

Mechanical strain promotes osteogenic differentiation of bone mesenchymal stem cells from ovariectomized rats via the phosphoinositide 3-kinase/Akt signaling pathway

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Abstract. Osteoporosis has become an overwhelming public health problem worldwide. As an elementary physiological factor to regulate bone formation and regeneration, mechanical strain may be used as a non-invasive intervention in osteoporosis prevention and treatment. However, little is known regarding the underlying mechanism. The aim of the current study was to investigate the effect of continuous mechanical strain (CMS) on osteogenic differentiation of bone mesenchymal stem cells (BMSCs) from ovariectomized rats (OVX BMSCs). In addition, involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in biomechanical signal transduction and its function were evaluated. The results demonstrated that OVX BMSCs subjected to CMS exhibited higher alkaline phosphatase (ALP) activity and deeper staining at 24 and 48 h. In addition, CMS upregulated the mRNA expression levels of ALP, collagen type I, runt related transcription factor 2 (Runx2), as well as the protein expression level of Runx2 in a time-dependent manner. The PI3K/Akt signaling pathway was rapidly activated by CMS, with its phosphorylation level reaching its maximum in a short duration and a large quantity of phosphorylated-Akt remaining in the nucleus. Pre-treatment with a selective blocker significantly blocked the strain-induced activation of PI3K/Akt and reduced the commitment of OVX BMSCs into osteoblasts, demonstrating a dominated regulative effect

of PI3K/Akt signaling in strain-induced osteogenesis. These results indicated that CMS induced the early differentiation of OVX BMSCs towards an osteogenic phenotype by activating the PI3K/Akt signaling pathway.

Introduction

Osteoporosis, a condition characterized by a reduction in bone mass and strength, is associated with increased risks for fracture. It has become an overwhelming public health problem worldwide, particularly in postmenopausal women (1). The average bone loss during the five years around the menopause or perimenopause reaches 15%, and postmenopausal women with low bone density are very likely to be predisposed to fractures (2,3). Currently, pharmacological interventions for osteoporosis are classified into antiresorptive agents that prevent bone resorption, such as hormone replacement therapy, bisphosphonates and denosumab, and anabolic agents, which help with formation of new bones, including strontium and teriparatide. However, the efficacy of certain drugs is limited by perceived intolerance and long-term adverse events (4-6). Mechanical strain is known as the elementary physiological factor that regulates bone formation and regeneration, as well as maintaining the integrity of bone structure and function. Evidence indicated that physical exercise may improve skeletal resistance to bone fracture, and delay the progress of osteoporosis by enhancing bone mass and strength (7,8). Therefore, physical activity may be used as a non-invasive intervention in osteoporosis prevention and treatment. However, little is known about the specific mechanism that regulates bone remodeling in osteoporosis.

Bone mesenchymal stem cells (BMSCs) are force-sensitive cells capable of detecting, transducing and responding to an extracellular stimulus, and thus differentiate into multiple cell lineages (9,10). Evidence indicates that the osteogenic ability of BMSCs is key in bone remodeling. The alterations in BMSCs associated with estrogen reduction may result in the attenuated regenerative ability of bone, which consequently results in osteoporosis. Additionally, BMSCs are proposed to be of great importance in the response of bone to mechanical stimulation (11-13). However, few studies focused on the signaling

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pathway involved in bio-mechanical transduction of BMSCs from ovariectomized rats (OVX BMSCs) *in vitro*. Thus, such studies regarding the effect of mechanical strain on OVX BMSCs may elucidate the mechanism of bone remodeling in osteoporosis.

It is well known that the signal transduction initiated by external chemical or mechanical stimulation is important in regulating bone development (14). The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is one of the most common signaling pathways that has been identified to be implicated in BMSC proliferation and differentiation by modulating the transcriptional activity of downstream genes (15). In addition, there is substantial evidence that the PI3K/Akt signaling pathway is essential for human and murine MSC osteogenesis *in vitro* (16,17). However, whether the PI3K/Akt signaling pathways is involved in the response of OVX BMSCs to mechanical strain has not, to the best of our knowledge, been thoroughly investigated. Therefore, by adopting an FX-4000TTM Tension PlusTM system, the mechanical environment of BMSCs *in vivo* was mimicked in the present study. Furthermore, the effect of continuous mechanical strain (CMS) on osteogenic differentiation of OVX BMSCs, and the involvement and function of the PI3K/Akt signaling pathway in biomechanical signal transduction were investigated.

