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Pertussis toxin-sensitive Gαi protein and ERK-dependent pathways mediate ultrasound promotion of osteogenic transcription in human osteoblasts[1]

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Abstract Bone cells respond to mechanical stimulation via mechanoreceptors and convert biophysical stimulation into biochemical signals that alter gene expression and cellular adaptation. Pulsed acoustic energy treatment raises membrane potential and induces osteogenic activity. How membrane-bound osteoblast mechanoreceptors convert physical ultrasound (US) stimuli into osteogenic responses is not fully understood. We demonstrated that low-intensity pulsed US treatment (200-μs pulse, 1 kHz, 30 mW/cm^2) elevated Cbfa1/Runx2 mRNA expression and progressively promoted osteocalcin mRNA expression in human osteoblasts. Pretreatment with pertussis toxin (PTX), but not with cholera toxin, suppressed US-augmented osteogenic transcription. This indicated that Gi proteins, but not Gs proteins, were involved in US promotion of osteogenic transcription. Further studies demonstrated US treatment could rapidly increase PTX-sensitive Gαi protein levels and subsequently enhanced phosphorylation of extracellular signal-regulated kinase (ERK). PTX pretreatment significantly reduced US promotion of ERK activation. Moreover, inhibition of ERK activity by PD98059 suppressed US augmentation of Cbfa1/Runx2 and osteocalcin mRNA expression. Membranous Gαi proteins and cytosolic ERK pathways acted as potent mechanosensitive signals in the response of osteoblasts to pulsed US stimulation.

Key words: Osteoblast; Pertussis toxin; Gαi protein; Extracellular signal-regulated kinase; Cbfa1/Runx2; Low-intensity pulsed ultrasound

1. Introduction

Bone cells perceive and respond to mechanical force via a mechanotransduction mechanism. Mechanoreceptors convert biophysical stimuli into biochemical responses that alter gene expression and cellular adaptation [1]. Mechanical intervention can elicit a proliferative response from bone cells. Because mechanical adaptive modeling response promotes bone tissue formation in fracture healing [2–4], extracorporeal shock waves, mechanical distraction and electromagnetic fields are regarded as non-invasive strategies for bone repair [5–7].

The acoustic energy of non-invasive, low-intensity pulsed ultrasound (US) has been shown to accelerate fracture healing [8–10]. US raises osteogenic responses of osteoblasts through induction of prostaglandin E_2 and osteogenic factors [11–13]. The role played by osteoblast mechanoreceptors in mediating physical US stimuli requires further study. Research has postulated that increasing current across the cell membrane by pulsed stimulation faeilitates tissue regeneration [14,15]. Cell membrane hyperpolarization is induced by mechanical strain and pulsed acoustic energy [16,17]. This implies that membrane perturbation potentially provokes membrane-bound signaling transduction, which in turn activates a cascade of osteogenic activity.

Guanine nucleotide regulatory proteins (G proteins) are a family of GTP-binding proteins that mediate signal transduction. Previous studies have demonstrated that G proteins were involved in strain-activated proliferation of human endothelial cells [18]. These molecules mediated steady/transient fluid shear stress-stimulated nitric oxide production [19,20], and flow-induced prostaglandin E_2 production by osteoblasts [21]. Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase family, mediated the transduction of mechanical stimulation into intracellular signals that regulate cell proliferation and differentiation [23,24]. We hypothesized that US promotes osteogenic activity through activation of membrane-bound G protein ERK.

The purposes of this study were to examine the effect of US on the osteogenic responses of human osteoblasts and to investigate whether US promotion of osteogenic activity is linked to activation of G proteins and ERK.

2. Materials and methods

2.1. Cell culture

Human fetal preosteoblastic cells (CRL-11372, American Type Culture Center, Manassas, VA, USA) were maintained in a mixture of phenol red-free Ham's F12 medium and Dulbecco's modified Eagle's medium (1:1) containing 10% fetal bovine serum and 2.5 mM L-glutamine (Life Technologies, Gaithersburg, MD, USA) in a 5% CO_2,

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34°C incubator for 6 days. Cells were harvested by trypsinization and resuspended in medium for studies.

