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The Distinct Effects of Estrogen and Hydrostatic Pressure on Mesenchymal Stem Cells Differentiation: Involvement of Estrogen Receptor Signaling

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Abstract—This study aimed to investigate the differential and synergistic effects of mechanical stimulation and estrogen on the proliferation and osteogenic or chondrogenic differentiation potential of bone marrow mesenchymal stem cells (BMSCs) and the roles of estrogen receptor (ER) in them. BMSCs were isolated and cultured using the whole bone marrow adherence method, and flow cytometry was used to identify the surface marker molecules of BMSCs. Cells were pre-treated with 1 nM 17β-estradiol or 1 nM of the estrogen receptor antagonist tamoxifen. Then, the cells were stimulated with hydrostatic pressure. Assessment included flow cytometry analysis of the cell cycle; immunofluorescent staining for F-actin; protein quantification for MAPK protein; and mRNA analysis for Col I, OCN, OPN and BSP after osteogenic induction and Sox-9, Aggrecan and Col-II after chondrogenic induction. Hydrostatic pressure (90 kPa/1 h) and 1 nM 17β-estradiol enhanced the cellular proliferation ability and the cytoskeleton activity but without synergistic biological effects. Estrogen activated ERKs and JNKs simultaneously and promoted the osteogenic differentiation, whereas the pressure just caused JNK-1/2 activation and promoted the chondrogenic differentiation of BMSCs. Estrogen had antagonism effect on chondrogenic promotion of hydrostatic pressure. Mechanobiological effects of hydrostatic pressure are closely associated with ERα activity. MAPK molecules and F-actin were likely to be important mediator molecules in the ER-mediated mechanotransduction of BMSCs.

Keywords—Hydrostatic pressure, Estrogen, Bone marrow mesenchymal stem cells (BMSCs), Differentiation, Estrogen receptor (ER), Mitogen-activated protein kinase (MAPK).

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ABBREVIATIONS

BMSCs	Bone marrow mesenchymal stem cells
TAM	Tamoxifen
ER	Estrogen receptor
OA	Osteoarthritis
Col-I	Collagen I
OCN	Osteocalcin
OPN	Osteopontin
BSP	Bone sialoprotein
Col-II	Collagen II
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
ERK	External signal-regulated kinase

INTRODUCTION

Adult stem cells are involved in the repair and replacement of injured or aged cells in response to traumatic events or natural cell turnover. This turnover and repair is important for maintaining physiological homeostasis.[2,5] Bone marrow mesenchymal stem cells (BMSCs) are the major cells involved in the repair of bone and cartilage defects in tissue engineering. Furthermore, BMSCs bear the pressure of the tissue fluid of cancellous bones when weight-bearing tissues are exposed to a functional load.[13,32] In addition, estrogen is also a key factor involved in the regulation of bone and cartilage remodeling.[18,24] Therefore, BMSCs, similar to mature osteoblasts and chondrocytes, are major targets for both mechanical stimulation and local estrogen action on the bone and articular cartilage during remod-

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eling and regeneration. In particular, there are many studies of the relationship between estrogen and bone metabolism in a mechanically stimulated environment. Frost proposed that when the mechanical stimulation exceeds the threshold of minimum effective stress, it can promote or inhibit bone resorption, but it was also found that estrogen could effectively reduce this threshold.[10] It has also been demonstrated that an increase in exercise intensity can ameliorate osteoporosis that occurs due to lack of estrogen,[28] suggesting that mechanical stimulation and estrogen levels may be correlated in the maintenance of bone tissue structure and function in the body. Studies have found that estrogen may promote bone formation by regulating the proliferation and differentiation characteristics of BMSCs,[12] which suggests the possibility of using estrogen to regulate the functions of multiple types of stem/progenitor cells to obtain "super stem cells" with a higher therapeutic value. Still, little is known about the relationship between estrogen and the remodeling, regeneration and repair of bone and cartilage that are similarly located in a mechanically stimulated environment. Researcher indicated that estrogen prevented injury-related cell death and GAG release, and it might be useful as a treatment option for cartilage-related sports injuries or osteoarthritis.[14] Our previous study on BMSCs reported that mechanical stimulation could induce the upregulation of estrogen receptor (ERα) expression in BMSCs, which may play an important role in mediating mechanical effects and promoting the mechanotransduction process in BMSCs.[36] However, the downstream mechanotransduction signal molecules that may be involved in differentiation process of BMSCs under estrogen clues or mechanical stimulations still kept unknown.

The present study uses 17β-estradiol (E_2) and/or hydrostatic pressure to stimulate BMSCs cultured *in vitro* to observe the effects of these factors alone or in combination on the proliferation and osteogenic/chondrogenic differentiation potential of BMSCs. Tamoxifen (TAM) was used to block the functions of ERα to further clarify the role of ERα in the mechanical signal transduction of BMSCs. The possible involvement of F-actin and the mitogen-activated protein kinase (MAPK) family proteins c-Jun N-terminal kinase (JNK) and external signal-regulated kinase (ERK) were evaluated to investigate the underlying signal mechanism of the above-mentioned biochemical or biomechanical regulation process.

MATERIALS AND METHODS

BMSCs Isolation, Culture and Identification

Adult female Sprague–Dawley rats (8 weeks) were sacrificed by cervical dislocation. The study was approved by the Committee on the Ethics of Animal Research of the Fourth Military Medical University (Xi'an, China). All of the surgeries were performed under pentobarbital sodium anesthesia, and every effort was made to minimize the animals' suffering. BMSCs were cultured using the whole marrow-adherence method. The cultured BMSCs (P3) were plated into 90-mm dishes at a density of 1×10^3 cells/well for the colony forming unit fibroblast (CFU-F) assays.

The cell aliquots were incubated with conjugated monoclonal antibodies recognizing CD29 (β1-integrin, BioLegend Inc., San Diego, CA), CD44 (receptor for hyaluronate and osteopontin, Abcam, Cambridge, U.K.), CD90 (Thy-1 glycoprotein, Epitomics, Burlingame, CA, USA), CD106 (vascular cell adhesion molecule-1, VCAM-1, BioLegend), CD34 (stem/progenitor hematopoietic cells, Abcam), CD45 (leukocyte common antigen, Epitomics) or conjugated isotype controls at 4 °C for 30 min. After a 30-min reaction with the secondary antibody, the cells were washed and resuspended in 0.5 mL of FC buffer for flow cytometry analysis (FACS Scan; Beckton Dickinson, Franklin Lakes, NJ), and the data were analyzed with Cellquest software. The osteogenic and adipogenic differentiation of BMSCs was measured according to the procedures reported by Pittenger *et al.*[21] Alizarin red and Oil Red O (Sigma-Aldrich, Buchs, Switzerland) staining were used for osteogenic and adipogenic differentiation detection, respectively. Photomicrographs were taken with a digital IX 50 microscope (Olympus, Tokyo, Japan).

