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A New Regulator of Osteoclastogenesis: Estrogen Response Element–Binding Protein in Bone

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Abstract
The heterogeneous nuclear ribonucleoprotein (hnRNP)–like estrogen response element–binding protein (ERE-BP) competes with estrogen receptor α (ERα) for occupancy of estrogen response elements (EREs). Here we report that ERE-BP potently stimulates osteoclastogenesis. ERE-BP mRNA and protein were found to be expressed ubiquitously in bone. Overexpression of ERE-BP in cultured osteoblasts stimulated expression of the receptor activator of NF-κB ligand (RANKL) and decreased osteoprotegerin (OPG). The effect of ERE-BP on RANKL was shown to be transcriptional in transient transfection assay and competed with via the ER. Constitutive expression of ERE-BP increased the sensitivity of cells toward 1,25-dihydroxyvitamin D3 stimulation of RANKL expression. In contrast, knockdown of ERE-BP in stromal ST-2 cells decreased basal RANKL promoter activity. Cocultures of ERE-BP lentivirus–transduced ST-2 cells with spleen monocytes induced formation of multinucleated osteoclasts (OCs) characterized by tartrate-resistant acid phosphatase, calcitonin receptors, and functional calcium resorption from bone slices. Although ERα competed with ERE-BP for an ERE in a dose-dependent manner, ERE-BP was an independent and potent regulator of RANKL and osteoclastogenesis. In preosteoclastic RAW cells, overexpression of ERE-BP increased RANK, upregulated NF-κB signaling, and enhanced differentiation toward a mature OC phenotype independent of RANKL. These results identify ERE-BP as a potent modulator of osteoclastogenesis. We hypothesize that ERE-BP may play a critical role in the regulation of bone homeostasis as a modulator of estrogen sensitivity as well as by direct action on the transcription of critical osteoclastogenic genes.

Keywords
OSTEOCLAST; ESTROGEN; hnRNP; RANKL; OSTEOCLASTOGENESIS; BONE

Introduction
Bone tissue constantly undergoes remodeling, a cyclic process of resorption and renewal. In the normal adult skeleton, osteoblastic bone formation and osteoclastic resorption are...
coupled to maintain skeletal mass. Several stimuli, including estrogen, have been identified that prevent excessive resorption by osteoclasts. Loss of estrogen at menopause or via ovariectomy, aromatase deficiency, or rare mutations in the estrogen receptor (ER) causes bone loss.\textsuperscript{(1–6)}

Osteoclasts (OCs) are the primary cell type contributing to bone loss in estrogen deficiency. Both increased differentiation of OCs and prolongation of their lifespan have been described as a consequence of estrogen deficiency.\textsuperscript{(7–14)} Loss of estrogen action via its genomic and nongenomic signal pathways activates a complex cascade in which elevated inflammatory cytokines modulate expression of the receptor activator of nuclear factor \( \kappa \)B ligand (RANKL) and its soluble decoy protein, osteoprotegerin (OPG).\textsuperscript{(15,16)} RANKL is expressed as a membrane-bound protein on stromal preosteoblasts and T cells. After binding the RANK receptor, RANKL activates a pathway that is similar to the canonical NF-\( \kappa \)B pathway through cytoplasmic assembly of a signalosome containing tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6) and nuclear factor of \( \kappa \) light polypeptide gene enhancer in B cells inhibitor (I\( \kappa \)B) kinases. I\( \kappa \)B kinases phosphorylate I\( \kappa \)B to liberate NF-\( \kappa \)B for nuclear translocation and subsequent transcriptional activation of RANKL-responsive genes.\textsuperscript{(17,18)} Activation of this pathway and the tempo of osteoclastogenesis depend on the OPG/RANKL ratio, which is modulated by estrogen.\textsuperscript{(19–22)} Little is known regarding the mechanism of interindividual sensitivity to estrogen or the reasons for differences in the skeletal-sparing potency of estrogen between men and women.\textsuperscript{(23)} These differences in estrogen sensitivity suggest the existence of modulators.