Materials and methods

Animals and cell culture. The current study was conducted in accordance with the regional Ethics Committee guidelines. Sixty female Sprague-Dawley rats (age, 6 weeks), weighing an average of 200 g, were obtained from Shanghai SLAC Experimental Animal Center (Shanghai, China). The animals underwent surgical ovariectomy according to FDA guidelines (18). The rats were then housed separately in a temperature-controlled room at 21°C with relative humidity at 60% under a 12-h light/dark cycle. Then, 12 weeks after ovariectomy, all rats were sacrificed. The humeri and tibiae were isolated from the OVX rats. The bone marrow was flushed out using Dulbecco's modified Eagle's essential medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone; GE Healthcare Life Sciences). Non-adherent cells were removed by replacing the medium after 72 h and it was subsequently refreshed every 3 days. On reaching 70–80% confluence, the cells were trypsinized with 10% trypsin-EDTA (Hyclone; GE Healthcare Life Sciences) and passaged. OVX BMSCs from passages 2 to 5 were used during the experiments.

Application of CMS. CMS of 10% elongation at a frequency of 1 Hz was applied using an FX-4000TTM Flexercell Tension PlusTM unit (Flexcell International Corp., Burlington, NC, USA). BMSCs were plated on Flexcell 6-well silicone rubber plates at a density of 2×10^4 /cm². After 24–48 h incubation, the cells had attached and reached ~80% confluence. The BMSCs were then subjected to CMS for 48 h.

Alkaline phosphatase (ALP) staining and relative ALP activity detection. The presence of ALP in the cell layers was assessed according to the manufacturer's protocol of the

5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium Alkaline Phosphatase Color Development kit (cat. no. C3206; Beyotime Institute of Biotechnology, Haimen, China) and described as follows. The OVX BMSCs were rinsed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 15 min. Coloration was then assessed and observed with a digital camera (Eclipse TS100; Nikon Corporation, Tokyo, Japan). The relative ALP activity was detected according to the manufacturer's protocol with the Alkaline Phosphatase Assay kit (Beyotime Institute of Biotechnology). After exposing to CMS for 24 and 48 h, samples from all groups were washed twice with double-distilled water and lysed via sonification. Cell lysates were incubated with p-nitrophenol phosphate (Beyotime Institute of Biotechnology) at 37°C for 1 h. The enzymatic reaction was stopped using 1 M sodium hydroxide and absorbance was measured at a wavelength of 405 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of the cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's recommended protocol. The RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) and cDNA was synthesized using a cDNA Synthesis Reverse Transcription kit (cat. no. RR037A; Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using a Light-Cycler system with SYBR Premix Ex TaqTM (RR420A, Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. The conditions of the qPCR were as follows: Denaturation at 95°C for 10 sec, and 50 cycles of 95°C for 10 sec and 60°C for 30 sec, with a final dissociation stage (95°C for 5 min) to complete the amplification procedure. β -actin served as an internal control. The data were analyzed using comparative C_q ($2^{-\Delta\Delta C_q}$) method and expressed as a fold-change respective to the control (19). Each sample was analyzed in triplicate. The primer sequences used in the current study are presented in Table I.

Western blotting. The cells were lysed on ice for 30 min in SDS lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors. For western blot analysis, 20 µg sample was resolved on a 10% SDS-PAGE gel and electro-transferred onto nitrocellulose membranes with a constant voltage of 90 V and duration of 70 min (Whatman, GE Healthcare Life Sciences). The following primary antibodies were used: Anti-runt related transcription factor 2 (Runx2; cat. no. 12256; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-Akt (cat. no. ab8805; 1:1,000; Abcam, Cambridge, MA, USA) and anti-p-Akt (cat. no. ab38449; 1:1,000; Abcam). For the normalization of protein loading, a GAPDH antibody (cat. no. 5174; Cell Signaling Technology, Inc.) was used at a dilution of 1:2,000. Horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:5,000 (cat. no. ab6721; Abcam). The antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Detection system (EMD Millipore, Billerica, MA), according to the manufacturer's protocols. Protein band intensities on the scanned films were compared to their respective controls using Alpha Image software.