Mechanical Stimulation of BMSCs

A self-designed, hydraulic pressure-controlling cellular strain unit following the model developed by Yousefian *et al.* was used here to simulate compressive stress on the cultured BMSCs.[33] By continuously compressing the gas phase (3% CO_2 in air) in a closed culture chamber (98% humidity) placed in a 37 °C incubator, we could exert accurate, adjustable and repeatable hydrostatic pressures on the cells,[3,35,37] imitating the compressive circumstances of the cells *in vivo*.

Experimental Grouping and Cell Treatment

The BMSCs used in this study were divided into 6 groups: Control group, cells without any treatment; group E, in which BMSCs were treated with 1 nM 17β-estradiol (Sigma-Aldrich, St. Louis, MO) for 12 h; group T, in which BMSCs were treated with 100 nM 4-hydroxytamoxifen (TAM, ERα antagonist,Sigma) for 1 h; group P, in which BMSCs were treated with a pressure of 90 kPa for 1 h; group P + E, in which

BMSCs were pretreated with 1 nM 17β-estradiol for 12 h and subsequently treated with 90 kPa for 1 h (As to the rational for the used loading regime, such as pressure and time of compression, and the 17β-estradiol concentration adopted in the present study, please see the Supplementary Materials.); and group P + T, in which BMSCs were pretreated with 100 nM TAM for 1 h and subsequently treated with 90 kPa for 1 h. At the beginning of the experiment, the medium was discarded and fresh DMEM medium without serum was added to each plate for 12 h to promote cell synchronization for the following cell cycle assay, F-actin observation and phosphorylation of MAPKs detection. Then, the cells received various treatments according to the experimental groups. All of the cells were collected after 13 h of culture since synchronization (including an initial 12-h treatment with 1 nM 17β-estradiol for the cells in the E and P + E groups, a treatment with 100 nM TAM for the last hour after the first 12 h for the cells in the T and P + T groups, and a 90 kPa pressure treatment for another 1 h for the cells in group P, group P + E and group P + T). For the analysis of osteogenic and chondrogenic differentiation, the experimental groups were the same as above. SD Rat MSC osteogenic differentiation medium (Cyagen Biosciences Inc., Santa Clara, USA) containing SD Rat MSC osteogenic differentiation basal medium, MSC-Qualified FBS (10 mL), penicillin–streptomycin (2 mL), glutamine (2 mL), ascorbate (400 μL), β-glycerophosphate (2 mL), and dexamethasone (20 μL) and SD Rat MSC Chondrogenic Differentiation Medium (Cyagen Biosciences Inc., Santa Clara, USA) containing SD Rat MSC chondrogenic differentiation basal medium, dexamethasone (10 μL), ascorbate (300 μL), ITS + supplement (1 mL), sodium pyruvate (100 μL), proline (100 μL), and TGF-β3 (1 mL) were, respectively, adopted for osteogenic or chondrogenic induction of BMSCs. Continuous osteogenic induction medium for 2 weeks or chondrogenic induction medium for 4 weeks was applied instead of culture medium, with 1 nM 17β-estradiol being added into the induction medium of the cells in E and P + E group, 100 nM TAM being added into the induction medium of the cells in T and P + T group, and with the cells in group P, group P + E and group P + T being subjected to 90 kPa for 1 h per day. The medium was changed every 3 days throughout the induction period.

Flow Cytometry-Based Detection of the Cell Cycle

At the beginning of each experiment, BMSCs were inhibited on the cell cycle G0 synchronization by serum starvation. At 30 min before detection, propidium iodide (PI) (Sigma) staining solution (100 μg/mL PI; 0.7 mg/mL RNase, Abcam) was added, mixed by shaking and the entire solution kept in the dark. The stained cells (10×10^4) were analyzed by flow cytometry, and the data were analyzed with Cellquest software. The indicators of cell proliferation include the S-phase fraction (SPF) of cells and the proliferation index (PI). We used the following formula for SPF:

$$\mathrm{SPF} = \mathrm{S}/(\mathrm{G0/1} + \mathrm{S} + \mathrm{G2/M}) \times 100\%;$$
$$\mathrm{PI} = (\mathrm{S} + \mathrm{G2/M})/(\mathrm{G0/1} + \mathrm{S} + \mathrm{G2/M}) \times 100\%$$

Fluorescent Staining of F-actin

After washing with phosphate-buffered saline (PBS), the cells (10×10^3) were fixed for 5 min in 3.7% formaldehyde in PBS. The cells were dehydrated with acetone and then permeabilised with 0.1% Triton X-100 in PBS. The slides were stained with a 50 μg/mL fluorescent phalloidin (Sigma) conjugate solution in PBS for 40 min at room temperature. The images were documented with a Leica SP2 laser-scanning confocal microscope (Leica Microsystems, Herb rug, Switzerland). Integrated optical density (IOD) characterizing the actin cytoskeletons was determined by application of Image-Pro Plus 6.0. (Media Cybernetics, Inc. Bethesda, MD, USA).

Western Blotting Analysis

The total cellular protein was quantified by the Bradford protein assay kit (Bio-Rad, Hercules, CA), and proteins were electrophoresed in a 12% SDS–polyacrylamide gel and transferred to a PVDF membrane (EMD Millipore Corporation, Billerica, MA, USA). The membranes were incubated with blocking buffer (TBS, 0.1% Tween-20 and 5% w/v nonfat dry milk) for 1 h at room temperature and then incubated with primary antibodies for ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 (Cell Signaling Technology Danvers, USA) (1:1000) overnight at 4 °C. After that, the membrane was washed three times in TBS-T for 5 min each and incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:2000, Cell Signaling). The bands were detected by ECL reagent (Thermo Fisher Scientific, Lausanne, Switzerland), and the intensities of the brands were quantified by the Photo-Image System (Gel-Pro Analyzer, Media Cybernetics Inc., Bethesda, MD, USA).

Osteogenic and Chondrogenic Gene Expression

Osteogenic Differentiation

The BMSCs (10×10^4) were cultured in 6-well plates at 37 °C in a 5% CO_2 humidified incubator. At

70–80% cell confluence, the medium was changed to osteogenic medium (Cyagen Biosciences Inc., Santa Clara, USA). The medium was changed every third day throughout the study. After 2 weeks of differentiation, the cells were fixed and stained with Alizarin red, and the total RNA of the induced cells was extracted for analysis of osteogenic gene expression by real-time PCR.

