

17 β -estradiol attenuates ovariectomy-induced bone deterioration through the suppression of the ephA2/ephrinA2 signaling pathway

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Abstract. The present study aimed to investigate whether 17 β -estradiol (E2) exerts protective effects on bone deterioration induced by ovariectomy (OVX) through the ephA2/ephrinA2 signaling pathway in rats. Female rats were subjected to OVX, sham surgery or OVX+E2 treatment. Levels of biomarkers were measured in serum and urine. Hematoxylin and eosin staining was performed on paraffin-embedded bone sections. Expression of genes and proteins was analyzed by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Bone mineral density (BMD) was analyzed by dual-energy X-ray absorptiometry. Trabecular bone microarchitecture was also evaluated. Osteoclastogenesis was induced by *in vitro* culturing with mouse receptor activator of nuclear factor κ B ligand (RANKL) and macrophage colony-stimulating factor 1. small interfering RNA was designed to knockdown ephA2 receptor and its ligand ephrinA2. Results of the present study demonstrated that E2 had suppressive effects on OVX-induced body weight gain and bone turnover factors in serum and urine. E2 inhibited the bone resorption function of osteoclasts by inhibiting the production of tartrate-resistant acid phosphatase-5b and RANKL, and induced bone formation function of osteoblasts by prompting runt-related transcription factor 2, Sp7 transcription factor and collagen alpha-1(I) chain expression in bone marrow cells. E2 treatment significantly increased

the tibia BMD and prevented the deterioration of trabecular microarchitecture compared with the OVX group. Moreover, E2 significantly decreased the OVX-stimulated expression of ephA2 and ephrinA2. EphA2 or ephrin A2 knockdown significantly suppressed osteoclastogenesis *in vitro*. In conclusion, E2 can attenuate OVX-induced bone deterioration partially through the suppression of the ephA2/ephrinA2 signaling pathway. Therefore EphA2/ephrinA2 signaling pathway may be a potential target for osteoporosis treatment.

Introduction

Constant bone mass is maintained by bone remodeling during adulthood and depends on the regulation of osteoblast-osteoclast coupling (1,2). Since resorption of old mineralized bone by osteoclasts is followed by new bone formation by osteoblasts, the osteoblast-osteoclast coupling can tightly regulate initiation, transition, and termination phases of bone resorption (3). Loss of the coupling and resulting disruption of bone homeostasis can result in several metabolic bone diseases, including osteopetrosis, osteoporosis and tumor-associated bone diseases (3). Therefore, therapies targeting these diseases mainly fall into two categories: Anabolic drugs, which stimulate bone formation, and antiresorptive drugs, which slow down bone resorption (4).

Estrogen, a steroid hormone, is well-known to affect the circulatory, reproductive and cardiovascular systems, and bone homeostasis through inhibition of bone resorption and enhancement of bone formation (5,6). Estrogen deficiency can cause early and late forms of osteoporosis in postmenopausal women (7). There are three types of estrogen: 17 β -estradiol (E2), estron and estriol. E2, secreted by the ovarium of adult women with a normal menstrual cycle, has the highest estrogenic potency (8). It has been demonstrated that E2 supplementation effectively stimulates the proliferative capacity of mesenchymal stem cells (MSCs) (9). Further investigation suggests that E2 induces bone formation by stimulating bone morphogenetic protein-2 gene transcription in MSCs (9). E2 inhibits senescence of MSC via the upregulation of telomerase, and improvement of osteogenic and adipogenic differentiation of MSCs (10,11).

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The impact of eph-ephrin bidirectional signaling on bone homeostasis provides an explanation for cellular and molecular mechanisms responsible for osteoblast-osteoclast coupling (1). Osteoblasts and osteoclasts can proliferate and differentiate from MSCs and macrophagocytes, and ephrins and ephs coupling regulates these cell-cell interaction processes (2). Ephrins are divided into two classes, ephrinAs (ephrinA1-A5) and ephrinBs (ephrinB1-B3). Ephs fall into the following two categories, ephAs (ephA1-A10) and ephBs (ephB1-B6) (12). A previous study reported that ephrinA2-ephA2 interaction facilitates the initiation phase of bone remodeling through enhancing osteoclast differentiation and suppressing osteoblast differentiation (1).