One clue to the mechanisms controlling estrogen sensitivity came from studies on estrogen-resistant New World primates (NWPs). These animals express high levels of an estrogen response element–binding protein (ERE-BP) that competes with the liganded ER\( \alpha \) for occupancy of estrogen-regulated gene promoters. NWPs have elevated serum levels of 17\( \beta \)-estradiol (E\( _2 \)) despite normal ER\( \alpha \) expression, a hallmark of hormone resistance. The ERE-BP is also expressed in Old World primates (including \textit{Homo sapiens}) at lower levels\textsuperscript{(24)} and has been shown to be a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) D and C-like subfamily.\textsuperscript{(25)} Twenty different hnRNPs have been described in mammalian cells, some of which contribute to target-organ resistance to other steroid hormones, including 1,25-dihydroxyvitamin D\( _3 \).\textsuperscript{(26,27)} The role of ERE-BP is not limited to competition with the cis-acting liganded ER. hnRNPs have been shown to function as single-strand intranuclear RNA-binding proteins that stabilize pre-mRNA.\textsuperscript{(28)} Recent studies also have shown that hnRNPs possess the potential to bind single- and double-stranded DNA and to regulate transcription and splicing.\textsuperscript{(26,27,29–31)}

The competition between ERE-BP and liganded ER\( \alpha \) was confirmed previously in vivo using transgenic mice that overexpress ERE-BP under the control of the breast whey acidic protein gene promoter (WAP-ERE-BP mouse).\textsuperscript{(32)} A gradient of impaired breast phenotype from near normal to failure of ductal development and lactation correlated with the relative level of transgene expression. This inhibition could be partially rescued by physiologic E\( _2 \) replacement, suggesting that the ERE-BP inhibitor was active within the physiologic range of estrogen levels.\textsuperscript{(33,34)} Thus ERE-BP may be a modulator of estrogen sensitivity in breast. The ubiquitous expression of ERE-BP suggested that it also might have a role in skeletal sensitivity to estrogen. Here we report that ERE-BP modulates the OPG/RANKL ratio, recruitment of OCs from their precursor cells, and osteoclastogenesis in bone. We hypothesize that ERE-BP may play a critical role in the regulation of bone homeostasis as a modulator of estrogen sensitivity and by direct action on the transcription of osteoclastogenic genes.
**Methods**

**Reagents**

Estrogen (E$_2$), ascorbic acid, and the tartrate-resistant acid phosphatase (TRACP) stain kit were purchased from Sigma–Aldrich (St. Louis, MO, USA). RANKL, macrophage colony-stimulating factor (M-CSF), and OPG-Fc were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies used were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Crystalline 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$; Biomol, Plymouth Meeting, PA, USA] was solubilized in 100% ethanol for addition to reaction mixtures.

**Animals**

All animal work was approved by the Animal Care and Use Committee at the VA Medical Center at Atlanta. The 7- to 12-week-old C57BL6/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA).

**Preparation of lentiviral cell lines**

The ERE-BP coding region was generated by PCR and cloned into the plenti6/V5-D-TOPO Expression Vector (Invitrogen, Carlsbad, CA, USA). The plasmid was purified and confirmed by sequencing. ERE-BP DNA then was cotransfected with ViraPower Lentiviral Packaging Mix (Invitrogen) into 293FT producer cells. After 48 hours of incubation, the viral supernatants were harvested, and viral particles were titered in the ST–2 or RAW 264.7 cell line. For transduction of lentiviral ERE–BP experiments, 1 × 10$^5$ ST–2 or RAW cells were grown in 12–well plates overnight, followed by the addition of 1 multiplicity of infection (MOI) of ERE-BP or control vector viral particles with 6 µg/mL of polybrene in 1mL of culture medium per well. After 24 hours at 37 °C, 5% CO$_2$ incubation, the medium was replaced by α-minimum essential medium (α-MEM) with 5 µg/mL of blasticidin to select stably transduced cells. The medium was replaced every 3 days thereafter. Transduction efficiency of stable ERE-BP cells was checked by real-time PCR.