Table I. Reverse transcription-quantitative polymerase chain reaction primer sequences for target genes.

| Gene | Forward (5'-3') | Reverse (5'-3') |
|-------------------------------------|-------------------------|-------------------------|
| β -actin | CACCCGCGAGTACAACCTTC | CCCATACCCACCATCACACC |
| Alkaline phosphatase | TATGTCTGGAACCGCACTGAAC | CACTAGCAAGAAGAAGCCTTTGG |
| Type I collagen | CAGGCTGGTGTGATGGGATT | CCAAGGTCTCCAGGAACACC |
| Runt related transcription factor 2 | ATCCAGCCACCTTCACTTACACC | GGGACCATTGGGAAGTATAGG |

Inhibition of the PI3K/Akt signaling pathway. In order to assess the role of the PI3K/Akt signaling pathway in the strain-induced differentiation of OVX BMSCs, the selective inhibitor, LY294002 was used. Preliminary experiments indicated that the optimum concentration of LY294002 was 10 μ M. Cells were pre-treated with inhibitors for 1 h prior to application of the strain stimulus, and they were present during the entire strain application.

Immunofluorescence analysis. Subsequent to mechanical loading, cells were fixed with 4% paraformaldehyde for 10 min, then washed with PBS and incubated in 0.1% Triton X-100 for 15 min at room temperature and then blocked with 5% bovine serum albumin for 1h at room temperature. The prepared samples were incubated overnight at 4°C with rabbit monoclonal anti-phosphorylated (p)-Akt (Ser473; dilution, 1:300) or rabbit monoclonal anti-Akt (dilution, 1:300) that were obtained from Cell Signaling Technology, Inc., and detected with Alexa 594 conjugate (dilution, 1:200; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. Nuclei were labeled with 1 mg/ml Hoechst for 10 min at room temperature (Roche Diagnostics, Basel, Switzerland). Slides were examined under an Olympus IX71 fluorescent microscope. At least three overview images were obtained from three independent experiments.

Statistical analysis. All experiments were performed a minimum of three times and data are expressed as means \pm standard deviation. Differences between two groups were identified using unpaired t-tests. Significant differences between the non-load and multiple stretch groups were determined using a one-way analysis of variance followed by the Least Significant Difference post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of CMS on osteogenic differentiation of OVX BMSCs. After exposure to CMS, OVX BMSCs demonstrated higher ALP activity and deeper staining at 24 and 48 h when compared with the non-loaded OVX BMSC group (Fig. 1A and B). CMS upregulated the mRNA expression levels of osteogenesis-associated markers of OVX BMSCs, ALP, type I collagen (COL I) and Runx2, as they began to increase significantly at 4 or 6 h after exposure to CMS, and reached to a peak value at 24 or 48 h (Fig. 1C). Additionally, the protein expression level of Runx2 was elevated in a time-dependent manner in OVX BMSCs compared with the non-loaded group, with a significant increase at 4 and 6 h (Fig. 1D and E).

Effects of CMS on induction of the PI3K/Akt signaling pathway. The activation time course of the PI3K/Akt signaling pathway was investigated in OVX BMSCs subjected to CMS. As demonstrated in Fig. 2A and B, Akt was significantly phosphorylated soon after the onset of stimulation and peaked at 15 min. The phosphorylation level subsequently declined gradually, but remained higher than the non-loaded group at 30 min. After 1 h of loading, the levels of p-Akt returned almost to baseline or were lower than the control group. The cellular localization of Akt and p-Akt was also examined by immunofluorescence analysis (Fig. 2C and D) and the nuclei were co-stained by Hoechst. Following CMS stimulation, Akt staining was performed with Akt antibodies, and observed in the cytoplasm and the nucleus at 1 and 4 h. p-Akt staining was more strongly expressed in the nucleus than in the cytoplasm at 1 h, and became markedly weaker at 4 h.

Effects of a PI3K/Akt inhibitor on CMS-induced OVX BMSCs. The above-mentioned findings indicate the activation of the PI3K/Akt signaling pathway by CMS. After demonstrating that PI3K/Akt may be involved in the mechanotransduction of CMS, its function in CMS-induced osteogenesis of OVX BMSCs was further investigated in the current study via pharmacological inhibition. LY294002 was used to block the activation of p-Akt in OVX BMSCs. Fig. 3A and B demonstrated that pre-treatment with Akt-specific inhibitor significantly blocked the phosphorylation of Akt and had no cytotoxic effect on the cells (data not shown). Immunofluorescence analysis of p-Akt co-confirmed that the activation of p-Akt was blocked by LY294002 treatment as p-Akt were predominantly stained in the cytoplasm (Fig. 3C and D).