The present study aimed to investigate whether E2 exerts its bone protective effects through the ephA2/ephrinA2 signaling pathway in bone deterioration induced by ovariectomy (OVX) in rats. Firstly, OVX was performed on rats and the effects of estrogen on OVX-induced body weight gain, bone turnover markers and key signaling molecules involved in the regulation of bone metabolism were evaluated. Effects of estrogen on BMD, bone histomorphology and trabecular bone microarchitecture were also detected. Finally, the mechanism underlying the effect of E2 on postmenopausal osteoporosis was explored. The present study demonstrated that E2 attenuates OVX-induced bone deterioration partially through the suppression of the ephA2/ephrinA2 signaling pathway, a result which aids the prevention and treatment of postmenopausal osteoporosis.

Materials and methods

Animals. A total of 45 Sprague-Dawley rats (12-week-old females; weighing 220-240 g) were obtained from the Animal Center of the Institute of Science and Technology (Shanghai, China) and were housed in a controlled environment laboratory for 2 weeks prior to the start of the experiment. Rats were kept at 22-24°C with a 12-h light/dark cycle and free access to food and water. All procedures involving animal welfare were reviewed and approved by the Ethical Committee of Pudan Hospital of Pudong New District (Shanghai, China).

Experimental protocols. Rats were divided into three groups: i) Sham operated (SHAM, n=15); ii) ovariectomized without treatment (OVX, n=15); and iii) ovariectomized rats treated with E2 (OVX+E2, n=15). All compounds were supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) unless stated otherwise. Rats were subcutaneously injected 5 times per week with 100 μ l medium (10% dimethyl sulfoxide, 90% sesame oil) in the SHAM and OVX groups, and with E2 (20 μ g/kg/day) in the OVX+E2 group. All three groups received treatment at the same time each day. All rats were weighed following 2, 4, 6 and 8 weeks of treatment.

Biochemical analysis. At 8 weeks of treatment, rats were deeply anesthetized with urethane (5 ml/kg) intraperitoneally and euthanized by exsanguination. Blood was collected following overnight fasting and serum was separated by centrifugation at 500 \times g for 15 min at 21°C and stored at -70°C. The level of serum calcium (Ca) was measured on the Ciba Corning 550 EXPRESS using Ciba Corning reagents (Ciba Corning

Diagnostic Ltd., Sudbury, UK) for the *in vitro* determination. The urine Ca and creatinine (Cr) concentrations were analyzed by the same method as the serum samples at 2, 4, 6 and 8 weeks following treatment. Serum bone-specific alkaline phosphatase (b-ALP) and bone resorption tartrate-resistant acid phosphatase-5b (TRAP-5b) were determined by ELISA (cat no. SB-TR103; Immunodiagnostic Systems, Boldon, UK) according to the manufacturer's protocol.

Bone mineral density (BMD) analysis. BMD of left tibiae was measured using dual energy X-ray absorptiometry (DEXA) and Lunar-DPX-IQ bone densitometry (GE Healthcare, Chicago, IL, USA).

Histopathological and histomorphometric analyses. Following 60 days of treatment, left tibiae were fixed by immersion in buffered formalin for 72 h at room temperature, then decalcified in 10% ethylenediaminetetraacetic acid for 4 weeks, dehydrated in a desiccator with graded ethanol (25, 50, 70, 90 and 100%), defatted in xylene and embedded in paraffin. Longitudinally oriented, 5-mm-thick sections were cut and stained with H&E at room temperature for 10 min for histopathological analysis, and with Safranin-O/Fast green dye for 10 min at room temperature for histomorphometrical analysis. Measurements were taken using a light microscope (magnification, $\times 4$; Leica Microsystems GmbH, Wetzlar, Germany) and an image analyzer (Image Pro-Express; Media Cybernetics Inc., Rockville, MD, USA). Static parameters including bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were calculated and expressed as previously described (13,14).

Osteoclast differentiation in vitro. Monocytes were isolated from the tibia and femur bone marrow of 8-10 weeks old rats, as previously described (15). Bone marrow cells were seeded in 96-well plates (3×10^4 cells/well) and washed with 50 nmol/l macrophage colony-stimulating factor 1 (M-CSF). After 4 days, the cells were considered bone-marrow-derived macrophages (BMMs). To induce osteoclastogenesis, BMMs were seeded in 48-well plates at a density of 15,000 cells/well and cultured with 100 nmol/l mouse receptor activator of NF- κ B ligand (RANKL) and 50 nmol/l M-CSF for 4-6 days in the presence or absence of the bone marrow sample. The medium was replaced every 2 days.

