

Estradiol-enhanced osteogenesis of rat bone marrow stromal cells is associated with the JNK pathway

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Abstract. Bone marrow stromal cells (BMSCs) can differentiate into osteoblasts. The present study investigated the osteogenic effects of estradiol, as well as the role of the c-Jun N-terminal kinase (JNK) signaling pathway in promoting estradiol-enhanced osteogenesis of rat (r)BMSCs. rBMSCs were treated for 7 days with or without estradiol and further treated with or without the JNK-specific inhibitor SP600125. The role of estrogen during rBMSC osteogenesis was evaluated by alkaline phosphatase activity and mineralized nodule formation using the Gomori method and Alizarin red S staining, respectively. Subsequently, the mRNA expression levels of transforming growth factor- β 1 (TGF- β 1) and core-binding factor α 1 (Cbfa1) were evaluated by reverse transcription-quantitative polymerase chain reaction, and TGF- β 1, Cbfa1 and phosphorylated (p)-JNK protein expression was detected by western blotting. All groups treated with SP600125 expressed low levels of TGF- β 1 and Cbfa1 mRNA and protein, and low p-JNK protein expression. Compared with the control cells, rBMSCs cultured with estradiol exhibited a significant upregulation in the expression levels of osteogenic genes and proteins. The present study demonstrated that estradiol enhanced osteogenic differentiation of rBMSCs and that the JNK signaling pathway was involved in this process, providing insights into the molecular mechanisms involved in rBMSC osteogenesis upon estradiol stimulation.

Introduction

Osteoporosis is a very common disease in postmenopausal women. It is frequently associated with bone fractures, which

are a major health problem worldwide (1). Osteoporosis is characterized by a reduction in bone mass caused by a disturbance in the ratio between bone formation and bone resorption. This imbalance may be associated with low blood serum estrogen levels in postmenopausal women (2). Estrogen deficiency is recognized as an important risk factor in the development of postmenopausal osteoporosis (3). Although prolonged exposure to relatively high doses of estrogen may result in sustained stimulation of osteoblastic activity in postmenopausal women (4), the precise mechanism by which estrogen enhances bone formation is not clear.

Osteoblast precursors are considered to be derived from marrow mesenchymal stem cells (MSCs) (5). It has been hypothesized that estrogen increases bone formation by enhancing the proliferation and differentiation of MSCs. Primary bone marrow stromal cells (BMSCs) are multipotent and capable of differentiating along the osteoblastic cell lineage (6). Rat BMSCs (rBMSCs) have been used in previous tissue engineering studies because they are strongly adherent, highly proliferative and readily available (7,8). Therefore, rBMSCs were used in the present study to examine the contribution of estrogen to osteogenic differentiation.

Mitogen-activated protein kinases (MAPKs) are involved in the regulation of gene expression, mitosis, metabolism, motility, survival, apoptosis and differentiation of stem cells. MAPK subfamilies mediate various biological effects: Extracellular signal-regulated kinase 1 (ERK1) and ERK2 regulate cell differentiation and proliferation; c-Jun N-terminal kinases (JNKs) have an important role in cell cycle regulation, apoptosis and cellular stress; and p38 MAPKs affect numerous biological processes, such as proliferation, differentiation and survival, and responses to stress and inflammation in certain cell types (9). A number of previous studies have focused on the functions of ERK1/2 and p38 MAPKs in other contexts, such as tumor and diabetes studies (10,11). MAPKs may also be activated during osteogenesis. The JNK signaling pathway has previously been demonstrated to serve a role in the regulation of osteogenic differentiation of MSCs (12). The present study hypothesized that estradiol may enhance the induction of osteogenesis by upregulating the JNK signaling pathway. To validate this hypothesis the underlying mechanisms of estradiol-induced osteogenesis in rBMSCs were investigated.

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Materials and methods

Ethics statement. A total of 5 male Sprague-Dawley rats (age, 6 weeks; weight 200±10 g) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). Rats were housed in a climate-controlled environment (temperature, 22±1°C; humidity, 50±5%) with a 12/12 h light/dark cycle and *ad libitum* access to food and water. All procedures were performed under 10% chloral hydrate anesthesia when necessary. The rats were sacrificed by asphyxiation with CO₂. All experimental procedures were approved by (permit no. 2011-167) and followed the guidelines of the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine (Shenyang, China).

