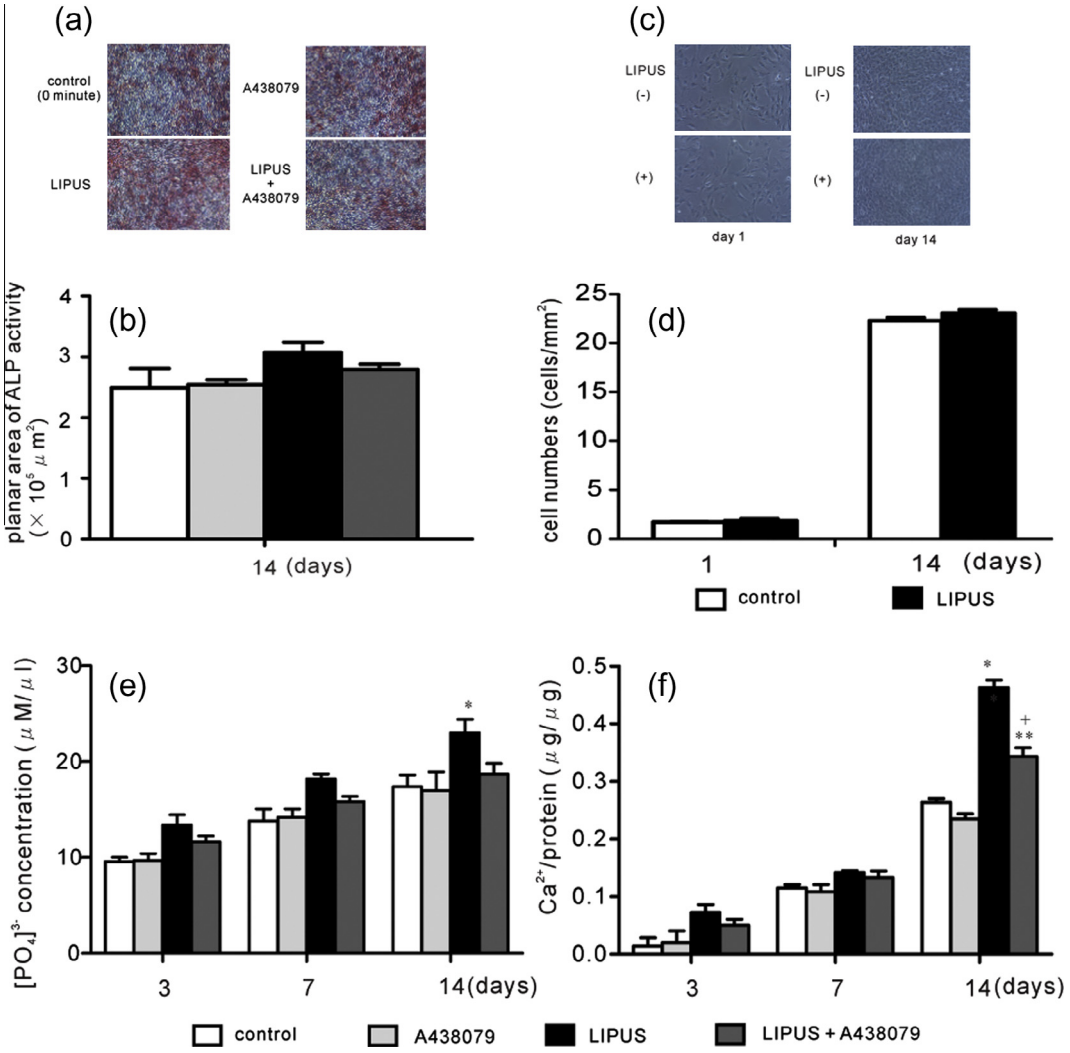


**Fig. 6.** Effects of LIPUS and A438079 on ALPase activity, extracellular phosphate concentration, cell viability, and calcium content in the ECM. MC3T3-E1 cells stimulated or not with LIPUS for 30 min per day during 14 days in the presence or absence of A438079 (10 μM) were fixed with 4% formalin and treated with 0.25 mM naphthol AS-MX phosphate and 1.25 mM Fast Red B salt to detect ALP activity. Cells were observed under a phase-contrast microscope (original magnification, × 100) (a); histograms show the percentage of ALPase activity under each condition (b). To determine cell viability, cells were stained with 0.4 w/v% trypan blue on days 1 and 14 of culture and observed under a phase-contrast microscope (original magnification, × 100) (c); histograms show the percentage of viable cells under each condition (d). Inorganic phosphate concentration was quantified using the Malachite Green Assay Kit (c). Calcium content in the ECM was determined using the Calcium E-Test kit (d). Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, LIPUS stimulation vs. control, $^{+}P < 0.05$, $^{++}P < 0.01$, LIPUS vs. LIPUS + A438079.