Small-interfering (si)RNA-based knockdown of ephA2 and ephrinA2. siRNA targeting ephA2 (5'-CAAUCACCGACCACC GGAC-3') or ephrinA2 (5'-CGTACGCGGAATACTTCGA-3') (all from Qiagen, Inc., Valencia, CA, USA) were used for transfection of cells using Lipofectamine RNAi MAX Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 500,000 cells were transfected with siRNA and harvested 48 h following transfection for the assessment of knockdown efficiency, or other subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) measurement of gene expression. At 8 weeks of treatment, rats were deeply anesthetized with urethane

(5 ml/kg) intraperitoneally and euthanized by exsanguination. Total RNA was extracted from bone marrow cells obtained from right tibiae using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reactions were performed in an ABI7300 Real-Time quantitative instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 31 sec. The expression levels of the internal control GAPDH, was used as a housekeeping gene, and the comparative $2^{-\Delta\Delta C_q}$ method (16) was used to quantify gene expression levels.

Western blot assay. Protein was collected from proximal tibiae that were lysed in radioimmunoprecipitation buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) containing protease inhibitors at 4°C for 30 min, and the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (30 μ g) were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare, Chicago, IL, USA). The membranes were blocked in 5% non-fat milk (Merck KGaA) overnight at 4°C. Transferred membranes were then stained with the following primary antibodies: Anti- epha2 (cat no. 507183; 1:1,000; Zhejiang Kangchen Biotech Co., Ltd.), anti- ephrinA2 (cat no. 123877; 1:2,000; R&D Systems, Inc., Minneapolis, MN, USA), and anti- β -actin (cat no. ab8227; 1:200; Abcam) overnight at 4°C. Subsequently, protein bands were detected by incubation with a horseradish peroxidase-conjugated secondary antibody (cat no. A50-106P; 1:1,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 1 h. Signals were detected using an enhanced chemiluminescence kit (cat. no. orb90504; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and exposed to Kodak X-OMAT film (Kodak, Rochester, NY, USA). Each experiment was performed at least three times and the results were analyzed using Alpha View Analysis Tools (AlphaView SA software, version 3.2.2; Protein Simple, Santa Clara, CA, USA).

Statistical analysis. Results of the present study were expressed as the mean \pm standard deviation. Data analysis was performed using SPSS (version 12.0; SPSS, Inc., Chicago, IL, USA). Differences between multiple independent groups were determined using one-way analysis of variance followed by a Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of E2 on OVX-induced body weight gain. In the present study, the initial body weights of the three groups were similar, 220 g. In the OVX group, body weight gain was consistently elevated compared with the SHAM group. Even though the daily food consumption in each group was nearly the same, the body weight of the OVX group was significantly increased compared with the SHAM group. Following E2 treatment, OVX-induced body weight gain was significantly suppressed in a time-dependent manner (Fig. 1). This outcome suggests that E2 had a suppressive effect on OVX-induced body weight gain.

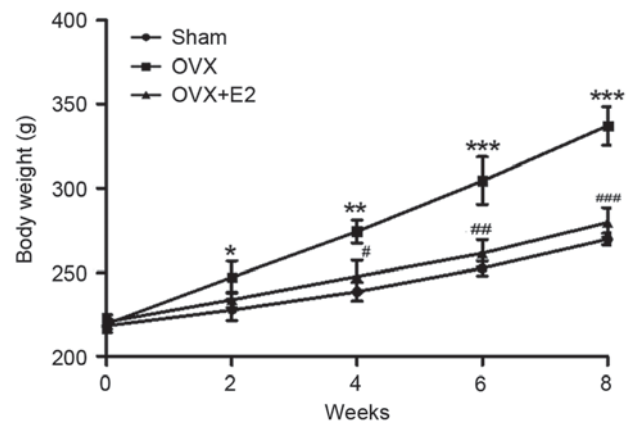


Figure 1. Effect of E2 on the body weight of OVX rats. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the SHAM group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the OVX group. Sham, placebo operated rats; OVX, ovariectomy; E2, 17 β -estradiol.

Effects of E2 on bone turnover markers. To further investigate the role of E2 in OVX-induced bone deterioration, markers for bone turnover, including serum Ca, urinary Ca/Cr, b-ALP and TRAP-5b were measured. Serum Ca concentration in the OVX+E2 group was significantly increased compared with the OVX group (Fig. 2A). Detection of urinary Ca/Cr for 8 weeks revealed increased levels in the OVX group compared with the SHAM group, while an immediate decrease was observed following E2 treatment (Fig. 2B). b-ALP and TRAP-5b concentrations in serum were also detected, revealing that E2 treatment effectively attenuated the enhancement of b-ALP and TRAP-5b concentrations induced by OVX in rats (Fig. 2C and D).