Primary culture of rBMSCs. rBMSCs were isolated from the bone marrow of the posterior tibias and femurs of 8-week-old Sprague-Dawley rats. Bone marrow cells were flushed out with α -minimum essential medium (α -MEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and centrifuged at 300 x g for 3 min. rBMSCs adhering to the plastic surface were selected for further experimentation. Primary cells (1x10⁷ cells/ml) were cultured with α -MEM containing 10% FBS (HyClone; GE Healthcare Life Sciences) for 5 days (37°C, 5% CO₂), changing the medium every 3 days. At 80% confluence, the cells were passaged at a ratio of 1:2. All experiments were performed using cells cultured to the fourth passage.

Flow cytometric analysis. Cells were grown to passage 4, washed twice with PBS and incubated with 0.25% trypsin for ~1 min; the reaction was stopped by adding PBS. Trypsinized cells were transferred to tubes at a density of 1x10⁶ cells/ml. The cells were then stained with antibodies (1:200) against cell surface markers CD29 (cat. no. 102215), CD90.1 (cat. no. 202515) and CD45 (cat. no. 202207) (BioLegend, Inc., San Diego, CA, USA) in the dark for 30 min at 4°C, followed by centrifugation at 300 x g for 5 min. Following centrifugation, cells were washed with PBS (1 ml), centrifuged at 300 x g for 5 min and resuspend in 300 μ l PBS. Cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and the FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Alkaline phosphatase (ALP) activity assay. A colorimetric p-nitrophenyl phosphate (pNPP) assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to evaluate ALP activity and to quantify the osteogenesis of rBMSCs cultured in either induction medium (IND group; 10⁻⁷ mol dexamethasone, 50 μ mol ascorbic acid and 10 mmol/l sodium β -glycerophosphate) or induction medium supplemented with estradiol (E2 group) at concentrations of 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹ mol. ALP activity was assayed at days 5, 7 and 9. Briefly, rBMSCs (5x10⁴ cells) were diluted in 0.2 ml of control (α -MEM supplemented with 10% FBS), IND or E2 medium and transferred to a 96-well plate (8 wells/experimental group). Triton X-100 (100 μ l; 0.1% Triton X-100) was then added and the cells were incubated at -20°C overnight. Following overnight incubation, cells were lysed in 100 μ l pNPP/diethanolamine buffer (1:1) at 37°C for 30 min. The

reaction was terminated by adding 80 μ l NaOH buffer (0.5 mol). All wells were analyzed in a microplate reader at an optical density of 415 nm.

ALP staining assay. The ALP staining assay comprised 3 experimental groups: i) A control group, which consisted of rBMSCs cultured in growth medium (10% FBS in α -MEM); ii) an IND group; and iii) an E2 group cultured in IND + estradiol (10⁻⁸ mol). Each group was further divided into 2 treatment groups in which the medium was supplemented with or without the JNK-specific inhibitor SP600125 (10 μ mol, 37°C, 30 min) (Merck KGaA). The 6 experimental groups were subjected to ALP staining assays using the Gomori method. Briefly, cells were seeded at a density of 1x10⁵ cells/well in 24-well plates with 3 wells/group. Following incubation for 14 days at 37°C, the cells were stained with fresh ALP incubation buffer (2% barbitone sodium, 2% anhydrous calcium chloride, 3% β -sodium glycerol-phosphate, 2% magnesium sulfate and 5 ml distilled water, pH 9.4) for 4 h at 37°C. The cells were rinsed with ultrapure water for 10 min, treated with 2% cobalt nitrate for 5 min, followed by 1% ammonium sulfide solution for 1 min. After drying, the stained cells were imaged under a light microscope and quantitative analysis was conducted using the BI-2000 digital image analysis system (Chengdu TME Technology Co., Ltd., Chengdu, China).

Alizarin red S staining. Alizarin red S staining was used to examine the mineralized nodule formation of rBMSCs. Cells were prepared as described for the ALP staining assay and cultured for 21 days. The cells were stained with alizarin red S [0.1% (w/v) in Tris-HCl] for 2 h at 37°C, then washed three times with PBS. Finally, mineralized nodules were imaged under an inverted light microscope and quantitative analysis was conducted using the BI-2000 digital image analysis system (Chengdu TME Technology Co., Ltd.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR analysis was performed on the 6 experimental groups described in the aforementioned ALP staining assay. Cells (3x10⁵) were seeded in flasks and cultured with the relevant incubation medium (control, IND or E2) with or without 10 μ mol SP600125 for 7 days. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and quantified with a spectrophotometer at 260/280 nm. Following the removal of genomic DNA using gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China), total RNA was reverse transcribed using a PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions.