**Isolation of monocytes**

Bone marrow cells were isolated from 6- to 9-week-old mouse tibias and femurs to serve as osteoclast precursors. Splenocytes were isolated simultaneously. Cells were washed twice, resuspended in α-MEM with 10% FBS and transferred to 100-mm dishes. After 1 hour, nonadherent cells from bone marrow were harvested, washed, and resuspended in α-MEM with 10% FBS and transferred to another plate as marrow stromal cells (MSCs). Adherent mononuclear cells were used for osteoclast formation experiments. The monocytes were incubated in α-MEM with 5 ng/mL of M-CSF for another 24 to 48 hours prior to use in the coculture system.

**Osteoclast formation in coculture**

ST–2-lentivirus control or lentivirus-ERE-BP-expressing cells were plated in 12-, 24-, or 96-well plates with α-MEM containing 10% FBS, 10 nM 1,25(OH)$_2$D$_3$, and 50 µg/mL of ascorbic acid. The following day, 10$^5$ monocytes per well from mouse bone marrow or spleen were overlayed for the coculture. For some experiments, where noted, osteoclast precursors were overlayed on MSCs. After 6 to 10 days of coculture, RNA was isolated for RT-PCR or cells were fixed and stained for TRACP.

**Osteoclast formation from RAW cells**

RAW 264.7-lentivirus control or lentivirus-ERE–BP-overexpressing cells were prepared similarly to the lentivirus-transduced ST–2 cells and cultured in α-MEM with 30 to 50 ng/mL of RANKL for 7 days, after which RNA was isolated for RT–PCR.
Calcium assay

Cells from coculture or RAW cell experiments were plated on bone slices (Lonza, Walkersville, MD, USA) in 96-well plates in α-MEM containing 10% FBS, 10 nM 1,25(OH)₂D₃, and 100 µg/mL of ascorbic acid. Supernatants were collected on days 7 to 10 for quantitative measurement of bone resorption by Calcein assay (Lonza).

Antibodies and immunoblotting assays

Cell extracts were subjected to electrophoresis on 4% to 20% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. Western blot analyses using RANKL antibody (Santa Cruz Biotechnology) were performed as described previously.⁹,¹²,³⁴

Real-time PCR analyses

Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was generated using the QuantiTect Reverse Transcriptase Kit (Qiagen) and 1 µg of RNA according to the manufacturer’s instructions. Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, USA) according to the manufacturer’s protocol, and 18S rRNA was used to normalize the target gene expression. The sequences for each primer set, which was validated by a single dissociation peak, were as follows: OPG, 5′-GCT GAG TGT TTT GGT GGA CAG TT-3′ (forward) and 5′-GCT GGA AGG TTT GCT CTT GTG-3′ (reverse); RANKL, 5′-TGC AGC ATC GCT CTG TTC C-3′ (forward) and 5′-CCG ACA ATG TGT GGC AGG TTT GCT CTT GTG-3′ (reverse); 18S, 5′-AAC CCG TTG AAC CCC ATT-3′ (forward) and 5′-CCA TCC AAT CGG TAG TAG CG-3′ (reverse); ERE-BP, 5′-CGC CAG TAA GAA CGA GGA A-3′ (forward) and 5′-GTC GCT GCT TCA ACT CCC CT-3′ (reverse); AND TRACP, 5′-ACA GCC CCC ACT CCC ACC CT-3′ (forward) and 5′-TCA GGT CTG GGT CTC CTT GG-3′ (reverse). Data were obtained as Cₜ values (cycle number at which PCR curves cross a calculated threshold line) and used to determine ΔCₜ values (Cₜ of target gene – Cₜ of 18S housekeeping gene). These values then were used to calculate mean ΔCₜ values ± SD for each extract for statistical comparisons. Visual representation of data was carried out by converting ΔCₜ values to fold-change data relative to ΔCₜ values for control cells using the equation 2ΔΔCₜ.