Effects of E2 on expression levels of bone metabolism-associated genes. ALP, TRAP, runt-related transcription factor 2 (Runx2), Sp7 transcription factor (osterix), collagen alpha-1(I) chain (colla1), osteoprotegerin (OPG) and RANKL are key signaling molecules involved in the regulation of bone metabolism. ALP is a marker for osteoblast activity and TRAP is a bone resorption factor. Runx2 is a master transcription factor of bone formation and osteoblast differentiation, and osterix, acting downstream of Runx2, regulates the transcription of bone-specific Colla1 gene (17,18). RANKL regulates osteoclast differentiation and function, and OPG is a soluble decoy receptor for RANKL, which competes with RANKL for binding to RANK and therefore suppresses osteoclast formation and activation (19,20).

E2 treatment effectively and significantly attenuated the upregulation of ALP and TRAP mRNA induced by OVX (Fig. 3A and B). Runx2, osterix and Colla1 were markedly downregulated in the OVX group compared with the SHAM and OVX+E2 groups (Fig. 3C). Additionally, E2 significantly suppressed the OVX-induced RANKL upregulation but had no evident effect on the OPG level (Fig. 3D). Therefore, the OPG/RANKL ratio was decreased in the OVX group compared with the SHAM group, indicating that OVX activated generation and differentiation of osteoclasts. The aforementioned results suggested that E2 inhibited bone resorption function of osteoclasts by inhibiting the production of TRAP and RANKL, and induced bone formation function of osteoblasts by inducing Runx2, osterix and Colla1 expression.

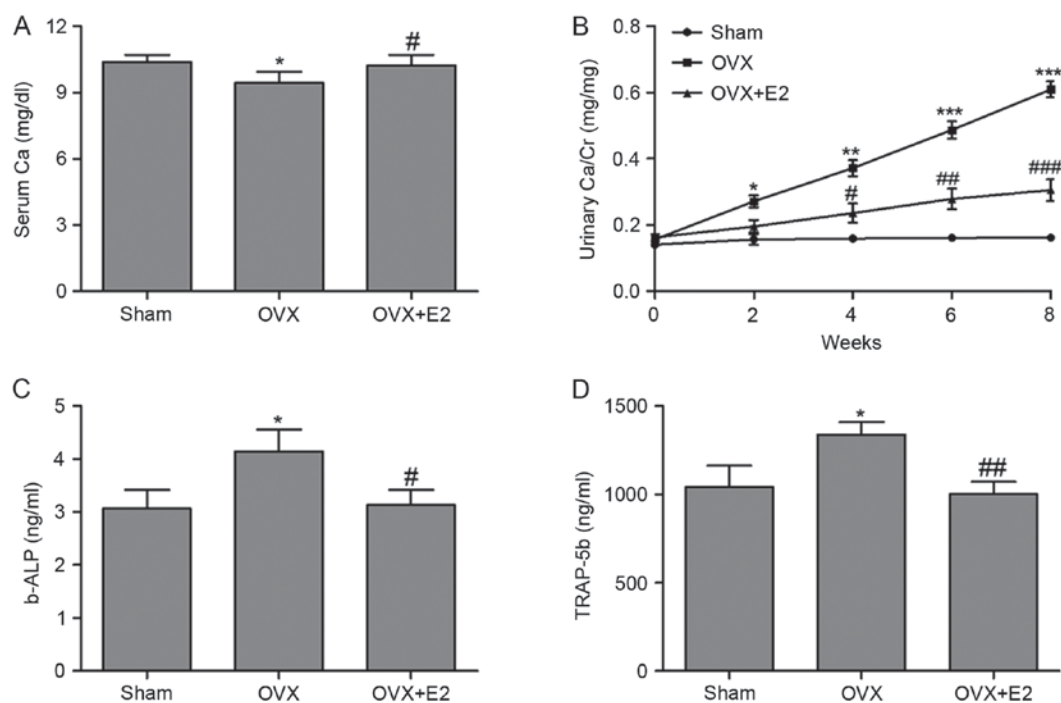


Figure 2. Effects of E2 on bone turnover markers. (A) Serum Ca at the end of the experiment. (B) Changes in urinary Ca/Cr levels. (C) b-ALP; and (D) TRAP-5b level at the end of the experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the SHAM group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the OVX group. Sham, placebo operated rats; OVX, ovariectomy; E2, 17 β -estradiol; TRAP-5b, tartrate-resistant acid phosphatase-5b; b-ALP, bone specific alkaline phosphatase.