2.2. Ultrasound treatment

Cells (5×10^5 cells/well, 6-well plate) were cultured for 48 h and were subjected to US with modifications as previously described [22]. Culture plates were floated in a thermostatically controlled water bath. A UV-sterilized transducer (Sonic Accelerated Fracture Healing System; Exogene, USA) that generated 1.5 MHz US in a pulsed-wave mode (200-μs pulse burst width with repetition frequency of 1 kHz at the intensity of 30 mW/cm^2) was immersed vertically into each culture well and placed to just touch the surface of the medium. The distance between the transducer and the cells was approximately 5–6 mm. Exposure time was 20 min for all cultures. Control samples were prepared in the same manner except that they were not exposed to US. Cells were harvested 30 min, 1, 3, 6, and 12 h after US treatment. To investigate the role of G protein in US promotion of osteogenic transcription, subconfluent cell cultures were pretreated with 2 ng/ml pertussis toxin (PTX) or cholera toxin (CTX; Sigma Chemicals, St. Louis, MO, USA) to block the G protein pathway for 24 h prior to US treatment. In some experiments, subconfluent cell cultures were pretreated with 20 μM PD98059 (Calbiochem, La Jolla, CA, USA) to inhibit ERK activity for 4 h before US treatment.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted and purified by the Tri reagent containing a monophasic solution of guanidine thiocyanate and phenol (Sigma Chemicals) from 10^6 cells with and without US treatment. One microgram of total RNA was reversely transcribed into cDNA, followed by PCR amplification using the following human gene-specific primers: Cbfa1/Runx2 (forward, 5′-GAA TGC TTC ATT CGC CTC ACA-3′; reverse, 5′-TGA CCT GCG GAG ATT AAC CAT-3′) (114-bp product expected); osteocalcin (forward, 5′-AAG AGA CCA AGG CGC TAC CT-3′; reverse, 5′-GCC GAT AGG CCT CCT TGA AAG-3′) (135-bp product expected); β-actin (forward, 5′-CGC CAA CCG CGA GAA GAT-3′; reverse, 5′-CGT CAC CGG AGT CCA TCA-3′) (168-bp product expected). The parameters for RT-PCR cycling were set as follows: RT reaction at 50°C for 2 min and at 60°C for 30 min; and PCR reaction at 95°C for 5 min; followed by 40 cycles of 94°C for 20 s and 60°C for 1 min. The PCR products were run on a 1.5% agarose gel electrophoresis containing ethidium bromide and visualized with UV-induced fluorescence. All signals were quantified by scan densitometry and the final value was obtained by calculating the Cbfa1/β-actin and osteocalcin/β-actin ratio values. The fold of promotion was calculated as the increase over the value of its corresponding control sample.

2.4. Preparation of membranous and cytosolic extracts

Membranous and cytosolic proteins of cell cultures were harvested as previously described [17]. Briefly, cells were lysed with ice-cold buffer C containing 10 mM Tris–HCl (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA and 0.7% NP-40 on ice for 10 min and then centrifuged at $500\times g$ for 5 min. Pellets were further lysed with buffer A containing 40 mM Tris–HCl (pH 7.9), 350 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM dithiothreitol (DTT), 2 μg/ml leupeptin and 1 μg/ml aprotinin on ice for 20 min and centrifuged at $12000\times g$, at 4°C for 10 min. Pellets were dissolved in buffer A and protein concentrations of extracts were determined by a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. ADP-ribosylation

ADP-ribosylation of membranous protein was determined as previously described [25]. 40 μg of membranous protein was incubated for 30 min at 25°C with 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 25 mM [γ-^{32}P]NAD (10 Ci/mmol; Amersham Life Sciences, Aylesbury, UK), 2 mM $MgCl_2$, 20 mM ADP-ribose and 20 μg/ml PTX (pre-activated with 10 μM DTT for 30 min at 32°C; Sigma Chemicals). The reaction was terminated by addition of 10 mM Tris–EDTA, 1 mM EDTA, and 50 mM K_2PO_4 (pH 8.0) and the membranous proteins were harvested by centrifugation at $16000\times g$ for 10 min at 4°C. Membranous proteins were dissolved in Laemmli buffer containing 200 mM Tris–HCl (pH 6.8), 10% glycerol, 4% SDS, 50 μM DTT and 0.05% bromophenol blue for 5 min at 95°C, and separated by electrophoresis through 12% polyacrylamide gels. Gels were then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Hoefer Pharmacia Biotech, CA, USA). Following transfer, membranes were rinsed, air-dried and autoradiographed with an intensifying screen. Gαi-1/3, Gαi-2 and Gαs proteins on the membranes were further recognized by specific mouse anti-Gαi-1/3, anti-Gαi-2 and anti-Gαs antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by goat anti-mouse horseradish peroxidase-conjugated IgG as the second antibody. Bands were visualized using chemiluminescence agents (SuperSignal®; Pierce, Rockford, IL, USA). Autoradiographs and Western blots from the same PVDF membranes were then aligned for band comparison and identification.