Effects of a PI3K/Akt inhibitor on osteogenic differentiation of OVX BMSCs. Subsequently, CMS-induced osteogenesis of OVX BMSCs was assessed using a PI3K/Akt inhibitor. Pretreatment with LY294002 inhibited CMS-stimulated ALP activity (Fig. 4A and B). Furthermore, the CMS-induced mRNA expression levels of ALP, Col I and Runx2 were significantly repressed at 24 and 48 h (Fig. 4C). Similarly, as presented in Fig. 4D and E, the CMS-induced Runx2 protein expression level was attenuated by LY294002. These results indicate that the PI3K/Akt signaling pathway is responsible for the CMS-induced osteogenesis of OVX BMSCs.

Discussion

Recent studies have demonstrated that mechanical stimuli are essential for the differentiation of stem cells into different lineages. Lack of mechanical stress significantly attenuates

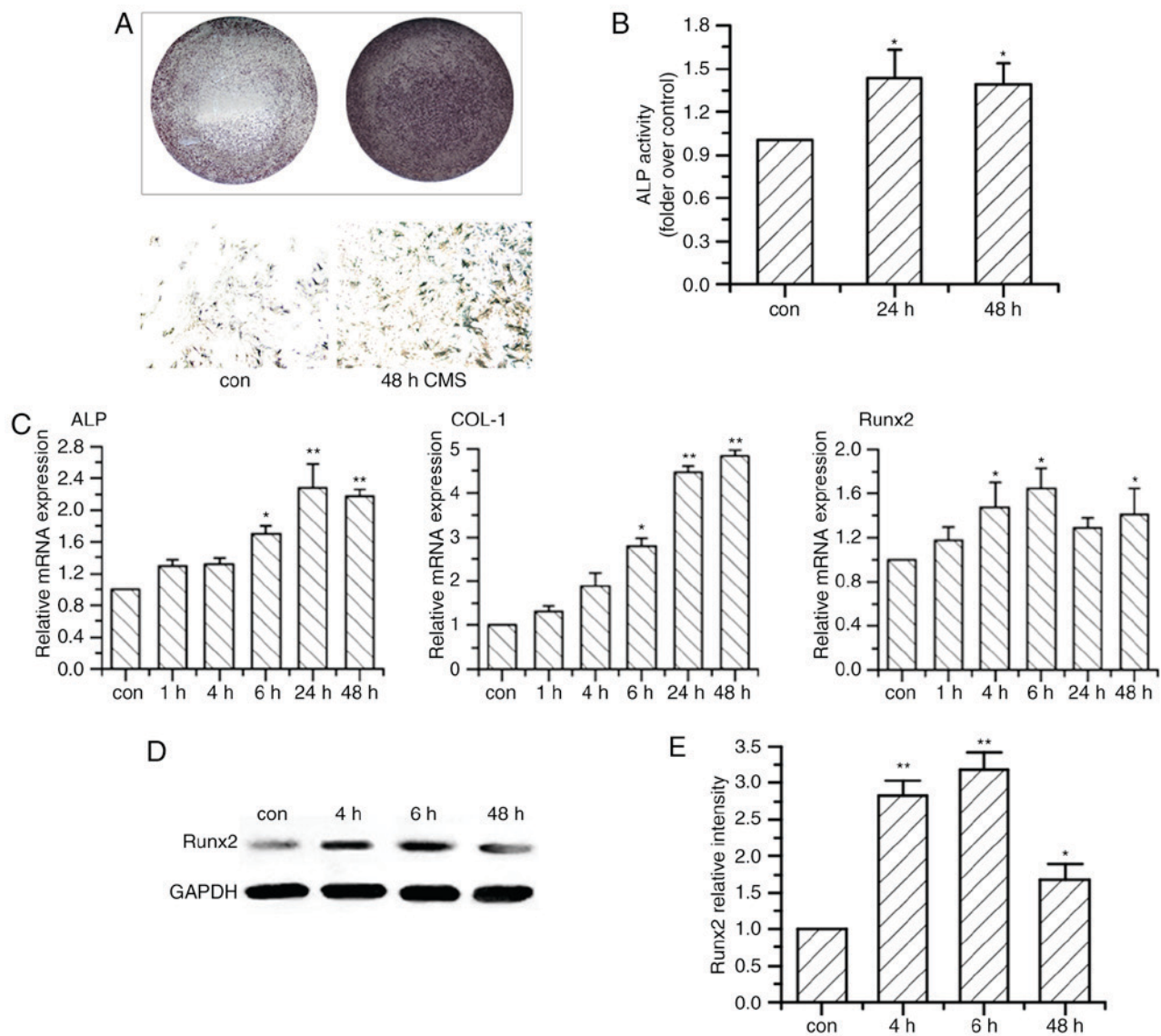


Figure 1. Effects of CMS on the osteogenic differentiation of OVX BMSCs. (A) ALP staining at 48 h vs. non-loaded control group (magnification, x200) and (B) ALP activity changes at 24 and 48 h vs. non-loaded control group. (C) mRNA changes of specific osteoblastic genes of OVX BMSCs at different time points following CMS. (D and E) Changes of Runx2 protein expression level following CMS. Each experiment was performed three separate times and the results are presented as means \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. non-loaded control group of OVX BMSCs. CMS, continuous mechanical strain; BMSCs, bone mesenchymal stem cells; OVX BMSC, ovariectomized rat BMSC; ALP, alkaline phosphatase; Runx2, runt related transcription factor 2; COL I, type I collagen; con, control group.

the differentiating capability of BMSCs into osteoblasts, which may lead to disuse osteoporosis (20,21). Characterized by decreased bone strength, osteoporosis is a chronic disease that easily predisposes individuals to fractures (22). As BMSCs are the progenitor cells of osteoblast cells, they are crucial in bone remodeling (23,24). The current study was designed to evaluate the effects and specific underlying mechanism of CMS on the osteogenic differentiation of OVX BMSCs, with the aim of improving treatment strategies for osteoporosis.

BMSCs from osteoporosis patients exhibited longer population doubling duration. In addition, ovariectomy alters the synthesis of mineralized matrix and gene expression markers associated with osteogenic differentiation in BMSCs, and thus results in the reduction of the osteogenic potential (25,26). Although our previous studies indicated that the ability of osteogenic lineage commitment of OVX BMSCs was

weaker than sham BMSCs under the exposure of intermittent mechanical strain; the current study demonstrated that OVX BMSCs exposed to CMS underwent osteoblastic differentiation when compared with non-loaded OVX BMSCs (27). ALP activity and expression levels serve as indicators of osteoblastic activity. Extracellular matrix molecules, such as COL I, are considered to be of great importance in osteoblast proliferation and differentiation. Additionally, Runx2 has been shown to be significant in regulating osteogenic differentiation (28). In the current study, the mRNA expression levels of ALP, COL I and Runx2 were enhanced in OVX BMSCs. Furthermore, OVX BMSCs subjected to CMS demonstrated higher ALP activities and deeper staining at 24 and 48 h when compared with the non-loaded OVX BMSC group. In addition, the protein expression level of Runx2 was increased at 4 and 6 h. These results demonstrated that OVX BMSCs underwent osteoblastic differentiation due to CMS.