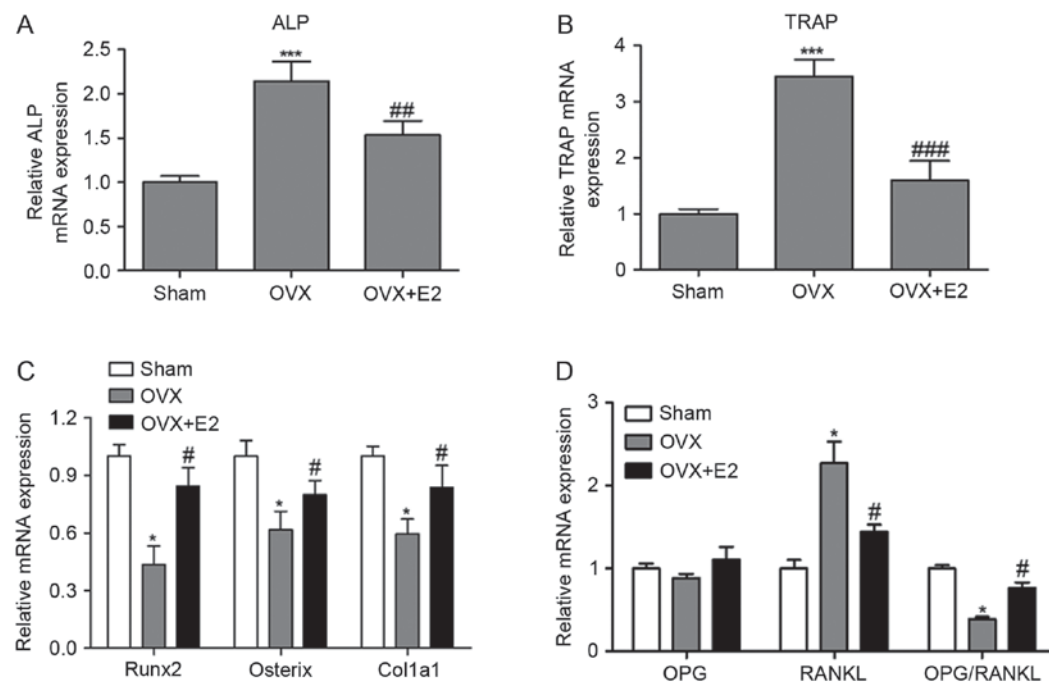


Figure 3. Effects of E2 on expression levels of bone metabolism-associated genes. (A) Relative ALP; (B) TRAP; (C) Runx2, osterix and colla1; (D) OPG and RANKL mRNA expression. Data are presented as the mean \pm standard deviation. * $P < 0.05$, *** $P < 0.001$ vs. the SHAM group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the OVX group. Sham, placebo operated rats; OVX, ovariectomy; E2, 17 β -estradiol; TRAP, tartrate-resistant acid phosphatase-5b; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; osterix, Sp7 transcription factor; colla1, collagen alpha-1(I) chain; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κ B ligand.

Effects of E2 on BMD and bone histomorphology of the experimental rats. As presented in Fig. 4A, the BMD of tibia differed significantly between the three groups at 4 and 8 weeks following treatment. Tibiae BMD decreased notably in the OVX compared with the SHAM group. E2

treatment significantly increased the OVX+E2 group tibia BMD compared with the OVX group.

H&E staining was performed to assess the effects of E2 on bone histomorphology. As presented in Fig. 4B, following OVX, the trabecular bone became thinner, sparse and interrupted,