2.6. Measurement of ERK activation

Cytosolic extracts were incubated with anti-ERK antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at 4°C. After incubation, immune complexes were precipitated with protein A (Sigma Chemicals) [26]. ERK activation was measured with an in vitro ERK phosphorylation kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, the ERK immunoprecipitate (20 μg) reacted with substrate buffer containing 20 μg myelin basic protein (MBP), 15 mM $MgCl_2$, 100 μM ATP and 5 μM protein kinase inhibitor for 30 min at 30°C. The reaction was stopped with Laemmli buffer for 5 min at 95°C. Mixtures were subjected to Western blot assay. Phosphorylated MBP on the blot was recognized by a specific mouse anti-phospho-MBP antibody, followed by goat anti-mouse horseradish peroxidase-conjugated IgG as the second antibody. The ERK activity was indicated by phosphorylated MBP detected by chemiluminescence agents [26].

2.7. Statistical analysis

All values were analyzed; Student's paired *t*-test was used to evaluate the difference between the sample of interest and its respective control. For time course analysis, multiple ANOVA was used. A *P* value of <0.05 was considered significant.

3. Results

3.1. US treatment increased Cbfa1/Runx2 and osteocalcin mRNA expression

We first determined whether US augmented Cbfa1/Runx2 and osteocalcin gene expression in osteoblasts. Cell cultures were treated with US for 20 min. RT-PCR results indicated that Cbfa1/Runx2 mRNA expression significantly increased within 1 h and 12 h after treatment, peaking at 3 h (Fig. 1). Similarly, osteocalcin mRNA expression of cell culture was significantly elevated 6 h after US treatment (Fig. 1).

3.2. Pretreatment with PTX, but not with CTX, reduced US promotion of osteogenic transcription

To determine whether the G protein pathway was involved in US promotion of Cbfa1/Runx2 and osteocalcin transcription, cell cultures were pretreated with PTX or with CTX to

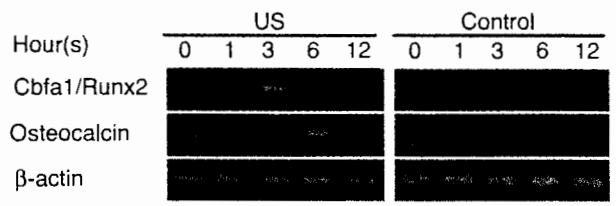

Fig. 1. Low-intensity pulsed US treatment promoted Cbfa1/Runx2 mRNA expression and subsequently increased osteocalcin mRNA expression. Human osteoblasts (5×10^5 cells/well, 6-well plate) were treated with 1.5 MHz US (200-μs pulse, 1 kHz, 30 mW/cm^2) for 20 min. Cells were subjected to assessment of Cbfa1/Runx2 and osteocalcin mRNA 0, 1, 3, 6 and 12 h after US treatment. After standardization of housekeeping gene expression, equal amounts of cDNA from each sample were subjected to 40 cycles of PCR to amplify Cbfa1/Runx2 and osteocalcin mRNA expressions.