Chondrogenic Differentiation

BMSCs (10×10^4) were resuspended in complete chondrogenic medium (Cyagen Biosciences Inc., Santa Clara, USA). Then, the cells were centrifuged at $150 \times g$ for 5 min at room temperature. The tubes were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Freshly prepared Complete Chondrogenic Medium was added to each tube every 2–3 days. Cell pellets were harvested after 4 w of culture for subsequent toluidine blue staining, glycosaminoglycan (GAG) quantification and PCR assays. Detection and quantification of GAG Cell pellets were harvested and digested in papain (0.56 U/mL in 0.1 M sodium acetate, 10 M cysteine HCl, 0.05 M EDTA, pH 6.0) at 60 °C for 16 h. Following digestion, constructs were evaluated for sulfated GAG content against a standard curve of chondroitin-6-sulphate using the 1,9-dimethylmethylene blue dye-binding assay.[6]

Real-Time PCR Assay

After induction, the total cellular RNA was isolated from the maintenance cell cultures by trizol (Invitrogen, Carlsbad, USA) to evaluate the expression of osteogenic markers including collagen I (Col-I), osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and chondrogenesis-related genes, including Sox-9, collagen II(Col-II) and Aggrecan by real-time PCR (TaKaRa Bio, Tokyo, Japan). The primer sequences for Col-I, OCN, OPN, BSP, Sox-9, Col-II and Aggrecan (Sango Biotech, Shanghai, China) are listed in Table 1. The reaction products were quantified by using a relative quantification tool (CFX Manager, Bio-Rad) with GAPDH as the reference gene. The reactions were performed under the following cycling conditions: 95 °C for 3 min, 39 cycles at 95 °C for 10 s and 60 °C for 35 s and a melting curve from 60 to 95 °C with increments of 0.5 °C for 5 s.

Statistics

All in vitro experiments were performed independently for each cell line and repeated at least three times. The results were expressed as the mean ± standard deviation and were compared using a one-way analysis of variance (ANOVA) combined with the Dunnett t test. A level of $p < 0.05$ was accepted as being statistically significant. The analyses were performed using SPSS 17.0 (SPSS, USA).

RESULTS

Isolation and Identification of BMSCs

The cultured cells displayed plastic-adherent, fibroblast-like morphological features. At 7–9 days, the cells grew rapidly, and the small colonies gradually fused with each other, displaying radiating growth (Fig. 1a). At the third passage, the cells were long and spindle-shaped, densely arranged and arranged in swirls (Fig. 1b). Single colonies were observed 12 days after being plated at low density, which confirmed the

TABLE 1. Primer sequences for real-time PCR.

Genes	Sequences
Col-I	F GGAGAGTACTGGATCGACCCTAAC
	R CTGACCTGTCTCCATGTTGCA
OCN	F TGCAAAGCCCAGCGACTCT
	R AGTCCATTGTTGAGGTAGCG
OPN	F AGACCATGCAGAGAGCGAG
	R ACGTCTGCTTGTGTGCTGG
BSP	F TGTGGAATGGTGCTACGGTCTC
	R GATCAACAGCCCTGATTTACGATG
Sox9	F AGAAAGACCACCCCGATTAC
	R TGCTGATGCCGTAACTGCC
Col-II	F GCCTCGCGGTGAGCCATGATC
	R CTCCATCTCTGCCACGGGGT
Aggrecan	F GCGGGTCATCGCTGCAGTG
	R GCTCGGTCAAAGTCCAGTGTG
GAPDH	F GGCAAGTTCAACGGCACAG
	R CGCCAGTAGACTCCACGACAT

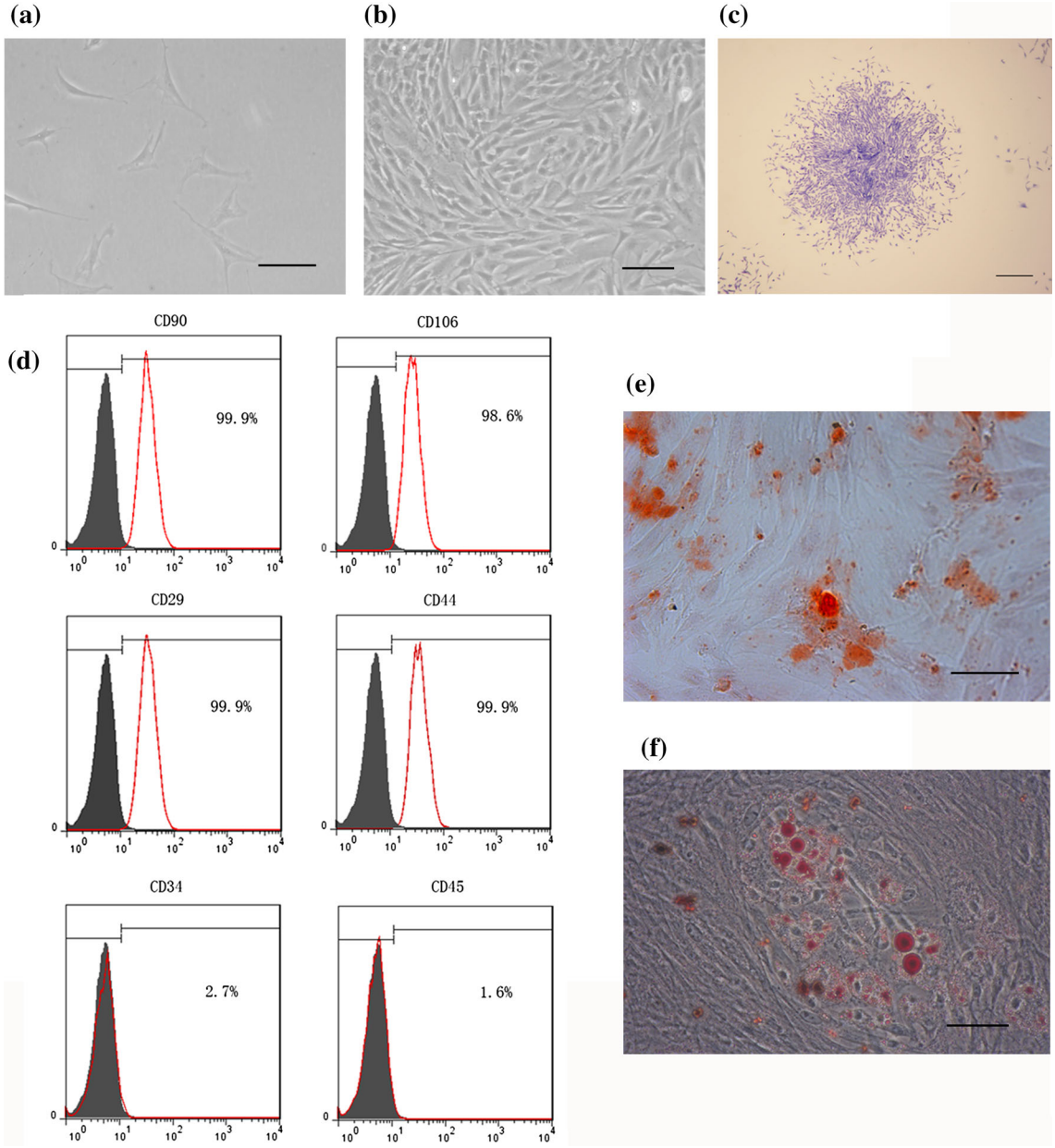


FIGURE 1. Identification and morphology of in vitro cultured BMSCs. (a) The cultured cells displayed plastic-adherent, fibroblast-like morphological features (7d) (bar = 100 μm). (b) Cells reached more than 90% confluence, had long spindle shapes and were arranged densely in swirls (P3). (bar = 100 μm) (c) Single colonies were observed after cells were plated at low density by toluidine blue stain (bar = 500 μm). (d) BMSCs were positive for CD90, CD106, CD29 and CD44 and negative for CD34 and CD45. (e) Alizarin red-positive mineral deposits indicate that the cultured cells have osteogenic potential (bar = 100 μm). (f) Oil Red O-positive cells indicate that the BMSCs have the capacity for adipogenic differentiation (bar = 100 μm).

