

# EFFECT OF ESTROGEN ON THE ACTIVITY AND GROWTH OF HUMAN OSTEOCLASTS *IN VITRO*

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## SUMMARY

**Objective:** Estrogen deficiency results in postmenopausal osteoporosis by increasing the rate of bone loss. The mechanism responsible for the effects of estrogen on osteoclasts is still unclear.

**Materials and Methods:** The potential of mononuclear cells from cord blood or bone marrow to differentiate into mature osteoclasts when co-cultured with human osteoblast cells was investigated. The effects of estrogen on osteoclastogenesis and osteoclast activity were also examined.

**Results:** Macrophage markers CD11b and CD14 were downregulated and vitronectin receptor was upregulated during 28 days' co-culture of mononuclear cells and human osteoblasts. Long-term co-culture resulted in the formation of numerous large tartrate-resistant acid phosphatase-positive multinucleated cells capable of resorption of bone slices. After incubation for 28 days, the addition of 17 $\beta$ -estradiol caused a significant decrease in the expression of vitronectin receptor and tartrate-resistant acid phosphatase-positive multinucleated cells in cultures derived from both bone marrow and cord blood. A significant decrease in bone resorption was also noted in the presence of estrogen.

**Conclusion:** Estrogen not only suppresses osteoclastogenesis but also inhibits the activity of osteoclasts. [*Taiwan J Obstet Gynecol* 2009;48(4):350-355]

**Key Words:** bone marrow, cord blood, osteoblast, postmenopausal osteoporosis

## Introduction

Bone metabolism is regulated by the balance between bone resorption by osteoclasts and bone formation by osteoblasts. Estrogen deficiency during menopause is associated with an increased rate of bone loss. However, the mechanism responsible for the effects of estrogen on osteoclasts is still unclear. Some reports suggest that estrogen inhibits bone resorption by mature isolated osteoclasts [1,2], whereas other studies have failed to detect such an effect [3,4].

A substantial body of evidence suggests that estrogen withdrawal following menopause leads to an increase in the production of hematopoietic growth factors, such as granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor (M-CSF), and proinflammatory cytokines, including interleukin 1, interleukin 6, and tumor necrosis factor from the stroma, monocytes and lymphoid cells [5-7]. Elevated levels of these factors are believed to stimulate the differentiation of myeloid precursor cells into osteoclasts [8]. Estrogen therapy suppresses the expression of these osteoclastogenic cytokines and reduces osteoclast formation [9,10]. This reduction in cytokine expression and a concomitant reduction in osteoclast formation have been suggested to account for at least part of estrogen's beneficial effects in preventing postmenopausal osteoporosis. These results suggest that estrogen could influence osteoclast formation, differentiation and activity.



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In this study, we investigated the ability of mononuclear macrophages derived from cord blood or bone marrow to differentiate into mature osteoclasts when co-cultured with human osteoblasts. We further examined the effects of estrogen on osteoclastogenesis and osteoclast activity during culture.

## Materials and Methods

### *Primary culture of human osteoblasts*

Human bone samples were obtained during orthopedic procedures from the upper femur of patients with no evidence of metabolic bone disease, following ethical approval. Bone explants were cultured according to a previously described method [11]. Connective tissue was carefully dissected from the bone fragments, which were then extensively washed with phosphate-buffered saline (PBS) and diced into small pieces (3–5 mm in diameter) using a scalpel. The PBS was decanted off, and the bone chips were transferred to a 50-mL polypropylene tube containing 15–20 mL of PBS. The tube was vortexed vigorously three times for 10 seconds, and then left to stand for 30 seconds to allow the bone fragments to settle. The supernatant containing hematopoietic tissue and dislodged cells was then decanted off. An additional 15–20 mL of PBS was added, and the bone fragments were vortexed as before. This process was repeated a minimum of three times, or until no remaining hematopoietic marrow was visible and the bone fragments assumed a white, ivory-like appearance.