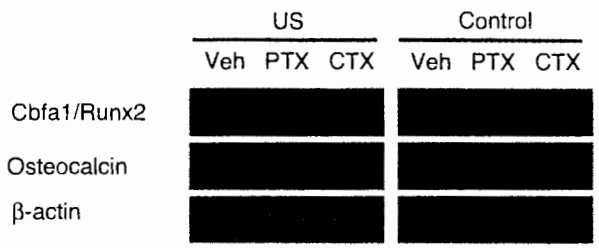

Fig. 2. Pretreatment with PTX, but not with CTX, significantly reduced US promotion of Cbfa1/Runx2 and osteocalcin mRNA expression. Osteoblasts were pretreated with or without 2 ng/ml PTX or CTX and subjected to US stimulation for 20 min. Expression of Cbfa1/Runx2 and osteocalcin mRNA was determined 3 and 6 h after US treatment, respectively.

block Gi or Gs protein activity. PTX pretreatment significantly reduced US-promoted Cbfa1/Runx2 mRNA expression in 3 h and osteocalcin mRNA expression in 6 h (Fig. 2). CTX pretreatment did not significantly alter US promotion of Cbfa1/Runx2 and osteocalcin mRNA expression (Fig. 2). This indicated that Gi proteins were involved in physical US promotion of osteogenic transcription.

3.3. *US treatment increased PTX-sensitive Gαi protein levels and ERK phosphorylation*

In vitro ADP-ribosylation was used to determine whether US increased PTX-sensitive Gi protein levels in osteoblasts. Membrane proteins were prepared and were subjected to ADP-ribosylation assay in the presence of [γ-^{32}P]NAD and activated PTX. Autoradiographs showed that US treatment significantly increased levels of PTX-sensitive 41-kDa molecules, but not of 46-kDa or 40-kDa molecules (Fig. 3A, left panel). We identified Gαi protein subunits and Gαs proteins by overlay and alignment of autoradiographs and Western blots from the same PVDF membranes. The 41-kDa, 40-kDa, and 46-kDa bands corresponded to Gαi-1/3, Gαi-2 (Fig. 3A, middle panels) and Gαs proteins (Fig. 3A, right panel), respectively. Our observation agrees with typical published values [18,25]. Kinetic changes in PTX-sensitive Gαi protein levels in US-treated osteoblasts are shown in Fig. 3B. PTX-sensitive Gαi protein levels were significantly increased within 30 min and 6 h after US treatment. Experiments were next carried out to determine which Gαi protein subunit (Gαi-1, Gαi-2 and Gαi-3) was involved in US-augmented PTX-sensitive Gαi proteins. Immunoblotting showed that osteoblasts subjected to US treatment had elevated Gαi-1/3 protein levels, which corresponded to the 41-kDa band. US stimulation did not alter Gαi-2 protein levels throughout the study period (Fig. 3C). Our recent study has demonstrated that acoustic energy stimulates ERK-dependent osteogenic differentiation of bone marrow mesenchymal cells in rats [26]. We investigated whether US stimulation could increase ERK phosphorylation of human osteoblasts. ERK immunoprecipitates harvested from cytosolic proteins were subjected to kinase activity assay using MBP as a substrate. Phosphor-