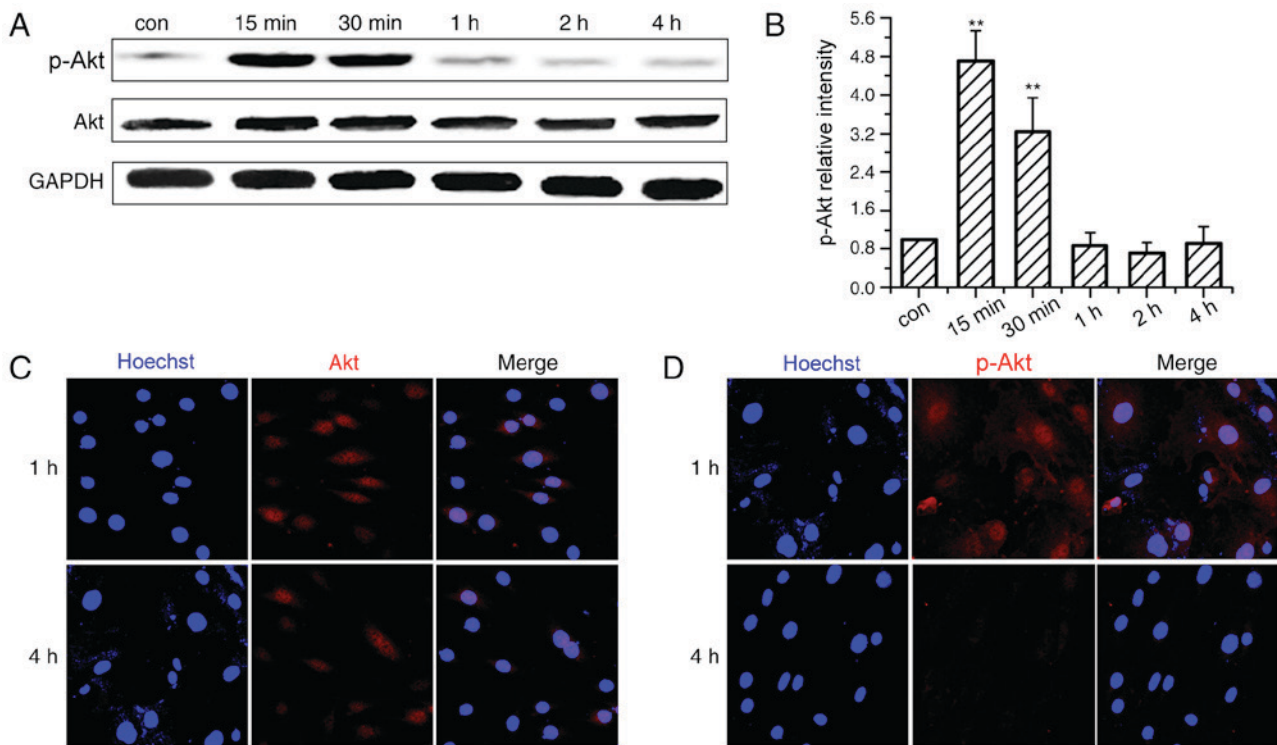


Figure 2. Effects of CMS on induction of the phosphatidylinositol 3-kinase/Akt signaling pathway. (A) Phosphorylation of Akt was examined by western blotting after CMS at the indicated time points. (B) Phosphorylated protein expression levels were normalized to their respective total protein levels. Immunofluorescent localization of (C) Akt and (D) p-Akt in ovariectomized rat bone mesenchymal stem cells subsequent to CMS (magnification, x200). Akt and p-Akt were labeled with an Alexa 594-conjugated antibody (red) and nuclei were counterstained with Hoechst (blue). The images demonstrate a typical example after 1 and 4 h of CMS. Values are presented as means \pm standard deviation (n=3). **P<0.01 vs. con group. CMS, continuous mechanical strain; p-, phosphorylated; con, control group.