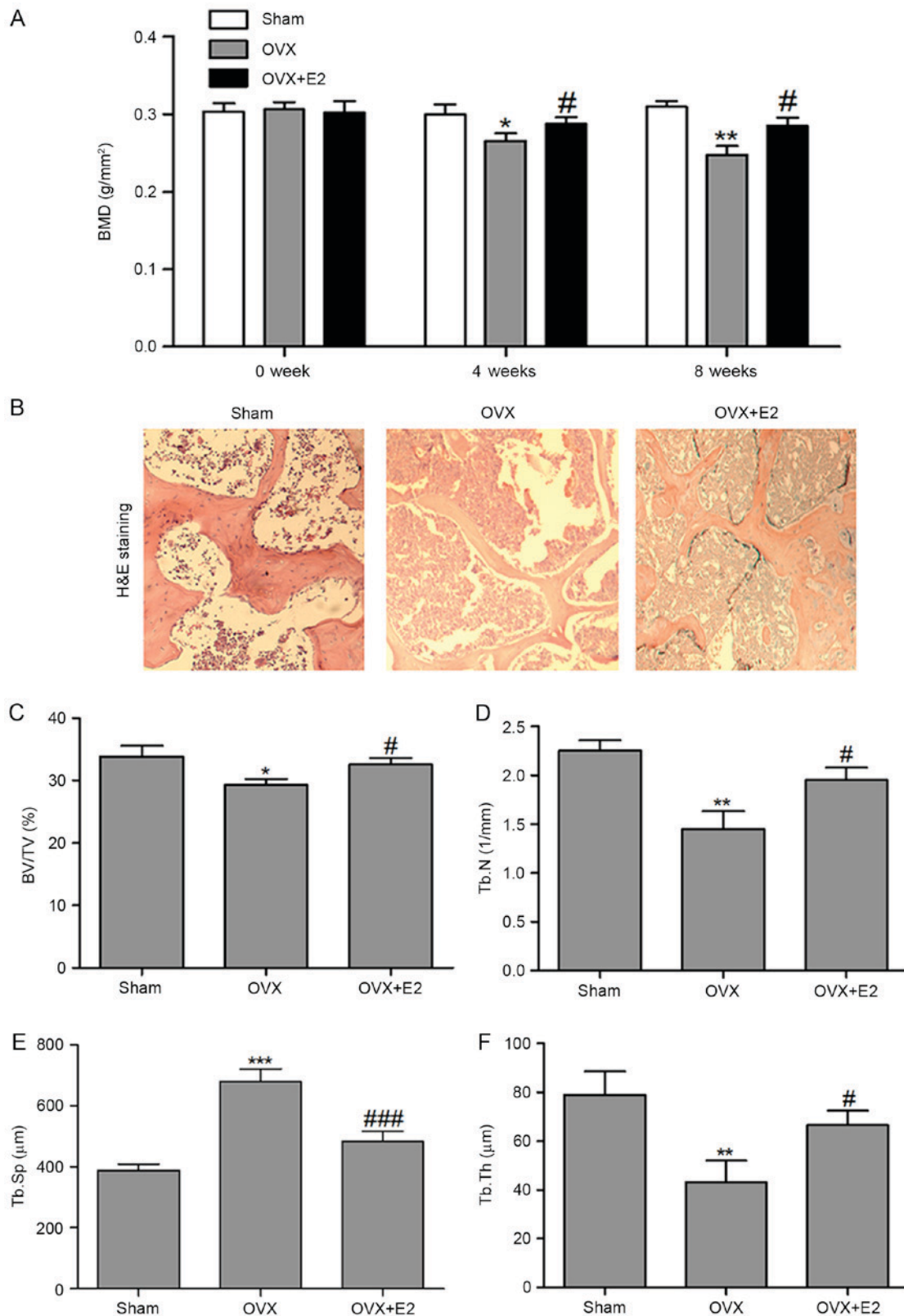


Figure 4. Effects of E2 on bone morphology. (A) Effect of E2 on BMD in left tibiae of OVX rats evaluated by DXA (B) Effect of E2 on bone histomorphology analyzed by H&E staining (magnification, x100). (C) Effects of E2 on the BV/TV; (D) Tb.N; (E) Tb.Sp; and (F) Tb.Th in the left tibiae of rats. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the SHAM group. # $P < 0.05$, ### $P < 0.001$ vs. the OVX group. DEXA, Dual-energy X-ray absorptiometry; Sham, placebo operated rats; OVX, ovariectomy; E2, 17 β -estradiol; BV/TV, bone volume per tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; BMD, bone mineral density; H&E, hematoxylin and eosin.

which showed reduced area in visual field/unit, and demonstrated enlargement of the medullary canal compared with the SHAM tissue. E2 reversed the effects of OVX stimulation on

the bone structure, and the photomicrograph of the trabecular bone in E2-treated rats appeared similar to the SHAM group trabecular bone.