The bone fragments were then digested for 2 hours at 37°C in a shaking water bath with crude bacterial collagenase at 1 mg/mL in Dulbecco's modified Eagle's medium (GIBCO, Maryland, NY, USA). The fragments were seeded into 75-cm<sup>2</sup> culture flasks and cultured in a calcium-free, phenol red-free medium (GIBCO) consisting of a 1:1 mixture of penicillin and streptomycin (100 U/mL and 100 µg/mL, respectively; GIBCO). The medium was replaced with an equal volume (10 mL) of fresh medium each week for the first 2 weeks and then twice weekly thereafter. At least 60% of the cells in all the cultures showed intense staining for alkaline phosphatase activity. Trypan blue was used to screen out dead cells after harvesting. The cultures were grown to confluence for about 4–6 weeks and were then passaged for experimentation.

### *Isolation of mononuclear cells from bone marrow and cord blood*

Human mononuclear cells were isolated from the cord blood of healthy volunteers ( $n=10$ ) using Ficoll-Paque

(Biochrom AG, Berlin, Germany) sedimentation and adherence. Each 4 mL of blood collected was diluted 1:1 with Eagle's minimal essential medium ( $\alpha$ MEM) (Invitrogen, Carlsbad, CA, USA), layered with 5 mL of Ficoll-Paque, and centrifuged at 510g for 20 minutes. The mononuclear cell-rich layer at the interface was removed and washed twice in  $\alpha$ MEM, and the pellet was then resuspended in  $\alpha$ MEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 2 mM L-glutamine. The number of cells in the cell suspension was finally counted in a hemocytometer after lysis of the red blood cells using a 5% (vol/vol) acetic acid solution. Bone marrow was aspirated from the iliac bone of patients ( $n=10$ ) undergoing bone graft surgery. The bone marrow cells were washed twice in  $\alpha$ MEM, and the pellet was resuspended in  $\alpha$ MEM supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 2 mM L-glutamine. The cell suspension was counted in a hemocytometer after lysis of the red blood cells using a 5% (vol/vol) acetic acid solution.

### *Preparation of co-cultures on coverslips and bone slices*

The monocyte/macrophage cell suspensions from cord blood or bone marrow were added to the 7-mm wells containing coverslips or bone slices seeded 48 hours earlier with human osteoblast-like cells. The cells were allowed to adhere for 1 hour at 37°C in 5% CO<sub>2</sub>, and the coverslips and bone slices were then removed from the wells, washed vigorously in  $\alpha$ MEM to remove non-adherent cells and placed in larger 16-mm wells containing 1 mL of  $\alpha$ MEM/FCS. These cultures were incubated for 28 days in the presence of 10<sup>-7</sup>M 1,25 (OH)<sub>2</sub>D<sub>3</sub>, 10<sup>-8</sup>M dexamethasone, and 25 ng/mL M-CSF, with or without 10<sup>-8</sup>M 17 $\beta$ -estradiol (E<sub>2</sub>). The culture medium was changed every 3–4 days.

### *Analysis of cell cultures for macrophage and osteoclast phenotypic markers and effects of estrogen on osteoclast formation*

The coverslips were removed from the co-cultures with and without 17 $\beta$ -E<sub>2</sub> after incubation for 24 hours and for 14 days and stained histochemically for the expression of tartrate-resistant acid phosphatase (TRAP), an osteoclast-associated marker, using a commercially available kit (Sigma-Aldrich, St. Louis, MO, USA). The cells were fixed in citrate/acetone solution and stained for acid phosphatase, with naphthol AS-BI phosphate as a substrate, in the presence or absence of 1.0 M tartrate; the product was reacted with Fast Garnet GBC salt. The cell preparations were then counterstained with hematoxylin. Cell preparations on coverslips were

also stained immunohistochemically using an indirect immunoperoxidase method with monoclonal antibodies BEAR1 and RMO52 (Coulter Immunotech, Marseille, France). These detected expression of the macrophage-associated antigens, CD11b and CD14, respectively, both of which are known not to be expressed by osteoclasts, while a monoclonal anti-vitronectin antibody (Sigma-Aldrich) was used to determine the expression of the vitronectin receptor (VNR), an osteoclast-associated antigen. When used in conjunction with lacunar bone resorption, VNR expression is an essential indicator of osteoclast differentiation.