PCR was performed using a SYBR Green kit (Takara Biotechnology Co., Ltd.) and an Mx3000P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following cycling conditions: 1 cycle of 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. Primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and were custom-made by Takara Biotechnology Co., Ltd. Gene expression was normalized relative to that of the housekeeping gene GAPDH. The following rat primers were used: GAPDH forward,

5'-TGTGTCCGTCGTGGATCTGA-3' and reverse, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; core-binding factor $\alpha 1$ (Cbf $\alpha 1$) forward, 5'-CACTGGCGGTGCAACAAGA-3' and reverse, 5'-ATGACGGTAACCACAGTCCCATC-3'; and transforming growth factor- $\beta 1$ (TGF- $\beta 1$) forward, 5'-GTGTGGAGCAACATGTGGAAGTCTA-3' and reverse, 5'-CGCTGAATCGAAGCCCCTGTA-3'. Relative mRNA expression levels were quantified using the $2^{-\Delta\Delta C_q}$ method (13).

Western blot analysis. rBMSCs were prepared as described in the ALP staining assay and proteins were extracted using a Mammal Tissue Protein Extraction kit (Boster Biological Technology, Pleasanton, CA, USA) with 1 mmol phenylmethanesulfonyl fluoride. Protein quantification was performed with a bicinchoninic acid protein assay. Protein samples (50 μ g) were separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with primary and secondary antibodies (37°C; 1 h). The primary antibodies were against GAPDH (cat. no. 5174), total JNK (cat. no. 9252), phosphorylated (p)-JNK (cat. no. 9251) (Cell Signaling Technology, Inc., Danvers, MA, USA), TGF- $\beta 1$ (cat. no. sc-146; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Cbf $\alpha 1$ (cat. no. ab76956; Abcam, Cambridge, UK). Protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) and exposed to X-ray films (Kodak, Rochester, NY, USA). Densitometric analysis was performed with AlphaView SA Software (ProteinSimple; Bio-Techne, Minneapolis, MN, USA) to analyze protein quantification. And the results were expressed as a ratio of the level of expression of the target protein to GAPDH expression. The level of JNK phosphorylation was represented by the ratio of p-JNK to JNK protein expression levels.

Statistical analysis. Results are presented as the mean \pm standard deviation. Multiple comparisons were performed by one-way analysis of variance followed by Fisher's least significant difference test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated at least 3 times.

Results

Morphological features and characterization of rBMSCs by flow cytometry. rBMSCs were imaged under an inverted microscope (Fig. 1A). Primary cells were circular or oval in shape. Cells were passaged when they reached 80% confluence at 8-10 days incubation. Cells at passage 4 were larger and spindle-shaped (Fig. 1B). Cells were allowed to grow to 80% confluence in a culture flask.

Cells were harvested and subjected to flow cytometry to examine cell surface markers. A total of 97.72 and 84.71% of cells were positive for the mesenchymal surface markers CD29 and CD90.1, respectively (Fig. 1C and D); whereas 2.81% were positive for the hematopoietic stem cell marker CD45 (Fig. 1E).

rBMSC ALP activity. ALP is an enzyme that is expressed during rBMSC osteogenesis and is considered a well-defined