Transfection and luciferase assay

Cells were plated at a density of 1 × 10⁵/well in 24-well tissue culture plates. After 24 hours, the cells were transfected with a luciferase promoter reporter construct²⁴,³⁵ as indicated for each experiment using Lipofectamine 2000 (Invitrogen) and pRL-TK (Renilla luciferase) as transfection efficiency control (Promega, Madison, WI, USA). Twenty-four hours after transfection, cells were harvested and assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Statistical analysis

Differences in means between experimental groups and their controls were tested using an unpaired Student’s t test. In experiments with more than two groups, ANOVA was used with multiple comparisons by the method of Tukey using GraphPad Prism software (La Jolla, CA, USA).
Results

ERE-BP is expressed in bone tissue and is regulated by \( E_2 \)

In common with other hnRNPs, ERE-BP is thought to be expressed ubiquitously. We used immunohistochemistry to visualize the protein in bone cells. Immunohistochemistry confirmed expression of ERE-BP in all bone cell types in murine tibias (Fig. 1A). ERE-BP also was found to be expressed in preosteoblast and preosteoclast clonal cell lines including murine MC3T3-E1, ST–2, and mouse osteoclast precursor monocytic RAW 264.7 cells (Fig. 1B–D). In view of the fact that expression of ERE-BP is high in NWPs with high circulating levels of \( E_2 \), we investigated whether expression of ERE-BP in bone is also elevated following treatment with \( E_2 \). Data show that expression of ERE-BP in osteoclast precursor RAW cells was unaffected following exposure to \( E_2 \) (Fig. 1B). In contrast, in OB precursor ST–2 and MC3T3 cells, \( E_2 \) upregulated expression of ERE-BP three- to fivefold in mRNA and protein levels (Fig. 1C, D). In the MC3T3 cells, upregulation of ERE-BP by \( E_2 \) was accompanied by an increase in the \( E_2 \)-responsive genes \( RANKL \) and \( OPG \). The latter was upregulated robustly by \( E_2 \), and there was a slight upregulation of RANKL, increasing the OPG/RANKL ratio fivefold.

ERE-BP stimulates \( RANKL \) gene expression

In order to study the effect of stromal cell ERE-BP expression on osteoclast differentiation, a lentivirus-ERE-BP-expressing stable osteoblastic precursor cell line was created from the ST–2 mouse bone marrow stromal cell line. ST–2 was used because it secretes the osteoclastogenic protein RANKL in response to treatment with \( 1,25(OH)_2D_3 \) and therefore can function to influence recruitment of osteoclasts from cocultured hematopoietic precursors. Figure 2A confirms the elevated expression of ERE–BP mRNA in the stably transduced cell line compared with a similarly made cell line infected with control lentivirus. As shown in Fig. 2B, increased ERE-BP in ST–2 cells was associated with a threefold elevation of \( RANKL \) mRNA. OPG was unchanged (data not shown). We then compared the dose response of \( 1,25(OH)_2D_3 \) in stimulating RANKL using both the ERE-BP-overexpressing and control cell lines. Treatment of both cell lines with \( 1,25(OH)_2D_3 \) (10 nM) for 48 hours resulted in a dose-dependent increase in the steady-state levels of \( RANKL \) mRNA, but this effect was much more pronounced in cells transduced with ERE-BP (Fig. 2C). Western blot analysis confirmed the dose-dependent induction of RANKL by \( 1,25(OH)_2D_3 \) and again indicated that this response was stronger in ERE-BP-overexpressing cells (Fig. 2D). Expression of ERE-BP did not change significantly with \( 1,25(OH)_2D_3 \) treatment in both cell lines. Thus ERE-BP was able to increase stromal cell RANKL expression at baseline by modulating RANKL expression sensitivity to \( 1,25(OH)_2D_3 \).