#### **Functional evidence of osteoclast differentiation and effects of estrogen on bone resorption**

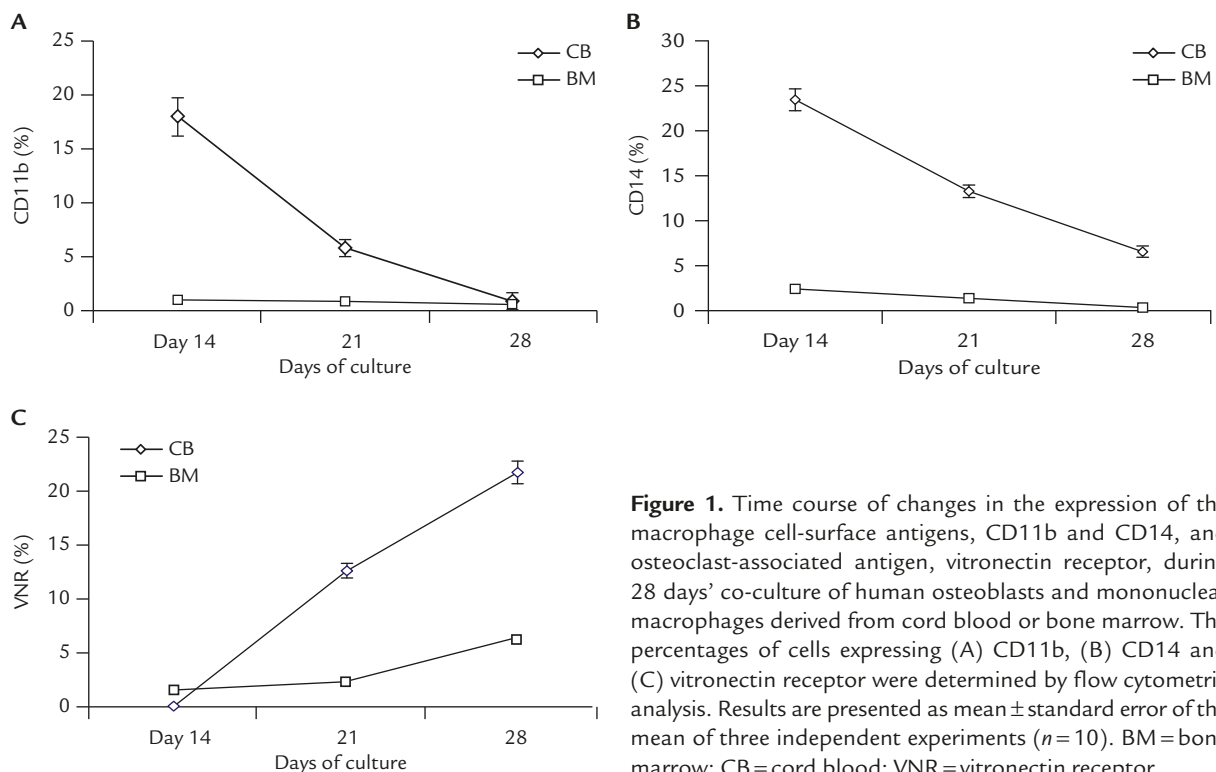
After 28 days' culture, the cortical bone slices on which human cord blood monocytes/bone marrow cells and human osteoblast-like cells had been cultured (with and without  $17\beta\text{-E}_2$ ) were removed from the wells, rinsed in PBS, and trypsinized for 15 minutes to remove the stromal cell layer. They were then washed vigorously in distilled water and left overnight in 0.25% ammonium hydroxide to remove the remaining adherent cells. After rinsing in distilled water, the bone tissues were pre-fixed with 1% glutaraldehyde (Electron Microscopy Science, Fort Washington, PA, USA) and 3% paraformaldehyde (Electron Microscopy Science) in 0.1M phosphate buffer (pH 7.4) for 3 hours and then post-fixed with