capacity of BMSCs to form CFUs (Fig. 1c). The detection of surface molecule expression revealed that the BMSCs were negative for hematopoietic markers, such as CD34 and CD45, but positive for mesenchymal markers, such as CD29, CD44, CD90 and CD106 (Fig. 1d). After 2 weeks of osteogenic induction, Alizarin red-positive mineral deposits could be observed, which indicates that the cultured cells have osteogenic potential (Fig. 1e). Similarly, Oil Red O-positive cells provided strong evidence for adipogenic differentiation potential after 2 weeks of adipogenic induction (Fig. 1f). However, the control cells did not have calcium salt precipitates after osteogenic induction, nor did they display lipid droplet formation after adi-

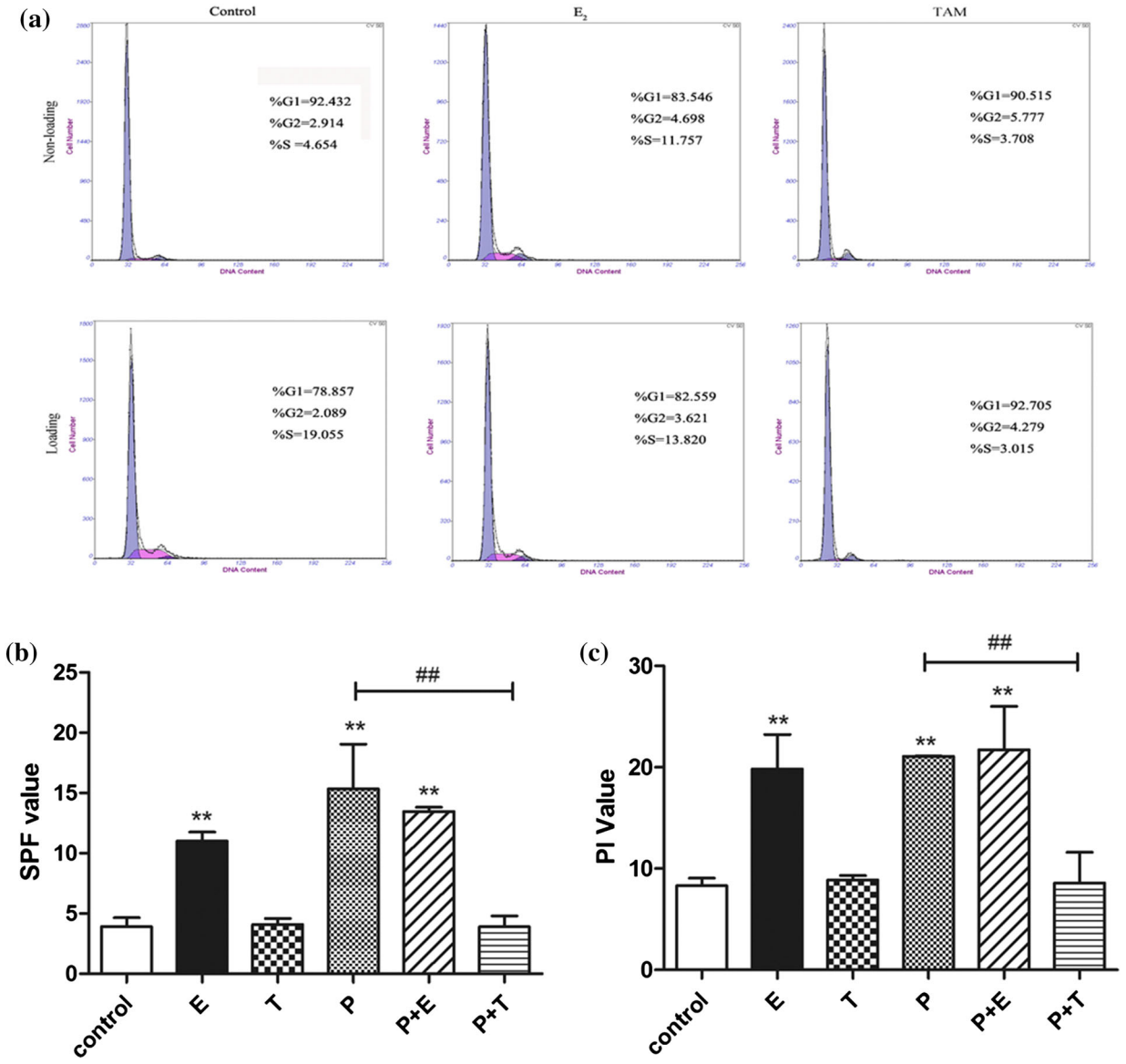


FIGURE 2. The DNA synthesis fraction and proliferation activity of BMSCs cultured in the presence or absence of hydrostatic pressure with 1 nM 17β-estradiol or 100 nM TAM. (a) Flow cytometry figure with cell cycle distribution and percentage of G0/G1, G2 and S phases measured by flow cytometry of each group. (b) The histogram of the S-phase fraction (SPF) showed that estrogen and pressure could both stimulate DNA synthesis of BMSCs, and treatment of TAM could weaken the promoting effect for cell proliferation of the pressure. (c) The proliferation index (PI) of the estrogen and/or pressure groups was significantly higher than that of the control group, and the increase in PI of the P group could be reversed by TAM treatment. ($p \leq 0.01$ vs. the control group, ##$p \leq 0.01$ vs. the P group).**

pogenic induction. These results suggest that the BMSCs were successfully obtained.