ERE-BP stimulates osteoclast differentiation

In the preceding experiments, we studied the expression of RANKL in ST–2 osteoblastic stromal cells. In bone, stromal cells support osteoclastogenesis by presenting RANKL to osteoclast precursors to stimulate differentiation toward a bone-resorbing phenotype. To further clarify the role of ERE-BP in osteoclastogenesis, we cocultured mouse monocytes from spleen or bone marrow, to serve as osteoclast precursors, with ST–2 osteoblastic cells that had been stably transduced with the lentivirus-ERE-BP or control lentivirus. To induce osteoclast formation, bone marrow cells were treated with 10 nM \( 1,25(OH)_2D_3 \). The effects of ERE-BP overexpression on the formation of osteoclasts were determined by staining for TRACP, a sensitive, specific marker of the osteoclast, and by real-time PCR of the markers of authentic osteoclasts, cathepsin K, and TRACP. As shown in Fig. 3A, spleen cells cultured on the ERE-BP-expressing ST–2 cells show accumulation of a larger number of OCs compared with those cultured on control ST–2 cells. Quantification of OC number was
done by counting TRAP\(^*\) multinucleated (>3 nuclei) osteoclasts, revealing a fourfold increase (Fig. 3B). The levels of mRNA for RANKL, OPG, TRACP, and cathepsin K were determined in the mixed population of cocultured cells. RANKL, TRACP, and cathepsin K were increased 4-, 64-, and 4-fold, respectively (Figs. 3C–F and 4A). To test the function of the osteoclasts, ERE-BP-transduced or control ST–2 cells were cocultured with spleen monocytes on human bone slices and treated with 10 nM 1,25(OH)\(_2\)D\(_3\) for 10 days. The cocultures that included ERE-BP-ST-2 cells expressed reduced OPG, resulting in a dramatic decrease in the OPG/RANKL ratio (Fig. 4A–C). Osteoclast-mediated bone resorption was determined by measuring calcium release into the day 10 supernatant. As seen in Fig. 4D, the level of supernatant calcium from the ERE-BP-overexpressing coculture system was 22% higher than control (\(p < 0.01\)). Furthermore, in order to confirm that the osteoclastogenic response to ERE-BP is RANKL-dependent, we added mouse OPG-Fc or neutralizing antibody to mouse RANKL to the coculture system. As expected, TRACP\(^+\) osteoclast numbers were decreased significantly with blockade of RANKL activity (Fig. 5A, B). The levels of mRNA for TRACP (Fig. 5C) and cathepsin K (not shown) were measured by real-time PCR. These results suggest that ERE-BP overexpression supports stromal cell upregulation of the osteoclastogenic RANKL and downregulation of OPG, thus stimulating the recruitment of functional osteoclasts from hematopoietic progenitors.

**ERE-BP acts directly on osteoclast precursors**

The preceding experiments supported a role for ERE-BP in stimulating osteoclastogenesis via actions in stromal osteoblastic cells. We sought to determine whether ERE-BP expression in osteoclast precursors also could modulate their differentiation to mature osteoclasts. Stable lentivirus-ERE-BP-overexpressing osteoclast precursors were prepared from mouse RAW 264.7 cells. ERE-BP expression was confirmed in this cell line (Fig. 6A). PCR revealed that the cell line had an increased expression of RANK, the RANKL receptor (Fig. 6B). Osteoclastogenesis then was stimulated with 50 ng/mL of RANKL for 7 days in the absence of a stromal cell feeder layer or 1,25(OH)\(_2\)D\(_3\) treatment, and markers of mature osteoclasts were measured by real-time PCR. Compared with the control cell line, ERE-BP and RANK remained elevated throughout the culture, and TRACP was increased fourfold in transfected cells (Fig. 6C). Cells also were layered on bone slices and cultured for 7 days to determine their functional capacity to resorb bone. Figure 6D shows that the calcium release in ERE–BP-overexpressing cell supernatant was 44% higher than from control supernatant, indicating that the OCs were functional (\(p < 0.001\)).