1% osmium tetroxide (Electron Microscopy Science, Fort Washington, PA, USA) in the same buffer for 2 hours. After dehydrating in an ethanol series, critical-point drying and coating with gold, the bone slices were examined by scanning electron microscopy (Hitachi S-2400; Hitachi, Tokyo, Japan).

## **Results**

### **Development of osteoclasts from bone marrow and cord blood in co-culture with osteoblasts**

Human osteoblasts and mononuclear macrophages derived from cord blood or bone marrow were co-cultured for 28 days. The expression of the macrophage cell-surface antigens, CD11b and CD14, was strong during the first 14 days and decreased progressively thereafter (Figures 1A and 1B). During the first 14 days, the co-cultures were largely negative for VNR, an osteoclast-associated antigen, and TRAP-positive multinucleated cells. VNR expression increased progressively from day 14 to day 28 of co-culture (Figure 1C). Although CD11b and CD14 were downregulated and VNR was upregulated in the osteoclasts differentiated from both cord blood and bone marrow cells at 28 days, their expression was more prominent in the cultures derived from cord blood than in those from bone marrow (Figure 1).

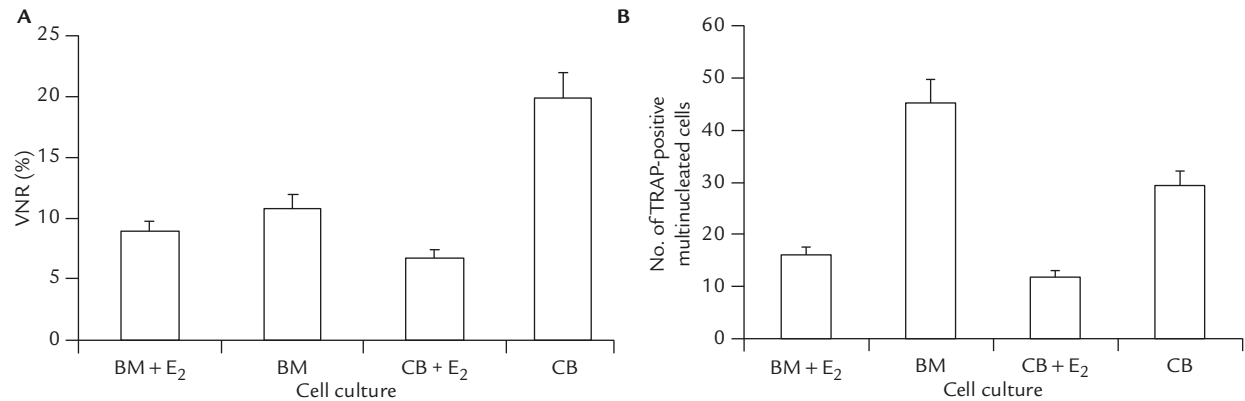


**Figure 1.** Time course of changes in the expression of the macrophage cell-surface antigens, CD11b and CD14, and osteoclast-associated antigen, vitronectin receptor, during 28 days' co-culture of human osteoblasts and mononuclear macrophages derived from cord blood or bone marrow. The percentages of cells expressing (A) CD11b, (B) CD14 and (C) vitronectin receptor were determined by flow cytometric analysis. Results are presented as mean  $\pm$  standard error of the mean of three independent experiments ( $n = 10$ ). BM = bone marrow; CB = cord blood; VNR = vitronectin receptor.