Cell Cycle Detection

Flow cytometry detection of the cell cycle revealed that the S-phase fraction (SPF) and the proliferation index (PI) of E, P and P + E groups were significantly higher than those of the control group (SPF: $p < 0.001$, PI: $p < 0.001$) (Figs. 2a and 2b), suggesting that 17β-estradiol and/or hydrostatic pressure can increase the number of cells entering into the DNA synthesis phase and thus accelerate cell proliferation. No significant SPF and PI differences were observed between the groups P and P + E (SPF: $p = 0.185$, PI: $p = 0.765$), and between the groups E and P + E (SPF: $p = 0.091$, PI: $p = 0.388$), which suggests that the estrogen and mechanical pressure did not exhibit significant synergistic or antagonistic effects in causing cells to enter the DNA synthesis phase or in stimulating proliferation. When pressure was applied to cells pre-treated by TAM for ERα blockage (P + T group),

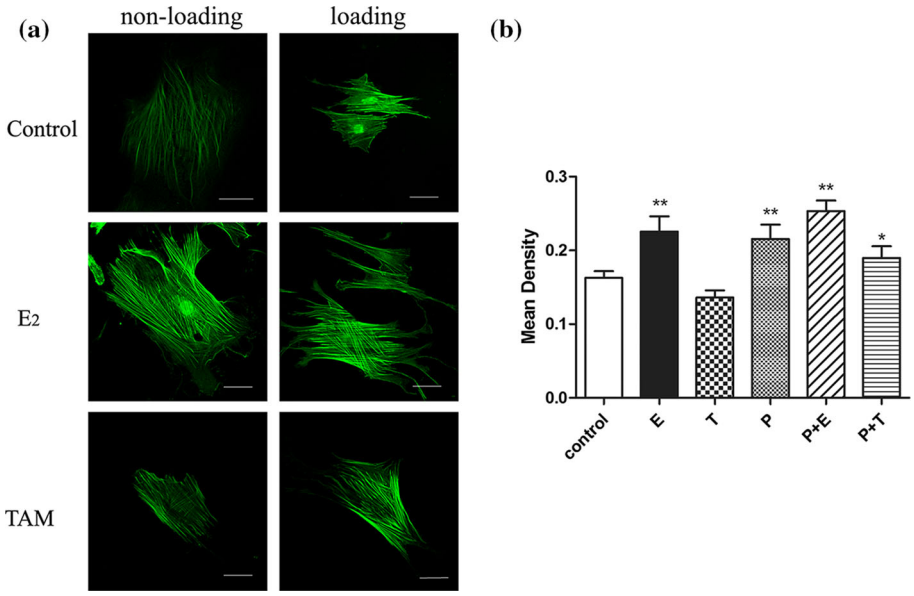


FIGURE 3. F-actin mapping stained by FITC-Phalloidin. stress fibers of BMSCs treated with 1 nM 17β-estradiol and 100 nM TAM under hydrostatic pressure or in controls during monolayer culture, as indicated by staining with. (a) F-actin fibers in the cells of estrogen and TAM treatment groups were closely packed, thickened and distributed along the long axis of the cell (Magnification: 400× bar = 50 μm) (n = 6). (b) The mean fluorescence was obtained by taking the integrated optical density (IOD) of the images. The IOD values for group E, P, P + E and P + T were significantly increased compared with the control group. (*$p \leq 0.05$, **$p \leq 0.01$ vs. the control group).

the SPF and PI of the cells were significant decreased compared with those of Group P (SPF: $p < 0.001$, PI: $p < 0.001$), and even reduced back to the levels that did not differ significantly from those of control (SPF: $p = 0.982$, PI: $p = 0.905$), indicating that ERα is involved in the process of hydrostatic pressure-mediated regulation of BMSC proliferation.

Cytoskeleton Staining

It was observed under laser confocal microscopy that the F-actin fluorescence staining in the control group was filamentous and primarily arranged along the long axes of the cells, and there was uniform fluorescence throughout the entire cell. Under 90 kPa/1 h hydrostatic pressure, F-actin fibers of the cells were closely packed and thickened, with clearly visible stress fibers distributed along the long axes of the cells (Fig. 3a). The mean fluorescence intensity of the E ($p = 0.001$), P ($p = 0.003$), P + E ($p < 0.001$) and P + T ($p = 0.034$) group were significantly higher than those in the control group. However, the difference between P and P + T group was not significant ($p = 0.205$) (Fig. 3b). These results indicate that the pressure-mediated promotion of enhanced F-actin expression and stress fiber assembly was not dependent on ERα.

Activation of the MAPK Pathway

The intensity of brands indicated that stimulation with 17β-estradiol alone could significantly enhance the level of ERK1/2 phosphorylation ($p = 0.004$, vs. control), whereas pressure alone ($p = 0.726$, vs. control) or pressure combined with estrogen ($p = 0.721$, vs. control) did not cause the activation of ERK1/2. There is significant difference of ERK1/2 phosphorylation between the groups E and P + E ($p = 0.007$) (Figs. 4a and 4b). Analysis of JNK protein expression and phosphorylation revealed that the JNK phosphorylation levels of the E ($p = 0.045$), T ($p = 0.024$), P ($p = 0.029$) and P + E ($p = 0.01$) groups were significantly higher than that of the control group. And there were no significant differences between P group ($p = 0.474$) or E group ($p = 0.314$) and P + E group. The JNK phosphorylation level of group P + T is significantly different than that of the P group ($p = 0.044$) (Figs. 4c and 4d), which suggests that JNK activation stimulated by pressure was mediated at least in part by ERα.

Detection of Osteogenic Genes

After the cells were cultured under osteogenic induction medium for 2 weeks, alizarin red staining revealed positive nodules of calcium deposition in each group (Fig. 5a). Real-time PCR revealed that pressure exerted no significant effects on expression of all the four osteogenic genes (Fig. 5b). Whereas, estrogen stimulation could significantly promote expression of the osteogenic indicators, OPN and BSP, in the BMSCs (OPN: $p < 0.001$, BSP: $p = 0.027$) (Fig. 5b). Under the combined stimulation of both mechanical