**ERE-BP regulates RANKL and NF-\(\kappa\)B transcriptional activity**

To investigate the molecular mechanism for ERE-BP-induced RANKL expression in osteoblastic cells, we transiently transfected a \(-7000/+ 111\) RANKL-luciferase reporter construct (gift from Dr O’Brien, University of Arkansas Medical Sciences, Little Rock, AR, USA),\(^{(35)}\) with ERE-BP, shERE-BP plasmid, or control vector into ST2 cells to measure transcriptional activity. Luciferase activity was increased in the ERE-BP-overexpressing cells (Fig. 7A). In contrast, luciferase activity was lower in cells in which ERE-BP expression was knocked down by a short-hairpin RNA interference construct (shERE–BP; Fig. 7A). RANKL signals through several pathways, including NF-\(\kappa\)B. To test whether ERE-BP had an effect on RANKL-stimulated NF-\(\kappa\)B, an identical experiment was done using an NF-\(\kappa\)B-luciferase reporter (BD Biosciences). Figure 7B shows that ERE-BP-expressing RAW cells had a higher level of NF-\(\kappa\)B-luciferase activity than control cells. Silencing of endogenous ERE-BP in control cells reduced NF-\(\kappa\)B-luciferase activity below baseline. To determine whether ERE-BP influences estrogen-mediated transcription in bone cells, a similar experiment was carried out using a consensus ERE-luciferase reporter\(^{(25)}\) in place of the RANKL- or NF-\(\kappa\)B-luciferase reporter. Overexpression of ERE-BP decreased ERE-luciferase activity by 35%, whereas shERE-BP had a small stimulatory effect (Fig.
When E₂ was added to culture, ERE-luciferase activity was increased, as expected, and E₂ partially blunted the inhibitory effect of ERE-BP (Fig. 7D). Transfection with an ERα-expressing plasmid revealed that ER competed with ERE-BP stimulation of RANKL-luciferase (Fig. 7E). E₂ treatment did not affect ERE-BP stimulation of RANKL-luciferase activity in the ST–2 cells without coexpression of ER (data not shown).

**Discussion**

We hypothesize that the constitutive expression of ERE-BP promotes a positive forward tempo of osteoclastogenesis that normally is held in check by the estrogenic milieu. ERE-BP therefore is responsible for a default state of bone resorption in estrogen deficiency. In support of this hypothesis, constitutive occupancy of a stimulatory ERE by ERE-BP has been demonstrated by ChIP assay. We predict that in vivo silencing of ERE-BP would cause a high-bone-density phenotype. Further work will be required to test this hypothesis.

ERE-BP is expressed ubiquitously in tissues and competes with the liganded ER for occupancy of estrogen-responsive cis elements. Competition of ERE-BP with ERα was reported previously in breast tissue, where increasing doses of E₂ or the E₂ antagonist tamoxifen were found to overcome ERE-BP inhibition of breast budding and milk protein production. We therefore expected ERE-BP to have an antiestrogenic effect in bone, resulting in increased expression of osteoclastogenic genes, increased osteoclastogenesis, and increased bone resorption. We focused on the effect of ERE-BP on the OPG/RANKL system, considering that it is a major determinant of osteoclastogenesis. RANKL is a membrane-bound protein presented on stromal cells and osteoblasts to stimulate differentiation of neighboring osteoclast precursors. Thus we initially studied the effect of ERE-BP in ST-2 cell presentation of RANKL to cocultured hematopoietic precursors. Our experiments revealed that ERE-BP increased RANKL gene expression in the stromal cells (Fig. 2). ERE-BP expression in the stromal cells was sufficient to stimulate osteoclast differentiation of the cocultured precursors. In addition, expression of ERE-BP in RAW 264.7 cells, a model of hematopoietic osteoclast precursors, also directly stimulated differentiation to functioning osteoclasts in part by increasing expression of RANK (Fig. 6). This dual action of ERE-BP in both stromal supporting cells and hematopoietic precursors ensures a high potency of the ubiquitously expressed ERE-BP in bone resorption. We also found suppression of the RANKL decoy protein OPG leading to a profound change in the OPG/RANKL ratio, an important determinant of osteoclastogenesis. In bone, the OPG/RANKL system appears to be a major target of ERE-BP because other osteoclastogenic factors such as FASL, FAS, and TNF receptors (TNFR1, TNFR2) in the ST-2 cells were not affected by ERE-BP, nor was osteoclast lifespan (data not shown).