mice deficient in RUNX2 and Osterix expression [28]. A438079, a selective antagonist of the P2X7 receptor [19,20], blocked the expression of RUNX2 and Osterix induced by LIPUS. In rats, A438079 inhibited BzATP-mediated changes in intracellular calcium concentration through P2X7 but not other P2 receptors, confirming A438079 specificity to P2X7 [19]. Moreover, Nakashima et al. [28] have reported that an osteocalcin-encoding gene regulated by Osterix, is downregulated in P2X7$^{-/-}$ calvarial cells.

Type I collagen is one of the major protein constituents of the bone ECM; it also functions as a scaffold for the nucleation of hydroxyapatite crystals during the calcification process. Non-collagenous matrix proteins such as bone sialoprotein, osteopontin, and osteocalcin have important roles in the organization of the collagen matrix and in the regulation of the formation and growth of hydroxyapatite crystals [29]. Bone sialoprotein, similar to type I collagen, serves as a nucleation center for the hydroxyapatite formation in the bone [30]. Osteocalcin is the most abundant non-collagenous bone matrix protein [31]; this small γ-carboxyglutamate protein is selectively expressed by osteoblasts and binds calcium ions [32]. The functions of osteopontin in the bone formation and remodeling are diverse and directly related to its fundamental roles in host defense and tissue repair [33].

ALPase hydrolyzes ester bonds in organic phosphate compounds under alkaline conditions and plays an important role in bone calcification [34]. Previous our study showed that a one of the mechanical stimuli tension force affects ALPase activity in mineralization of osteoblasts [35]. In this study, we observed that LIPUS enhanced ECM protein expression and increased phosphate concentration and calcium content in the ECM and that A438079 blocked these LIPUS effects. Furthermore, P2X7 gene silencing inhibited LIPUS stimulatory effects on the expression of the genes encoding ECM proteins. However, LIPUS did not affect ALPase activity important in bone mineralization. LIPUS and tension force are different kinds of mechanical stimuli. Thus, it may cause different process of mineralized nodule formation induced by these stimulations.

Extracellular ATP concentrations depend on the balance between ATP release and its degradation by ectonucleotidases. The ATP-related increase in phosphate levels and mineral deposition in MC3T3-E1 cells observed here and in a previous

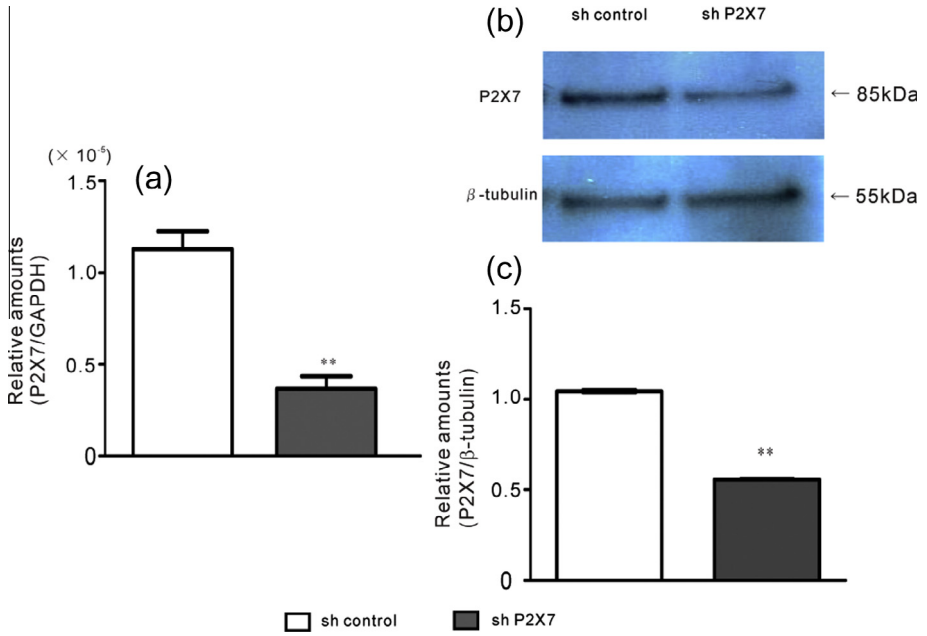


**Fig. 7.** Effects of P2X7-specific shRNA on P2X7 gene silencing in MC3T3-E1 cells. MC3T3-E1 cells were transfected with Lentiviral Particles containing P2X7-specific shRNA (shP2X7) or control scrambled shRNA (sh control). Total RNA and protein was purified from confluent cells, and the expression of P2X7 mRNA and protein was determined by real-time PCR (a) and Western blotting (b); histograms show the intensity of Western blotting bands under each condition (c). Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, sh P2X7 vs.sh control.