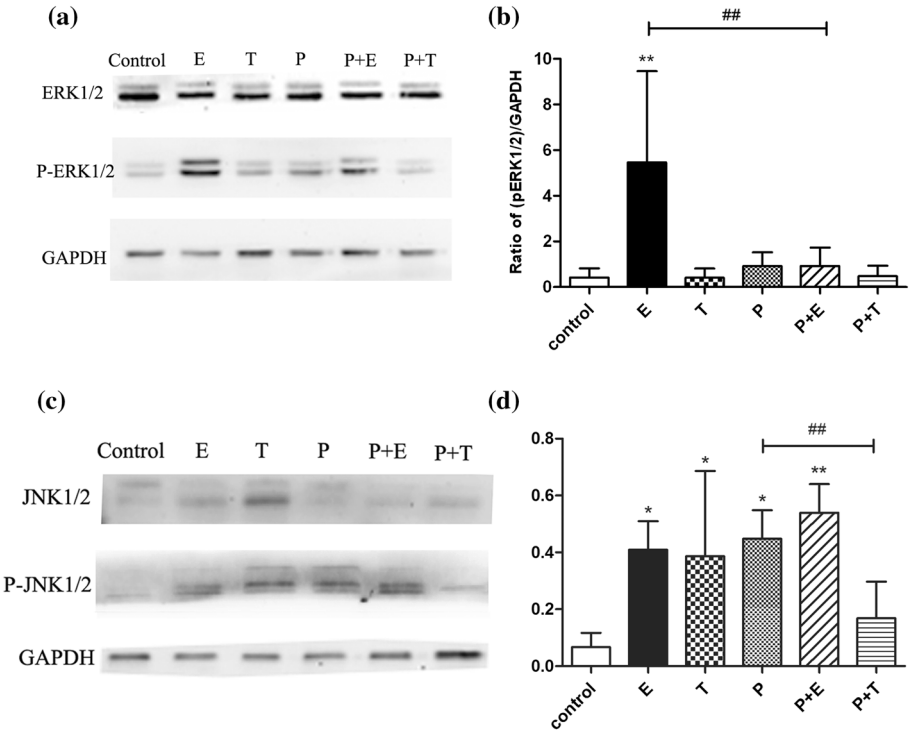


FIGURE 4. Phosphorylation of ERK1/2 and JNK1/2 in cultured BMSC under estrogen and/or hydrostatic pressure. Western blots are shown for the phosphorylated (lower panel) and unphosphorylated (upper panel) forms of ERK1/2 (a) and JNK1/2 (c). Relative expression of pERK1/2 (b) and pJNK1/2 (d) derived from Quantity One density analysis. The 17β-estradiol stimulation group displayed significant ERK1/2 phosphorylation ($p \leq 0.01$ vs. the control group, $^{\#\#}p \leq 0.01$ E group vs. the P + E group) (b) and the JNK phosphorylation levels of all treatment groups were higher than that of the control, and the JNK phosphorylation levels in the P group were significantly higher than in the P + T group (*$p \leq 0.05$ vs. the control group, **$p \leq 0.01$ vs. the control group, #$p \leq 0.05$ vs. the P group, ##$p \leq 0.01$ vs. the P group) (d). Data are expressed as the mean ± SD.**

pressure and estrogen, there was no increase in the osteogenic differentiation markers of the cells (Col-I: $p = 0.073$, OCN: $p = 0.080$, OPN: $p = 0.152$, BSP: $p = 0.592$, vs. control), which indicates that pressure significantly reversed the estrogen-mediated promotion of BMSC osteogenic differentiation at 2 weeks.

Detection of the Chondrogenic Genes

BMSCs were cultured under chondrogenic induction medium for 4 weeks, and toluidine blue staining revealed blue metachromatic granules in each group (Fig. 6a), suggesting the chondrogenic differentiation of stem cells and the secretion of a cartilage matrix. Biochemical analyses showed that the GAG content in the P and P + E group were significantly higher than those of the control ($p < 0.001$). Compared with the P group, the GAG content in the P + E and P + T groups were significantly decreased ($p < 0.001$) (Fig. 6b). Real-time PCR was used to further analyze the chondrogenic genes in various groups. The assay revealed that biomechanical stimulation significantly upregulated all the 3 chondrogenic markers (Sox-9: $p < 0.001$, Aggrecan: $p < 0.001$, Col-II: $p < 0.001$), which were also significantly higher than those of the P + E group (Sox-9: $p < 0.001$, Aggrecan: $p < 0.001$, Col-II: $p < 0.001$) and P + T group (Sox-9: $p < 0.001$, Aggrecan: $p < 0.001$, Col-II: $p < 0.001$) (Fig. 6c). The results indicated that estrogen could significantly antagonize the chondrogenic differentiation of BMSCs stimulated by 90 kPa/1 h hydrostatic pressure. In addition, ERα is an important signaling molecule mediating the promotion of chondrogenic differentiation caused by hydrostatic pressure.

DISCUSSION

Estrogen treatment has been demonstrated by many studies to be effective in promoting the self-renewal and differentiation of BMSCs.[9,19,20,23] In the present study, we found that estrogen can significantly promote the proliferation and induce the osteogenic differentiation of BMSCs and that it did not stimulate (and even inhibited) the expression of chondrogenic genes in BMSCs. These results indicated that estrogen may not be involved in chondrogenesis but rather osteogenesis and its modulation.[16] Extrinsic mechani-

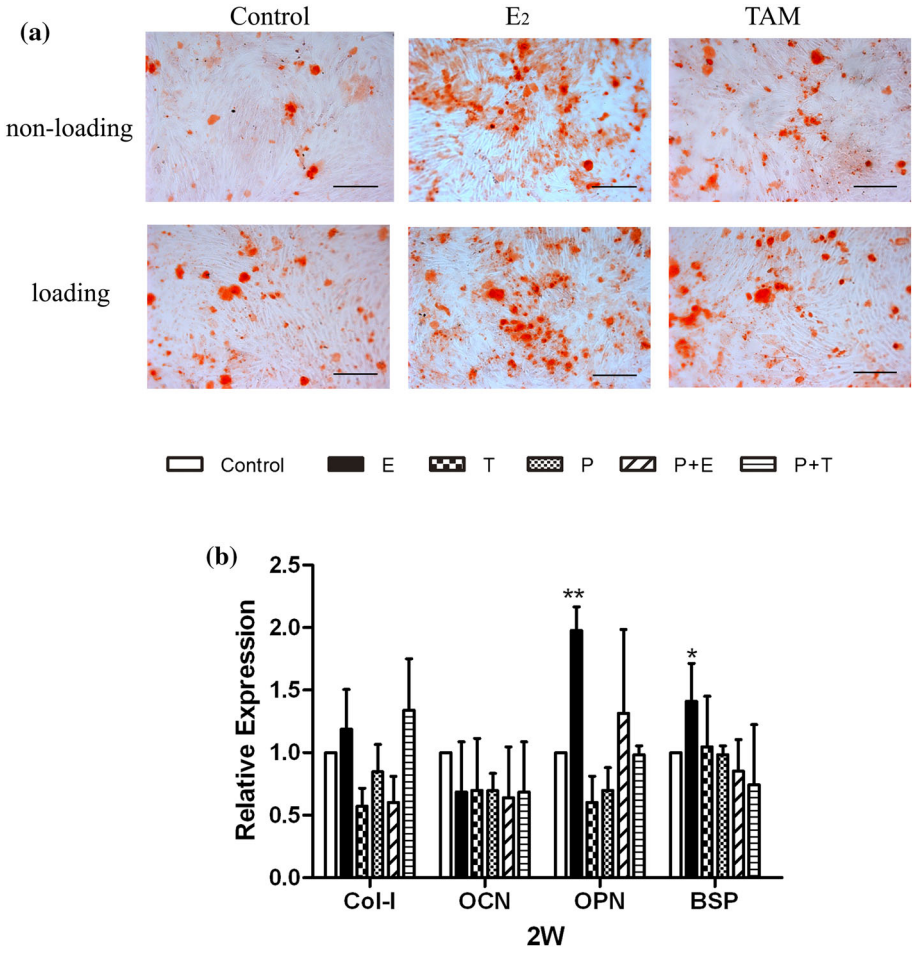


FIGURE 5. Effects of the estrogen and/or hydrostatic pressure treatment on osteogenic differentiation of BMSCs. (a) Mineral deposits were observed by alizarin red staining after 2 weeks of osteogenic differentiation (bar = 400 μm). (b) Expression levels of osteogenic markers (Col I, OCN, OPN and BSP) after 2 weeks of osteogenic differentiation under 1 nM E_2 or 100 nM TAM treatment with or without hydrostatic pressure stimulation were analyzed. All gene expression levels were normalized to the endogenous control GAPDH. (*$p \leq 0.05$ vs. the control group, **$p \leq 0.01$ vs. the control group).