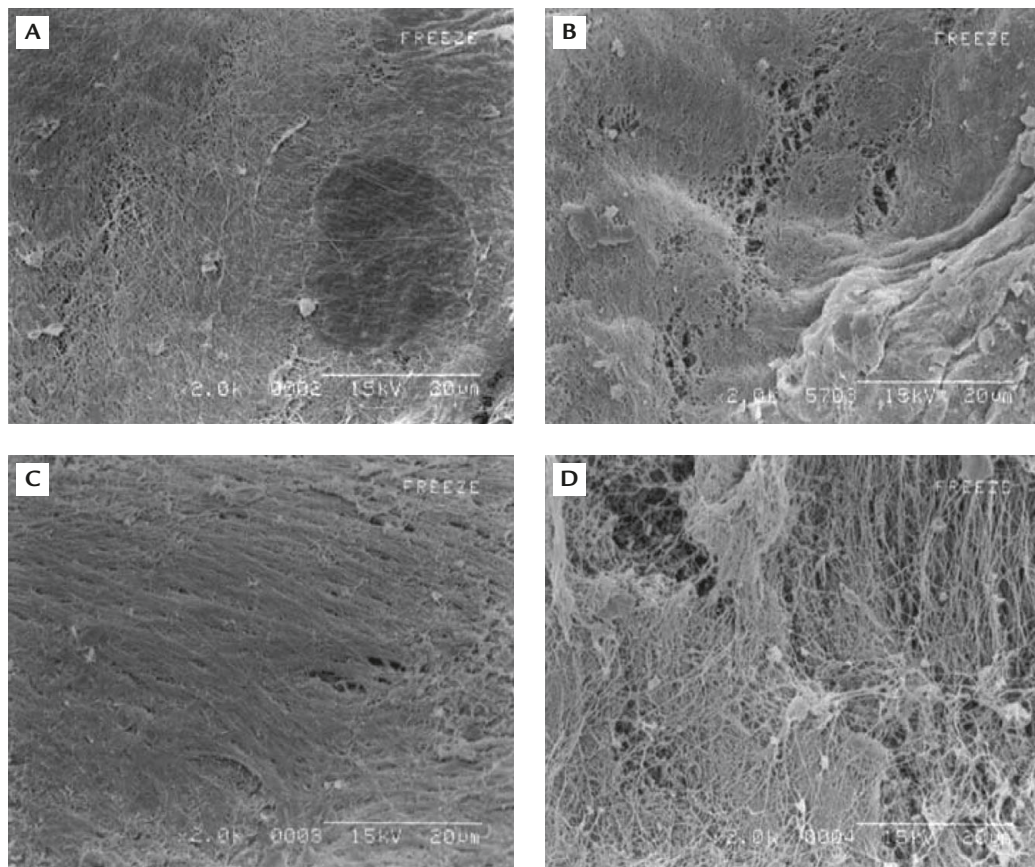
### Effects of estrogen on osteoclastogenesis and osteoclast activity

The addition of  $17\beta$ -E<sub>2</sub> after 28 days' incubation caused a significant decrease in VNR expression (Figure 2A) and TRAP-positive multinucleated cells (Figure 2B) in

cultures derived from both bone marrow and cord blood cells. Compared with the cultures without  $17\beta$ -E<sub>2</sub>, the extent of bone resorption on bone slices was also decreased in the presence  $17\beta$ -E<sub>2</sub> in the 28-day cultures (Figure 3).



**Figure 2.**  $17\beta$ -estradiol (E<sub>2</sub>) inhibited the development of osteoclasts. The expression of (A) vitronectin receptor and (B) tartrate-resistant acid phosphatase-positive multinucleated cells were evaluated after co-culture of human osteoblasts and mononuclear macrophages derived from cord blood or bone marrow with or without  $17\beta$ -E<sub>2</sub> for 28 days. The percentage of cells expressing vitronectin receptor was determined by flow cytometric analysis. Results are presented as mean  $\pm$  standard error of the mean of three independent experiments ( $n=10$ ). BM=bone marrow; CB=cord blood; TRAP=tartrate-resistant acid phosphatase; VNR=vitronectin receptor.