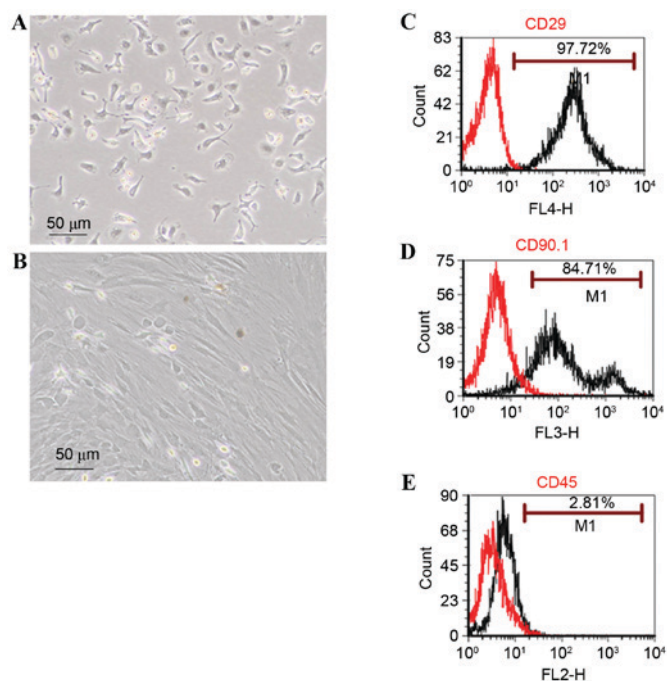


Figure 1. Morphology and cell surface markers of rBMSCs. Morphology of primary rBMSCs at (A) 5 days and (B) passage 4 rBMSCs at 6 days (magnification, $\times 100$). Flow cytometric analysis of rBMSCs for (C) CD29, (D) CD90.1 and (E) CD45. rBMSCs, rat bone marrow stromal cells.

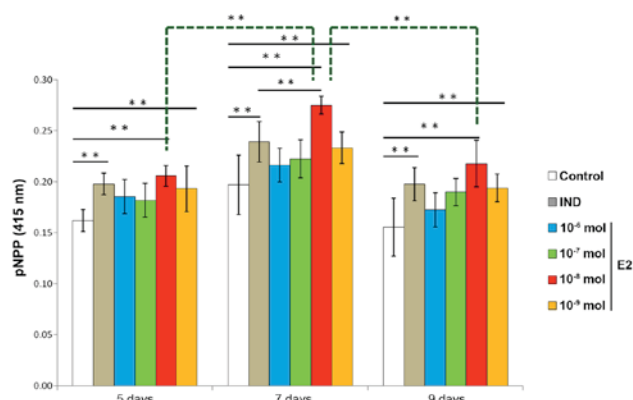


Figure 2. ALP activity of rBMSCs treated with various concentrations of E2. The optimal E2 concentration and incubation time were determined as 10^{-8} mol and 7 days, respectively ($^{**}P < 0.01$). E2, estradiol; IND, induction medium; pNPP, p-nitrophenyl phosphate; rBMSCs, rat bone marrow stromal cells.

and early marker for their differentiation. ALP activity was examined to evaluate the optimal concentration and time required for estradiol-induced rBMSC osteogenesis. Compared with the control group, two of the E2 groups (10^{-8} mol and 10^{-9} mol estradiol) and the IND group exhibited higher levels of ALP on days 5, 7 and 9. Compared with the IND group, the 10^{-8} mol E2 group had significantly higher levels of ALP on day 7 ($P < 0.01$); rBMSCs treated with 10^{-8} mol estradiol also had significantly higher levels of ALP on day 7 than those cells receiving the same treatment on days 5 and 9 ($P < 0.01$). Therefore, estradiol induction of osteogenesis was considered optimal at a concentration of 10^{-8} mol estradiol and 7 days incubation (Fig. 2).