cal signals imposed upon cells *via* the extracellular environment play a very important role in determining the fate of BMSCs.[1,8,11,17,20,22,31,34] In our study, pressure stimulation of 90 kPa for 1 h had no effects on the expression of osteogenic markers by BMSCs but it did significantly increase their expression of chondrogenic markers. This result has been confirmed by many studies.[15,25,30] This finding suggests that changes in the biomechanical environment of joints may affect the degradation and repair of cartilage. These effects may explain why physical activity is beneficial for the articular cartilage and may contribute to decreasing the symptoms and improving the functionality of OA patients.[7,29]

Our study showed the differential roles of estrogen and biomechanical stimulation in regulating the differentiation of BMSCs. What is the connection between the estrogen level and the intrinsically generated mechanical forces within the cells in response to their extracellular environment? Our research has shown that mechanical pressure can upregulate the expression of the estrogen receptor (ERα), which may play an important role in mediating the mechanobiological effects on BMSCs and enabling the mechanotransduction process in these cells.[36] In this study, it was confirmed that ERα is an important molecule in mediating the mechanical and biological effects of hydrostatic pressure on BMSCs because the pressure-mediated effects of promoting cell-cycle initiation and chondrogenic differentiation of BMSCs were significantly reduced by blocking ERα using TAM. Based on these observations, we believe that ERα played an important regulatory role in the mechanobiological response of BMSCs.

In conjunction with the results mentioned above, estrogen and hydrostatic pressure appear to play different roles in determining the fate of BMSCs. We found that estrogen can effectively activate ERKs and

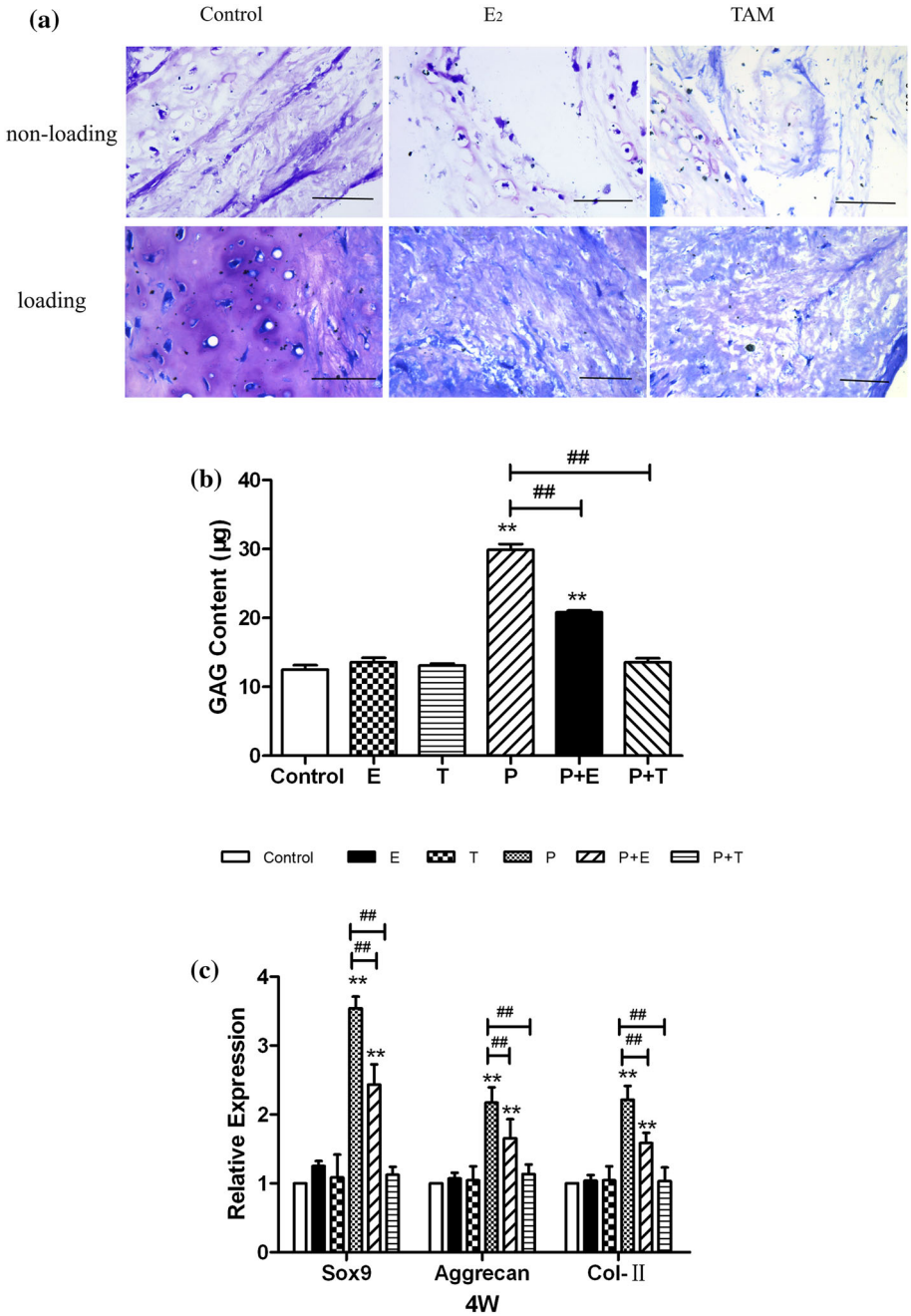


FIGURE 6. Effects of the estrogen and/or hydrostatic pressure treatment on chondrogenic differentiation of BMSCs. (a) Cell pellets were sectioned and stained using toluidine blue (bar = 50 μm). (b) Biochemical analyses showed that the GAG content in the P and P + E group were significantly higher than those of the control. (c) Expression levels of chondrogenic markers (Sox9, Col-II and Aggrecan) after 4 weeks of chondroblast differentiation under 1 nM E_2 or 100 nM TAM treatments with or without hydrostatic pressure stimulation were analyzed. All gene expression levels were normalized to the endogenous control GAPDH. (*$p \leq 0.05$ vs. the control group, **$p \leq 0.01$ vs. the control group, $^{\#\#}p \leq 0.01$ vs. the P group).