**Figure 3.** (A) Culture with  $17\beta$ -estradiol (E<sub>2</sub>), day 14; (B) culture with  $17\beta$ -E<sub>2</sub>, day 28; (C) culture without  $17\beta$ -E<sub>2</sub>, day 14; (D) culture without  $17\beta$ -E<sub>2</sub>, day 28. The extent of bone resorption on the cortical bone slices incubated in co-cultures of human osteoblasts and mononuclear macrophages derived from cord blood or bone marrow, with or without  $17\beta$ -E<sub>2</sub> for 28 days. Distinctive excavations were noted in the bone surface in the cultures (A, B) with and (C, D) without  $17\beta$ -E<sub>2</sub>.



## Discussion

In this study, we used an *in vitro* osteoclast formation model and found that mononuclear osteoclast precursors were present in both the bone marrow and cord blood. Co-culture with human osteoblasts was found to support the formation of human osteoclasts. Cells isolated from bone marrow and cord blood initially expressed a predominantly macrophage phenotype. After long-term co-culture with human osteoblasts, the expression of VNR and TRAP-positive multinucleated cells increased. In addition, we found that estrogen not only suppressed osteoclastogenesis in bone marrow and cord blood, but also inhibited bone resorption by osteoclasts.

Cells from bone marrow and cord blood expressed the macrophage markers CD11b and CD14, which were downregulated during the differentiation of osteoclasts. The expression of VNR, an osteoclast-associated antigen, was upregulated during osteoclast differentiation. These results clearly indicate that cells from the bone marrow and cord blood are both macrophages and progenitors of osteoclasts. However, during osteoclast formation, we found that the expression of CD11b and CD14 in the first 14 days of co-culture, and also the expression of VNR after 28-days' co-culture, were more prominent in cells from cord blood than in those from bone marrow. It is possible that there are fewer osteoclast precursors in bone marrow than in cord blood. Some reports have shown that approximately 4.3–5% of bone marrow cells respond to M-CSF [12,13] and, therefore, represent osteoclast progenitors.

Estrogen deficiency is responsible for increased bone turnover during the menopausal period. Kawamoto et al [14] demonstrated that the number of TRAP-positive cells in the rat periodontium was significantly higher in ovariectomized rats than in intact rats, or in ovariectomized rats treated with  $17\beta$ -E<sub>2</sub>. They, therefore, suggested that estrogen deficiency induced osteoclastogenesis in the rat periodontium and that quantitative changes in osteoclastogenesis could be prevented by estradiol infusion. In the present study, estrogens suppressed the expression of VNR and TRAP-positive multinucleated cells in both bone marrow cell- and cord blood cell-derived cultures for 28 days. Similar results were also reported by Ramalho et al [15], who found that  $17\beta$ -E<sub>2</sub> and raloxifene significantly decreased the number of TRAP-positive multinucleated cells produced by bone marrow cells cultured for 15 days. This suggests not only that estrogens suppress osteoclastogenesis but also that osteoclast precursors are direct targets of estrogen action. The mechanism of regulation of osteoclastogenesis by estrogens has been evaluated in some studies [16,17], which showed that estrogen

downregulated osteoclastogenesis by directly decreasing the responsiveness of osteoclast precursors to osteoclastogenic factors. However, in the present study, we found that estrogens did not interfere with the process of osteoclast formation from precursors in the bone marrow and cord blood.