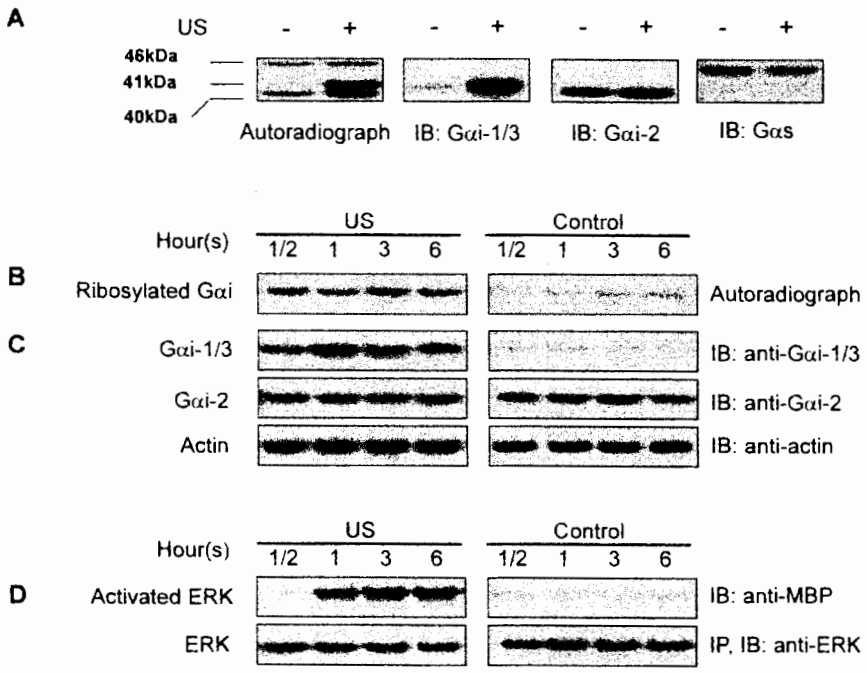

Fig. 3. Increase of membranous PTX-sensitive Gαi protein levels and activation of cytosolic ERK. A: US increased levels of PTX-sensitive 41-kDa molecules, but not 40-kDa or 46-kDa molecules (left panel). The 41-kDa, 40-kDa and 46-kDa molecules corresponded to Gαi-1/3, Gαi-2 (middle panels) and Gαs proteins (right panel), respectively. Membranous proteins were PTX catalyzed ADP-ribosylation in the presence of [γ-^{32}P]NAD. Reaction products were separated by SDS-PAGE. Gels were electrotransferred onto PVDF membranes. Membranes were subjected to autoradiography. Gαi-1/3, Gαi-2 and Gαs proteins on the same membranes were probed by anti-Gαi-1/3, anti-Gα-2 and anti-Gαs antibodies and were visualized using chemiluminescence agents. B: US enhanced PTX-sensitive Gαi protein levels in 30 min. C: US elevated Gαi-1/3 protein levels in 30 min. US did not alter Gαi-2 protein expression throughout the study period. Immunoblotting of actin showed equal loading and transfer for all lanes. D: US progressively increased cytosolic ERK phosphorylation in 1 h. In vitro assay of phosphorylated MBP was used to measured phosphorylation of cytosolic ERK immunoprecipitate. Immunoblotting of ERK showed equal loading and transfer for all lanes.

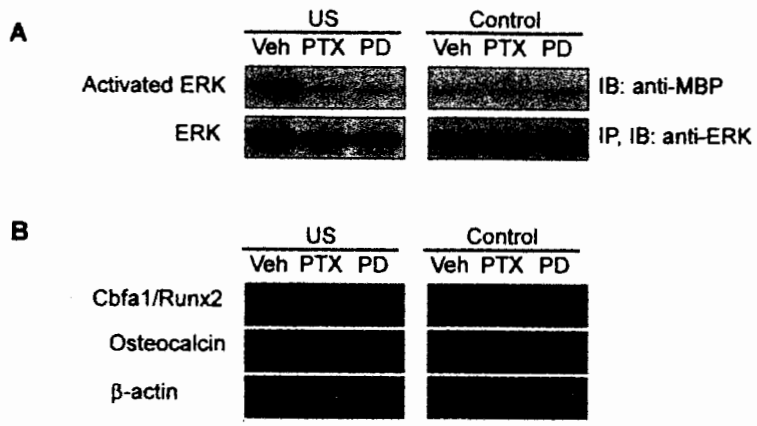

Fig. 4. Effects of PTX and PD98059 on ERK activation and osteogenic transcription of US-treated osteoblasts. A: Pretreatment with PTX and with PD98059 (PD) suppressed US promotion of ERK phosphorylation. In vitro assay of phosphorylated MBP was used to measure cytosolic ERK phosphorylation. B: PTX and PD98059 significantly reduced Cbfa1/Runx2 and osteocalcin mRNA expression as determined by RT-PCR.

ylated MBP immunoblotting indicated that US subsequently increased cytosolic ERK activation in 1 h (Fig. 3D).

3.4. Pretreatment with PTX and with PD98059 blocked US-augmented ERK activation

We elucidated the role of Gαi proteins and ERK in the US promotion of osteogenic transcription. PTX pretreatment decreased US-augmented ERK phosphorylation. PD98059 pretreatment blocked US-stimulated ERK activity (Fig. 4A). PTX inhibition of Gαi protein and PD98059 blockade of ERK activity downregulated US-promoted Cbfa1/Runx2 and osteocalcin mRNA expression (Fig. 4B). These results suggest that Gαi proteins have an important role in mediating ERK-dependent US enhancement of osteogenic transcription.