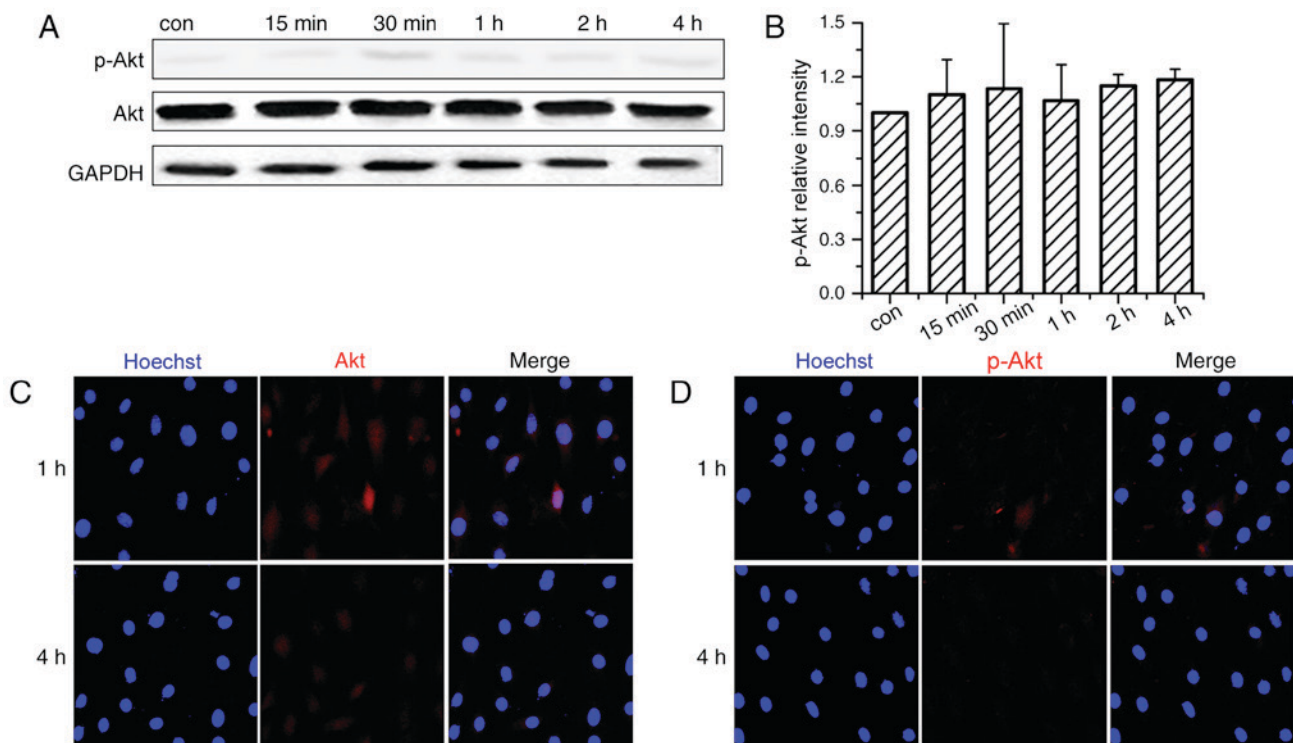


Figure 3. Involvement of a phosphatidylinositol 3-kinase/Akt inhibitor in OVX BMSCs under CMS. (A) Protein expression levels of Akt and p-Akt in OVX BMSCs treated with LY294002 were visualized by western blotting at the indicated time points. (B) Phosphorylated protein expression levels were normalized to their respective total protein levels. Immunofluorescent localization of (C) Akt and (D) p-Akt in OVX BMSCs upon CMS with the selective inhibitor, LY294002 (magnification, x200). The Akt and p-Akt was labeled with an Alexa 594-conjugated antibody (red), and nuclei were counterstained with Hoechst (blue). The images demonstrate a typical example after 1 and 4 h of CMS. Values were obtained from three individual experiments and presented as means \pm standard deviation (n=3). BMSCs, bone mesenchymal stem cells; OVX BMSC, ovariectomized rat BMSC; CMS, continuous mechanical strain; p-, phosphorylated.