It is well established that estrogens play a central role in the regulation of bone resorption by osteoclasts. However, the mechanism by which estrogens exert their effects on osteoclasts is not known. The aforementioned results suggest that estrogens could inhibit bone resorption by reducing osteoclast formation, as indicated by suppression of VNR expression and reductions in the numbers of TRAP-positive multinucleated cells in the cultures. Whether or not estrogen directly interferes with the bone resorptive process of mature osteoclasts remains controversial. In the present study, the extent of bone resorption on bone slices was obviously suppressed in the presence of estrogens, compared with the level of resorption in cultures without estrogens. In long-term cultures, although the expression of VNR was increased, bone resorption on bone slices was still suppressed in the presence of estrogens. Sarma et al [18] suggested that estradiol inhibits bone resorption by reducing the number of osteoclasts, but does not exert its effect directly on mature osteoclasts. The study by Sarma et al [18] used bone marrow cultures derived from both healthy men and postmenopausal women, and further studies are needed to evaluate the effects of sex on estrogen-induced effects.

In conclusion, estrogen prevents bone loss through multiple effects on bone cells and their precursors, resulting in decreased osteoclast formation and a reduced capacity of mature osteoclasts to resorb bone. Further studies are needed to investigate changes in the bone microenvironment in women with early postmenopausal bone loss or postmenopausal osteoporosis.

## References

1. Hughes DE, Dai A, Tiffée JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF- $\beta$ . *Nat Med* 1996;2:1132–6.
2. Mano H, Yuasa T, Kameda T, et al. Mammalian mature osteoclasts as estrogen target cells. *Biochem Biophys Res Commun* 1996;223:637–42.
3. Tobias JH, Chambers TJ. The effect of sex hormones on bone resorption by rat osteoclasts. *Acta Endocrinol (Copenh)* 1991;124:121–7.
4. Horowitz MC. Cytokines and estrogen in bone: anti-osteoporotic effects. *Science* 1993;260:626–7.
5. Manolagas SC, Jilka RL. Bone marrow, cytokines, and bone remodeling: emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 1995;332:305–11.

6. Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. *J Bone Miner Res* 1996;8:1043-51.
7. Turner RT, Riggs BL, Spelsberg TC. Skeletal effects of estrogen. *Endocr Rev* 1994;15:275-300.
8. Roodman GD. Cell biology of the osteoclast. *Exp Hematol* 1999;27:1229-41.
9. Kimble RB, Bain SD, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. *J Bone Miner Res* 1997;12:935-41.
10. Cosman F, Lindsay R. Selective estrogen receptor modulators: clinical spectrum. *Endocr Rev* 1999;20:418-34.
11. Chen FP, Lee N, Wang KC, Soong YK, Huan KE Effect of estrogen and  $1\alpha,25(\text{OH})_2$ -vitamin  $\text{D}_3$  on the activity and growth of human primary osteoblast-like cells in vitro. *Fertil Steril* 2002;77:1038-43.
12. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmez SH. CSF-1—a mononuclear phagocyte lineage-specific hemopoietic growth factor. *J Cell Biochem* 1983;21:151-9.
13. Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. *J Bone Miner Res* 2000;15:1477-88.
14. Kawamoto S, Ejiri S, Nagaoka E, Ozawa H. Effects of oestrogen deficiency on osteoclastogenesis in the rat periodontium. *Arch Oral Biol* 2002;47:67-73.
15. Ramalho AC, Couttet P, Baudoin C, Morieux C, Graulet AM, de Vernejoul MC, Cohen-Solal ME. Estradiol and raloxifene decrease the formation of multinucleate cells in human bone marrow cultures. *Eur Cytokine Netw* 2002;13:39-45.
16. Shevde NK, Bendixen AC, Dienger KM, Pike JW. Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proc Natl Acad Sci U S A* 2000;14:7829-34.
17. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF- $\kappa\text{B}$  ligand (RANKL)-induced JNK activation. *J Biol Chem* 2001;276:8836-40.
18. Sarma U, Edwards M, Motoyoshi K, Flanagan AM. Inhibition of bone resorption by  $17\beta$ -estradiol in human bone marrow cultures. *J Cell Physiol* 1998;175:99-108.