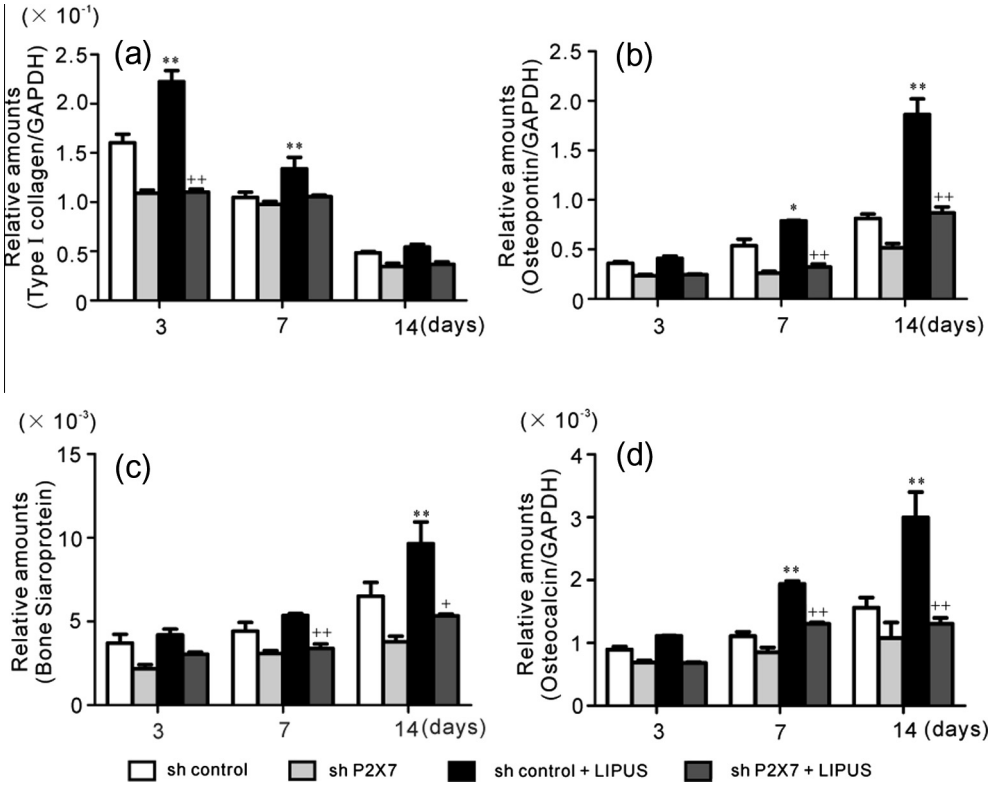


**Fig. 8.** Effects of LIPUS on mRNA expression of ECM proteins in P2X7-deficient osteoblasts. MC3T3-E1 cells transfected with P2X7 shRNA (sh P2X7) or scrambled shRNA (sh control) were stimulated or not with LIPUS for 30 min per day during 14 days and mRNA expression of type I collagen (a), bone sialoprotein (b), osteopontin (c), and osteocalcin (d) was determined by real-time PCR on days 3, 7, and 14 of culture. Data are expressed as the mean ± S.E.M. of three independent experiments performed in triplicate; $^{*}P < 0.05$, $^{**}P < 0.01$, sh control + LIPUS vs. sh control, $^{+}P < 0.05$, $^{++}P < 0.01$, sh P2X7 + LIPUS vs. sh control + LIPUS.

study, suggests that ATP-hydrolyzing enzymes such as transglutaminase 2 (TG2), contribute to the elevation of phosphate concentration. Indeed, ATPases have been shown to mediate mineralization without ALPase activity [36]; TG2 acts as ATPase in a calcium-dependent environment [37]. We observed that A438079 blocked phosphate deposition induced by LIPUS,

probably because its inhibition of the P2X7 receptor results in calcium decrease in the environment.

In summary, LIPUS induced the release of extracellular ATP, expression of osteogenesis-related transcription factors, and deposition of ECM proteins through the P2X7 receptor in osteoblast-like cells. Furthermore, LIPUS increased inorganic phosphate and mineral accumulation in the ECM; however, the activity of ALPase was not affected. Overall, our results suggest that in osteoblasts, LIPUS-induced ATP promotes bone formation via the P2X7 receptor independently of ALPase activity.

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