4. Discussion

Elevation of osteogenic transcription followed pulsed US treatment that activated membrane-bound Gαi protein and cytosolic ERK. While a number of studies have suggested that US promotes proliferation and differentiation of osteogenic cells [11,12,27], little has been done to define the role of G proteins in mediating physical US stimuli. Our findings provide the first indication that the Gαi protein → ERK → Cbfa1/Runx2 → osteocalcin pathway is involved in US promotion of osteogenic transcription. Raising US-sensitive signal transduction and osteogenic transcription of osteoblasts is proposed as a further explanation for the clinical success of US treatment on promotion of fracture healing.

Runt domain-related Cbfa1/Runx2 is a critical component in bone formation and an important transcriptional regulator in osteoblasts [28]. Activation of Cbfa1/Runx2 increases osteocalcin and collagen I gene expression [29,30]. This molecule is also a target of a mechanical signal by which physical stimulation dictates the cellular and metabolic activities of osteoblasts [31]. In this study, we have demonstrated that osteoblasts respond to US treatment by increasing Cbfa1/Runx2 and osteocalcin mRNA expression. This suggests that osteoblasts convert physical US stimuli into biological responses that increase osteogenic transcription. Our findings provide prominent molecular evidence that explains US-promoted osteogenic differentiation of osteoblasts and mesenchymal stem cells.

The mechanisms underlying US-augmented mechanotransduction of osteogenic activity in osteoblasts are not well understood. In our study, we provide the first evidence that US stimulation increases PTX-sensitive Gαi protein levels. Gαi-1/3 proteins are, at least in part, involved in US-augmented osteogenic activity. Previous studies have reported that G proteins act as potential mitogenic regulators of fibroblasts [32,33]. G protein activation has been found to be involved in catecholamine- and fluoroaluminate-stimulated alkaline phosphatase activity and proliferation of osteoblasts [34,35]. In the current study, we found that PTX suppressed US-stimulated osteogenic transcription, indicating that Gαi proteins mediate the effects of physical US stimuli by acting as potent mechanoreceptors for osteoblasts. It is not presently known by which mechanisms US activates Gαi proteins in osteoblasts. A previous study has postulated that pulsed stimulation by US raises current across the cell membrane to increase anabolic responses in cells [36]. We have previously reported that pulsed acoustic energy induces membrane hyperpolarization of osteogenic cells [17], implying that alteration of membrane potential may activate membrane-bound Gi proteins. Physical stimulation of membrane perturbation has been reported to activate mechanosensitive molecules such as ion channels and integrins [37,38]. These bio-active molecules are probably involved in activating Gαi proteins in osteoblasts after US stimulation.

We also found that US treatment rapidly increased membrane-bound Gαi protein activation and subsequently transduced stimuli to activate cytosolic ERK. Previous studies have demonstrated that ERK plays a critical role in regulating fluid movement-stimulated osteoblast proliferation [39]. Gi proteins are involved in ERK activation in growth factor-stimulated osteoblast proliferation [40]. Our research further indicates that PD98059 inhibition of ERK significantly reduces US-augmented osteogenic transcription, suggesting osteoblasts convert pulsed acoustic energy into osteogenic signals via ERK activation. These findings agree with those in our previous studies, which demonstrated that bone marrow mesenchymal stem cells responded to pulsed acoustic energy by increasing ERK phosphorylation and enhancing osteogenic

differentiation [17,26]. Moreover, intracellular signals that promote osteoblast growth and differentiation, including those mediated by bio-active radicals such as nitric oxide, prostaglandins and calcium, may occur in response to cellular homeostatic disturbance induced by US [11–13,22]. These data indicate that multiple signal transduction pathways are responsible for US-promoted osteogenic activity of osteoblasts. Mesenchymal stem cells including osteogenic precursor cells and perivascular progenitor cells have the potential to differentiate into bone-forming cells [41–43]. Nevertheless, we cannot exclude the possibility that pericytes in mesenchymal stem cells contribute to the US promotion of osteogenesis. Future studies need to evaluate what roles these molecules and cell populations play in the regulation of osteogenic transcription by osteogenic cells in response to pulsed US treatment.

In summary, pulsed US treatment rapidly activated membrane-bound Gαi protein and cytosolic ERK. These molecules play an essential role in regulating US-promoted osteogenic transcription of osteoblasts. Physical US treatment provides a promising regimen for regulating osteogenic activity. US stimulation may not only be applicable for enhancing osteogenesis but also provide an alternative non-invasive method for ex vivo extension of mesenchymal stem cells and tissue regeneration in the future.

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