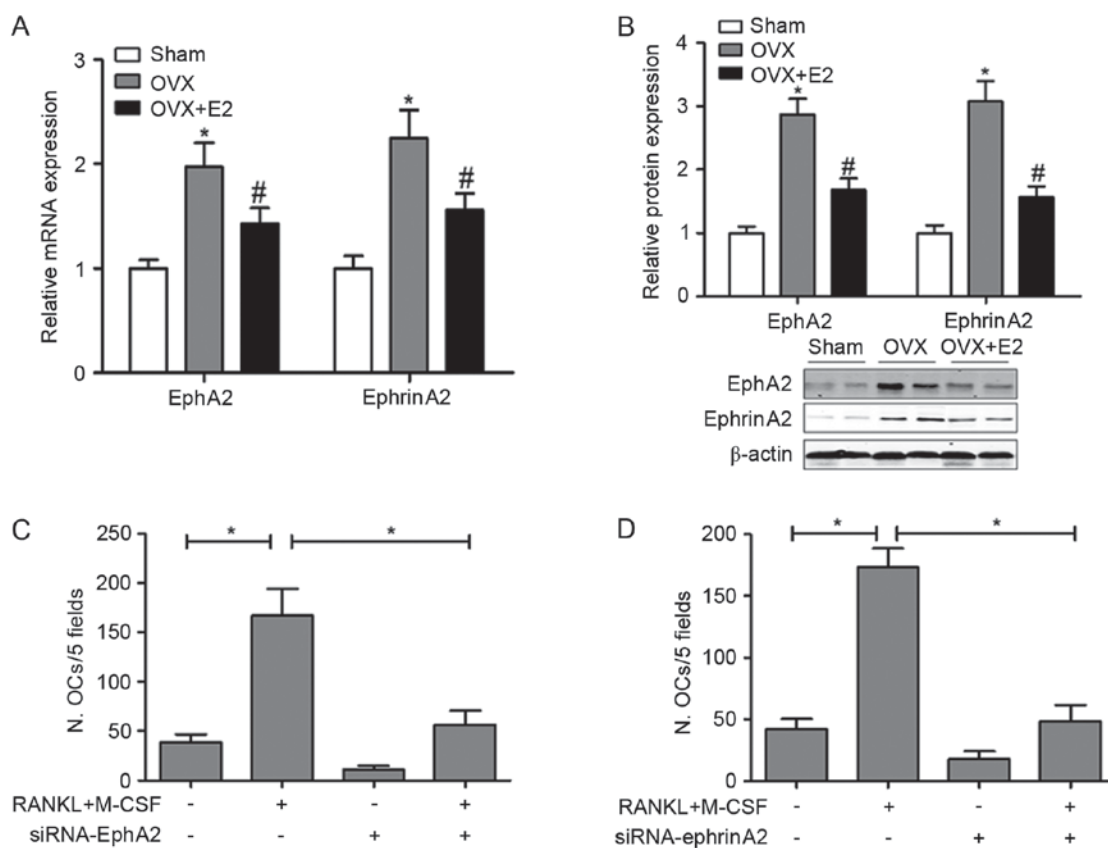


Figure 5. Effect of E2 on OVX-induced bone deterioration through the suppression of the ephA2/ephrinA2 signaling pathway. (A) Reverse transcription-quantitative polymerase chain reaction; and (B) western blot analyses demonstrated that E2 significantly decreased the OVX-stimulated expression of ephA2 and ephrinA2. (C) EphA2; and (D) ephrinA2 knockdown significantly suppressed osteoclastogenesis, indicating their otherwise stimulating role on osteoclast differentiation. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. the SHAM group. # $P < 0.05$ vs. The OVX group. Sham, placebo operated rats; OVX, ovariectomy; E2, 17 β -estradiol; siRNA, small interfering RNA; RANKL, receptor activator of nuclear factor κ B ligand; M-CSF, macrophage colony-stimulating factor 1.

Histomorphometry was performed to evaluate bone quality and architecture. As presented in Fig. 4C-F, OVX rats had a reduced BV/TV, Tb.N and Tb.Th compared with the SHAM group. E2 treatment reversed those reductions and prevented the increase of the Tb.S P-value, which differed significantly between the OVX and E2 groups. Low BV/TV, Tb.N and Tb.Th, and high Tb.Sp in the OVX group indicated bone loss, mainly due to trabecular perforation and thinning, and loss of trabecular connectivity. All these effects were attenuated by treatment with E2.