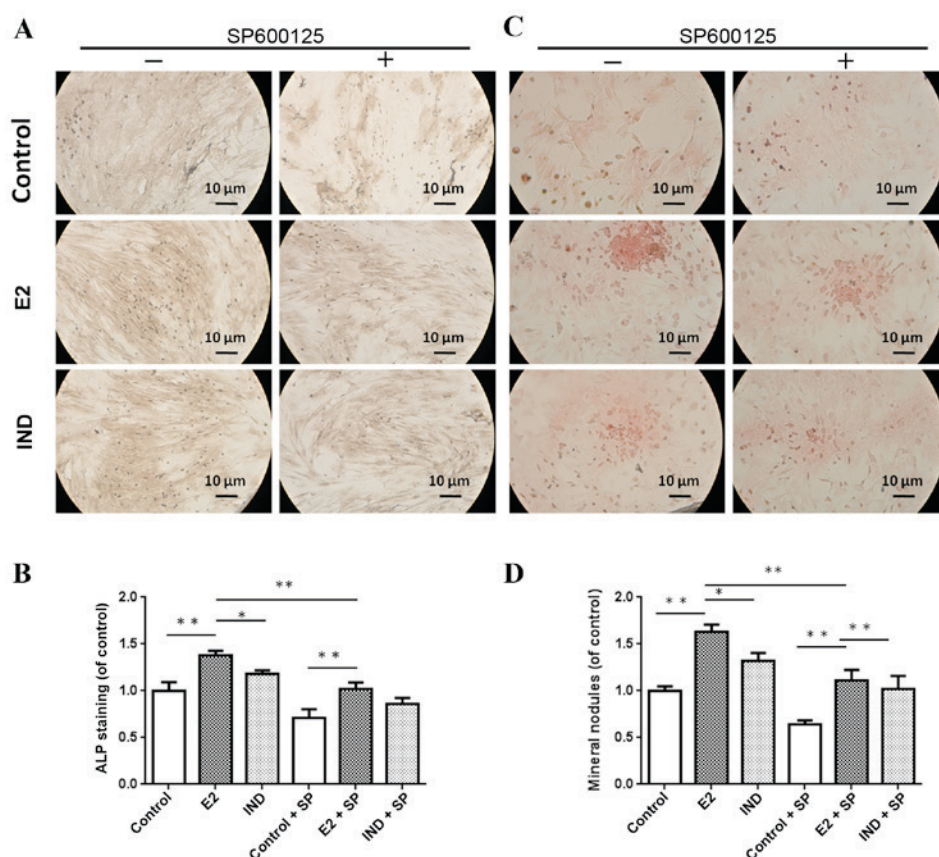


Figure 3. ALP staining and mineral nodule formation of rat bone marrow stromal cells. (A and B) ALP staining was performed using Gomori's method. (C and D) Mineral nodule formation was determined by Alizarin red S staining. * $P < 0.05$ and ** $P < 0.01$. ALP, alkaline phosphatase; E2, estradiol; IND, induction medium; SP, SP600125.

ALP staining and mineral nodule formation. Cell cultures were imaged with an inverted light microscope (Fig. 3). The E2 and IND rBMSC groups had significantly higher levels of ALP compared with the control group ($P < 0.01$) (Fig. 3A and B). The level of ALP in rBMSCs of the E2 group was significantly higher compared with cells in the IND group ($P < 0.05$).

Alizarin red S staining was used to examine the formation of mineralized nodules in the various rBMSC cultures. As shown in Fig. 3C and D, the number of nodules forming in the E2 and IND groups were significantly larger compared with the control group, and those of the E2 group were significantly larger compared with the IND group at 21 days ($P < 0.01$ or $P < 0.05$). Treatment with the JNK inhibitor SP600125 reduced the osteogenesis-stimulating effects of estradiol; however, nodule formation remained significantly higher in the E2 and IND groups, compared with the control group.

JNK phosphorylation. The relative level of p-JNK compared with the total JNK protein expression in rBMSCs was assayed by western blotting (Fig. 4). Compared with the E2 group, the control and IND groups had lower levels of p-JNK ($P < 0.01$). Addition of the JNK inhibitor SP600125 significantly reduced the levels of JNK and p-JNK protein expression under all conditions. However, the expression levels of p-JNK in the E2 and IND groups co-cultured with SP600125 remained higher compared with the control group treated with the inhibitor ($P < 0.05$).

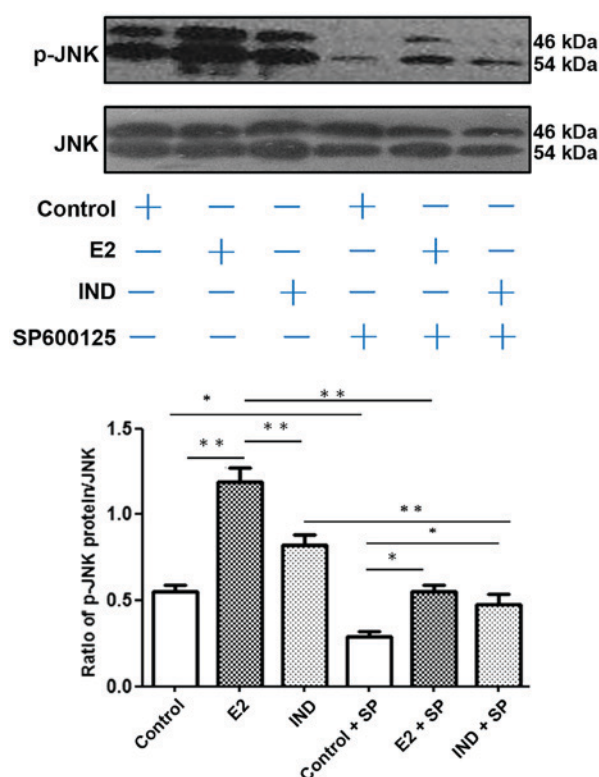


Figure 4. Western blot analysis of JNK and p-JNK protein in rat bone marrow stromal cells. Significant differences among each group are noted by * $P < 0.05$ and ** $P < 0.01$. E2, estradiol; IND, induction medium; JNK, c-Jun N-terminal kinase; p, phosphorylated; SP, SP600125.