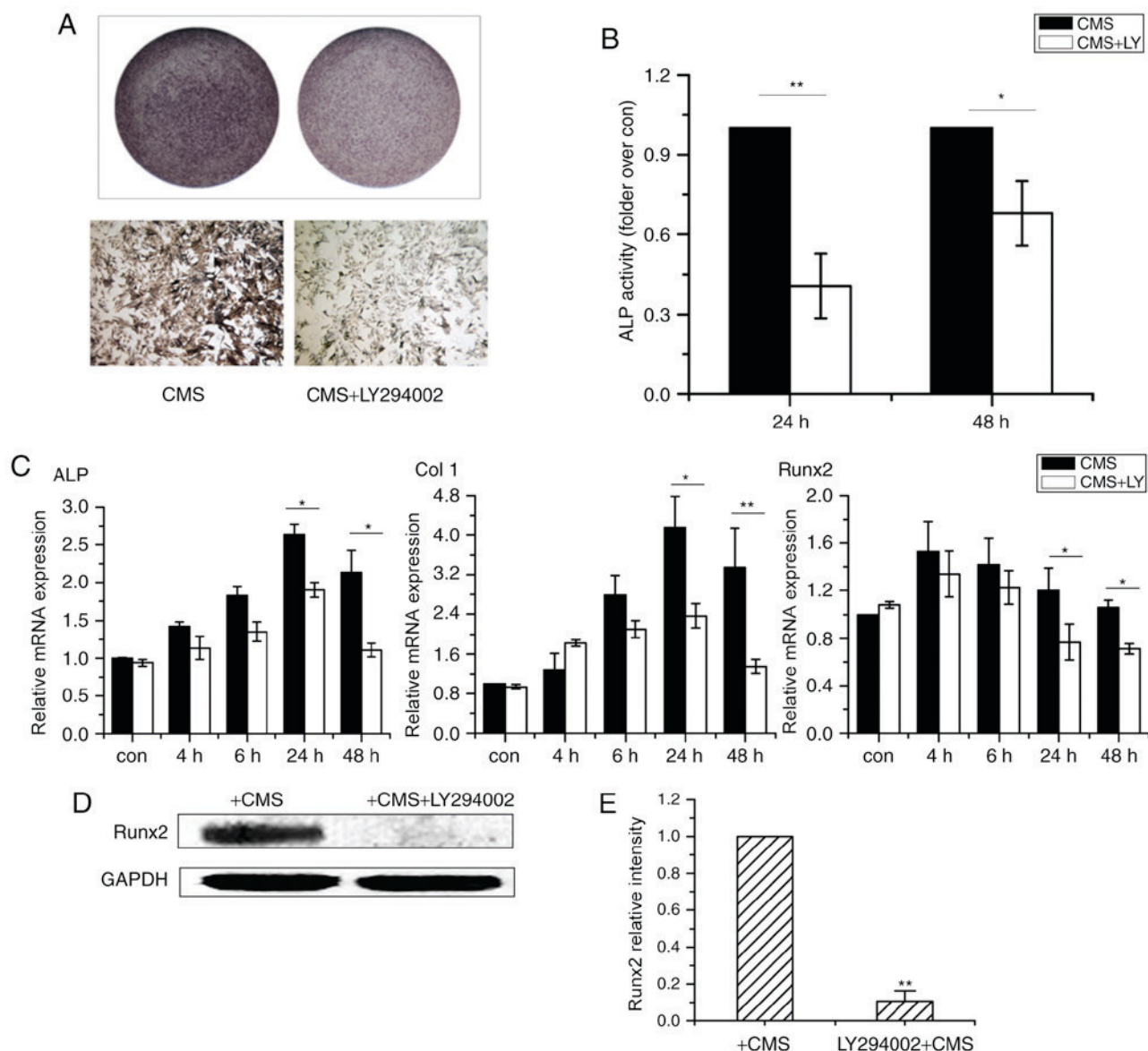


Figure 4. Effects of phosphatidylinositol 3-kinase/Akt signaling on osteogenic differentiation of OVX BMSCs following application of CMS. (A) Effects of an Akt-specific inhibitor on ALP staining at 48 h (magnification, x200) and (B) ALP activity at 24 and 48 h. (C) Changes of CMS-induced specific osteoblastic genes in BMSCs pre-treated with LY294002 at the indicated time points. (D) Protein expression levels of Runx2 were determined by western blotting in the presence of CMS or LY294002 pre-treatment 48 h after CMS. (E) Runx2 expression levels were normalized to their respective total protein levels. Data were obtained from three individual experiments and presented as means \pm standard deviation. * $P < 0.05$ or ** $P < 0.01$. BMSCs, bone mesenchymal stem cells; OVX BMSC, ovariectomized rat BMSC; CMS, continuous mechanical strain; ALP, alkaline phosphatase; Runx2, runt related transcription factor 2; COL I, type I collagen; con, control group.

The PI3K/Akt signaling pathway is key in the physiology and pathophysiology of various types of cell, exerting profound effects on processes, including proliferation, migration, metabolism and differentiation (29). In the current study, Akt was phosphorylated under the stimulation of CMS, with phosphorylation levels peaking at 15 min and then gradually declining; however, the level remained greater than that of the unloaded group. Meanwhile, as indicated by immunostaining, OVX BMSCs subjected to CMS demonstrated greater accumulation of p-Akt in the nucleus, indicating that mechanical strain enhances phosphorylation and nuclear translocation of the Akt protein. After confirming the activation of Akt, the OVX BMSCs were pre-treated with an inhibitor of the Akt signaling pathway (LY294002) to determine whether their strain-induced osteogenic commitment was dependent