E2 suppresses ephA2 and ephrinA2 expression in the experimental rats. It is commonly accepted that bone homeostasis is regulated by various genes and signaling pathways in response to osteotropic agents. Among these pathways, ephA2-ephrinA2 signaling was reported to stimulate osteoclast and inhibit osteoblast differentiation (12). Therefore, the present study investigated whether the effect of E2 on bone formation/resorption regulation is associated with the expression of ephA2 and ephrin A2. RT-qPCR and western blot assays demonstrated that E2 significantly decreased the OVX-stimulated expression of ephA2 and ephrinA2 protein (Fig. 5A and B).

EphA2 or ephrinA2 knockdown significantly suppresses osteoclastogenesis. The involvement of the ephA2-ephrinA2 signaling pathway in the E2-induced effects on bone metabolism,

especially bone resorption function, was evaluated. Osteoclast differentiation was induced by RANKL and M-CSF *in vitro* and the number of osteoclasts in 5 fields was detected when ephA2 or ephrinA2 were knocked down. As presented in Fig. 5C and D, osteoclast differentiation was successfully induced by RANKL and M-CSF. However, ephA2 or ephrinA2 knockdown significantly suppressed osteoclastogenesis, indicating their stimulating role on osteoclast differentiation under normal conditions.

Discussion

The results of the present study indicate that E2 can attenuate OVX-induced bone deterioration partially through the suppression of the eph A2/ephrin A2 signaling pathway. E2 demonstrated suppressive effects on OVX-induced body weight gain and increased bone turnover. E2 inhibited the bone resorption function of osteoclasts and induced the bone formation function of osteoblasts by stimulating the expression of bone metabolism-related genes. E2 treatment significantly increased the tibia BMD and prevented bone loss compared with the OVX group. The underlying mechanism was mediated, at least partially, by the suppression of the ephA2/ephrinA2 signaling pathway.

As demonstrated in previous studies, OVX rats exhibit significantly higher body mass compared with sham-operated rats, mainly due to the estrogen deficiency that stimulates

fat deposition (21,22). Previous studies have demonstrated that increased body mass provides an additional stimulus for bone neo-formation, acting as a partial protection against the osteopenia of long bones (23). Excess body weight gain was completely prevented E2 treatment in the present study, which is consistent with previous studies (24,25).

Biochemical markers of bone turnover are important research tools to measure the effects of various agents on bone remodeling (26). Results of the present study demonstrated a reduction in the bone turnover rate following the treatment with E2, evidenced by alterations in the serum Ca, urinary Ca/Cr, b-ALP and TRAP-5b concentrations, which are consistent with a previous study (22). The protective role of E2 on bone homeostasis was exerted through inhibition of bone resorption via suppression of the production of TRAP and RANKL and induction of bone formation function by promoting Runx2, osterix and Colla1 expression, which are consistent with previous studies (7,27). Decreased bone mass is one of the major factors jeopardizing bone integrity, leading to reduced bone strength and an increased susceptibility to fractures (28). Therefore, E2 treatment prevents the deterioration of trabecular microarchitecture and enhances the bone strength.

The present study demonstrated that mRNA and protein expression of ephA2 and ephrinA2 was significantly decreased in the OVX+E2 group, and ephA2 or ephrinA2 knockdown significantly suppressed osteoclastogenesis. Similar to the effect of the ephA2-ephrinA2 signaling, the interaction between homeobox protein P pHb1/B3 and ephrin B1 suppressed osteoblast differentiation (29). By contrast, ephB4-ephrinB2 signaling inhibited osteoclast differentiation and promoted osteoblast differentiation, inducing a shift from bone resorption to bone formation (30).

In osteoblastogenic cultures, ephA2 was found to inhibit differentiation and mineralization of osteoblasts (31). The present study indicated that ephrinA2 and other osteoclast-efferent factors negatively regulated bone formation. Furthermore, reverse signaling through ephrinA2 into osteoclasts enhances osteoclastogenesis, most likely via phospholipase C γ 2 activation (12,31). In addition to osteoclast-osteoblast interactions, osteoblast-osteoblast or osteoclast-osteoclast interactions through ephrinA2, ephA2 and A4 have also been reported (12,31).

In conclusion, the present study indicates that E2 can attenuate OVX-induced bone deteriorations partially through the suppression of the ephA2/ephrinA2 signaling pathway. The ephA2/ephrinA2 signaling pathway maybe a potential target for osteoporosis treatment.

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