JNKs simultaneously, similar to the findings of Dai *et al.*[4] In contrast, the hydrostatic pressure significantly stimulated JNK activation in BMSCs without promoting ERK phosphorylation, which is consistent with the results of Sakao.[26] However, the results also showed that the hydrostatic pressure may blocks the activation of ERK1/2 which induced by estrogen. And there was no synergistic effect between estrogen and hydrostatic pressure in JNKs activation. It suggested that the chemical and physical stimuli for BMSCs may have very different underlying mechanisms. They may play a joint role in the activation of some certain molecules while play independent roles in activating some other signal molecules. Combining the Real-

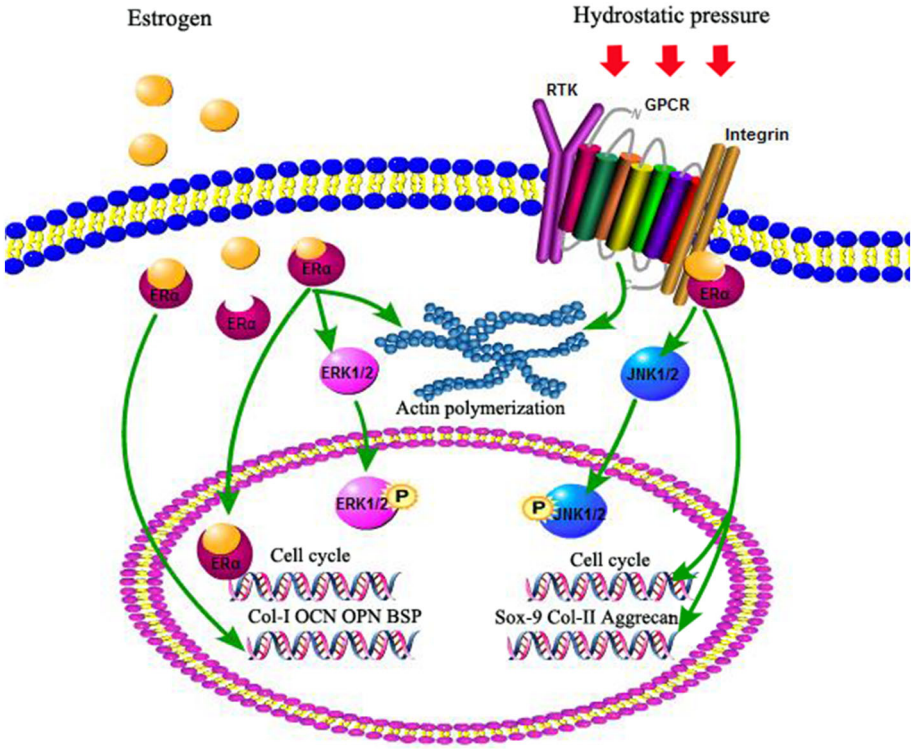


FIGURE 7. Schematic diagram showing the details of the ERα, F-actin, ERK, and JNK-related signal transductions triggered by estrogen or hydrostatic pressure in bone marrow mesenchymal stem cells in regard to its proliferation and differentiation regulation. Hydrostatic pressure and estrogen enhance the cellular proliferation ability and the cytoskeleton activity. In addition, the hydrostatic pressure causes JNK-1/2 activation and promotes the chondrogenic differentiation of BMSCs, whereas estrogen acts strong activation of ERK and stimulates the osteogenic differentiation of BMSCs. The estrogen receptor ERα plays an important role in the promotion of cell proliferation, JNK-1/2 phosphorylation and chondrogenic differentiation by mechanical pressure.

time PCR results that only estrogen led to the upregulation of osteogenic differentiation, and similar variation trend in ERKs activation among several groups, it could be concluded that ERK activity dictates lineage commitment toward osteogenesis in BMSCs under estrogen stimulation. On the other hand, hydrostatic pressure led to the upregulation of chondrogenic differentiation. Additionally, hydrostatic pressure greatly promoted JNKs activation of BMSCs with or without estrogen. It indicated that JNK activity could dictate lineage commitment to chondrogenesis under hydrostatic pressure. These results differed from the findings of Simmons *et al.*, who used a combination of tension force and an osteogenic inducing agent on mesenchymal stem cells and found that tension can activate ERKs and P38 without any effect on JNK phosphorylation.[27] We believe that the difference in the results may be attributed to the different types of mechanical stimulation adopted. In addition, the combined biological effects of pressure and estrogen are far from simple synergistic or antagonistic effects, and combination treatments may have distinct biological effects for different signal molecules within the same MAPK family.

Based on these observations, we believed that ERα played an important regulatory role in the mechanobiological response of BMSCs. Upon further exploration of the molecular mechanisms of this pathway, we found that after blocking ERα function, the pressure-mediated effects on F-actin activation and JNK protein activation were significantly reduced, which indicated that the mechanobiological effects of hydrostatic pressure are closely associated with ERα activity. MAPK molecules and F-actin were likely to be important mediator molecules in the ER-mediated mechanotransduction of BMSCs. However, our results can only explain the role of ER in MAPK activation. The detailed molecular regulation mechanism, specifically the type of signal molecules that mediate between the ER and MAPK molecules, needs further study. Our previous work revealed that the activation of the JNK pathway by hydrostatic pressure may be negatively regulated by RhoA and Rac1.[38] From these observations, together with the present results of this study, we hypothesized that, as a common pathway of a plurality of the mechanotransduction signaling system, MAPK could be regulated by more than one upstream signaling molecule, and these upstream molecules may also have crosstalk with each other.

Therefore, the regulation mechanism between ER and MAPK are worthy of further investigation by further specifically designed experiments.

In the present study, we focused only on the role of hydrostatic pressure in BMSC activities. Nevertheless, it should be noted that for bone and cartilaginous tissues, hydrostatic pressure is only one component of the mechanical environment that they endure. Many tissues of interest experience *in vivo* stressful states that differ substantially from the application of simple hydrostatic pressure, although a dilatational component can always be identified for any given loading regime.[1] Further research is required to determine if estrogen has a synergistic effect with other types of mechanical stimulation that BMSCs may encounter *in vivo* whether they are present in bone marrow or as seed cells in tissue-engineered grafts.

CONCLUSIONS

Based on our results, we disclose the distinct effects of estrogen and hydrostatic pressure on BMSC differentiation. Pressure promotes the chondrogenic differentiation of BMSCs, with JNK-1/2 activation being involved, and estrogen promotes osteogenic differentiation of BMSCs, with ERK activation being involved. ERα plays an important regulatory role in the mechanobiological response of BMSCs. MAPK molecules and F-actin are likely to be important signal molecules in the ER-mediated mechanotransduction of BMSCs (Fig. 7).

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AUTHOR CONTRIBUTIONS

YZ. and F-Z.Y. made the acquisition of data and most of the analysis of the data, and were also involved in the drafting of the manuscript. Y-H.Z. and Y-J.C. helped with the interpretation of the data. H.M. and M.Z. was involved in the conception and design of the study, and did the most of the manuscript drafting and revising. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial competing interests related to this study.

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