on Akt activation. Following treatment with LY294002, the strain-induced gene expression of osteogenic markers and Runx2 protein expression decreased significantly. Previous studies demonstrated that Akt was particularly important in bone formation and was activated early in the transcriptional activation of osteogenesis (15,30,31). Substantial evidence indicated that PI3K/Akt signaling was required for murine osteogenesis *in vitro*, including mouse embryonic fibroblasts, murine BMSCs, and in the mouse MSC line, C3H10T1/2 (17). Nuclear translocation of activated Akt may lead to the phosphorylation of key transcription factors, which in turn affects the levels of certain proliferation or differentiation-associated genes (32). Additionally, Akt is the mechanically activated kinase responsible for numerous other interventions. For example, the PI3K/Akt signaling pathway participates in

matrix metalloproteinase-2 expression by 10% mechanical stretch in vascular smooth muscle cells and by 18% in human aortic smooth muscle cells (33,34). Furthermore, ultrasound stimulation promotes bone formation in osteoblasts via the integrin/protein tyrosine kinase 2/PI3K/Akt and extracellular-signal-regulated kinase signaling pathway (35). Studies also indicated that mammalian target of rapamycin complex 2 was required for mechanical activation of Akt and that mechanical inhibition of glycogen synthase kinase was dependent on Akt activation (36). However, as Akt is a pleiotropic signaling molecule with downstream targets that are differentially regulated depending upon the nature of the activating input, further studies investigating the downstream targets of strain-induced osteogenic commitment on Akt activation are required.

In conclusion, continuous short-term mechanical strain induced the early differentiation of OVX BMSCs towards an osteogenic phenotype, and CMS may activate the PI3K/Akt signaling pathway during osteoblastic differentiation. The present study may provide a promising strategy for regulating strain-induced bone remodeling in osteoporosis, however, further research is required regarding the downstream target and the *in vivo* conditions.

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