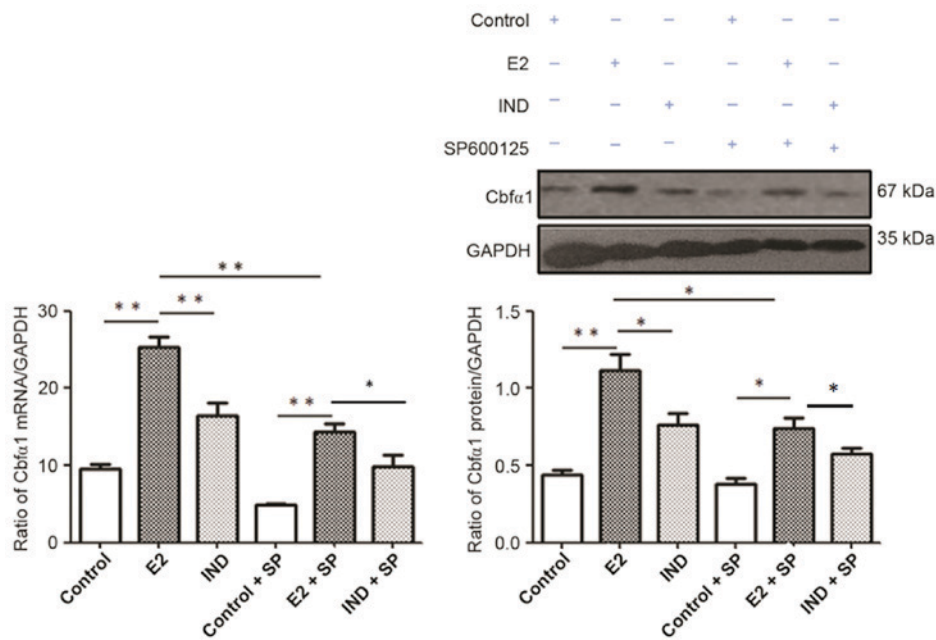


Figure 5. Cbfa1 mRNA and protein expression levels in rat bone marrow stromal cells. Reverse transcription-quantitative polymerase chain reaction and western blot analysis of Cbfa1 mRNA and protein expression, respectively. GAPDH blot is the same as in Fig. 6, as Cbfa1, TGF- β 1 and GAPDH were all probed for on the same membrane. *P<0.05 and **P<0.01. Cbfa1, core-binding factor α 1; E2, estradiol-treated group; IND, induction medium group; SP, SP600125.

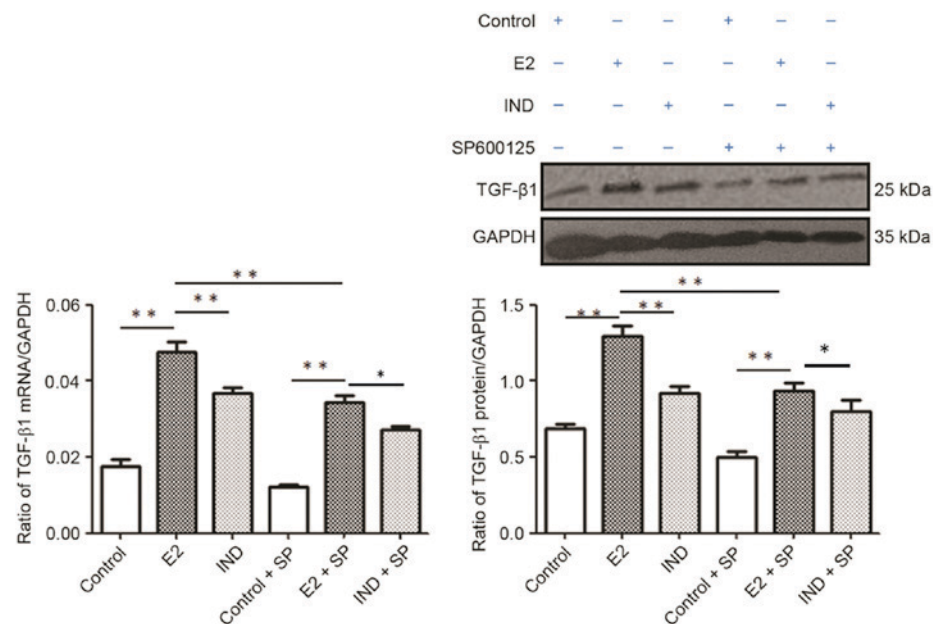


Figure 6. TGF- β 1 mRNA and protein expression levels in rat bone marrow stromal cells. Reverse transcription-quantitative polymerase chain reaction and western blot analyses were used to detect TGF- β 1 mRNA and protein levels, respectively. GAPDH blot is the same as in Fig. 5, as Cbfa1, TGF- β 1 and GAPDH were all probed for on the same membrane. *P<0.05 and **P<0.01. E2, estradiol group; IND, induction medium; SP, SP600125; TGF- β 1, transforming growth factor- β 1.

Cbfa1 mRNA and protein expression. Cbfa1 mRNA and protein expression levels were significantly higher in rBMSCs from the E2 group compared with the control and IND groups (P<0.01 or P<0.05; Fig. 5). Although the levels of Cbfa1 mRNA and protein expression were lower in all groups following the addition of SP600125, they remained higher in the E2 group compared with the IND and control groups (P<0.01 or P<0.05).

TGF- β 1 mRNA and protein expression. The E2 group had significantly higher levels of TGF- β 1 mRNA and protein expression compared with the control and IND groups (P<0.01; Fig. 6). The expression levels of TGF- β 1 mRNA and protein were subsequently lower in each group when co-treated with SP600125; in addition, the levels were significantly higher in the E2 group compared with the control or IND groups (P<0.01).

Discussion

The present study investigated the effects of the estrogen steroid sex hormone estradiol on the osteogenic differentiation of rBMSCs. Estradiol treatment was associated with an increase in osteogenic induction, as determined by examining markers of osteogenesis, such as ALP activity and expression, and the formation of mineralized nodules. ALP is an early stage marker, whereas the formation of mineralized nodules is an indicator of middle- and late-stage osteogenesis (14,15). The present study determined the optimal concentration of estradiol treatment in rBMSC culture was 10^{-8} mol and the optimal incubation time was 7 days. Results indicated that osteogenesis in rBMSCs treated with estradiol was more robust compared with those treated with IND media, as indicated by higher levels of ALP and stronger formation of mineralized nodules.

Animal models of osteoporosis, such as ovariectomized mice, are widely used to study the signaling cascades that control rBMSC osteogenesis, including research on postmenopausal osteoporosis (16). However, it remains unclear how estrogen contributes to the osteogenic differentiation of stem cells. A previous study demonstrated that the JNK signaling pathway is involved in the regulation of osteogenesis (12). Guicheux *et al* (17) reported that bone morphogenetic protein 2 regulated osteoblastic differentiation of stem cells through activation of the JNK and p38 MAPK signaling pathways. Therefore, the present study explored the role of JNK signaling in the estrogen-mediated regulation of osteogenic differentiation of rBMSCs. The results demonstrated that estradiol is associated with higher mRNA and protein expression levels of p-JNK and Cbfa1 in rBMSC cultures. Cbfa1 is a transcription factor that regulates the expression of numerous genes relevant to osteogenesis (18,19). It is a major factor in the initiation and regulation of early osteogenesis, and serves a crucial role in the late mineralization stages (20). Notably, the present study revealed that the E2 group had relatively high levels of p-JNK and Cbfa1 mRNA and protein following treatment with the JNK-specific inhibitor SP600125, compared with cells in the control and IND groups. These data indicated that JNK signaling may serve a crucial role during estrogen-induced osteogenesis of rBMSCs.

Furthermore, results from the present study demonstrated that the mRNA and protein expression levels of TGF- β 1 were higher in the E2 group compared with the IND and control groups, and were decreased in the presence of SP600125. TGF- β 1 may stimulate the phosphorylation of JNK, which enables the signal to be transferred into the nucleus to regulate the osteogenic differentiation of rBMSCs. In conclusion, the present study provides evidence to suggest that estradiol may promote rBMSC osteogenesis by activating the JNK signaling pathway. These data support the hypothesis that estradiol-induced JNK signaling contributes to osteogenesis and the prevention of postmenopausal osteoporosis.

Acknowledgements

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