ERE-BP action on the RANKL promoter could be blocked by expression of ERα, similar to the competition in breast whey proteins. However, we were not able to show consistent effects of E₂ on the RANKL promoter without coexpression of ERα, suggesting differences in the cell types tested or the potency of E₂ in the breast whey protein and RANKL promoters. Our experiments used a ~7000 RANKL promoter that may not contain additional upstream ERE, which may have conferred optimal sensitivity to E₂. We did find that E₂ or overexpression of ER reduced ERE–BP inhibition of a consensus ERE–luciferase reporter in the same cells. Thus the same fundamental competition between ERE-BP and ER was occurring, similar to that reported for breast whey protein promoters. The RANKL promoter appeared to be a very sensitive target to ERE-BP activation. Our results revealed that ERE-BP is a direct stimulator of osteoclastogenesis independent of competition with the ER because silencing of endogenous ERE-BP in the absence of added E₂ decreased basal expression of RANKL. ERE-BP therefore acts as a potent trans-acting regulator of the RANKL promoter.
Additional mechanisms contributed to ERE-BP potency in osteoclastogenesis. Osteoclast precursor RAW cells expressing ERE-BP showed enhanced sensitivity to RANKL and upregulation of the NF-κB pathway downstream of RANK, as revealed by the increase in an NF-κB-reporter assay. ERE-BP stimulated basal NF-κB reporter activity, an effect that was abrogated by overexpressing ER. Since the NF-κB reporter contained no ERE, ERE-BP may be interacting directly with components of the canonical NF-κB signaling pathway. Several modulators of this pathway are known, including IκB and extrinsic modulation via MAPK, some of which are influenced by E2. The increased sensitivity of RAW cells to RANKL was associated with an ERE-BP stimulation of RANK expression, which also may have contributed to the enhanced NF-κB response to RANKL.

ERE-BP is itself upregulated by E2, indicating that ERE-BP may serve in an intracellular negative-feedback capacity. We found that E2 increased the expression of ERE-BP in osteoblasts, as described previously in breast tissue, but not in preosteoclasts, suggesting differential regulation of ERE-BP in bone cell types. The differential regulation suggests that osteoclasts may be more sensitive to E2 than osteoblasts because they lack induction of the ERE-BP intracellular negative feedback.

Estrogen is known to inhibit the NF-κB pathway by sustaining levels of IκB. ERE-BP may block this effect of E2 or ERα. Cotransfection of ER abrogated the stimulatory effect of ERE-BP on NF-κB and RANKL promoter activity, confirming the competitive nature of the ER–ERE-BP interaction. We cannot rule out the possibility that overexpressed ER downregulated NF-κB activity by competition with other transcription factors (in vitro squelching), but previous studies show restoration of breast ductal development in vivo through ligand activation of ER in ERE-BP-expressing mice, suggesting that the competition between ER and ERE-BP is a physiologic phenomenon. Additional work will be required to better define this interaction.

ERE-BP is itself upregulated by E2, indicating that ERE-BP may serve in an intracellular negative-feedback capacity. We found that E2 increased the expression of ERE-BP in osteoblasts, as described previously in breast tissue, but not in preosteoclasts, suggesting differential regulation of ERE-BP in bone cell types. The differential regulation suggests that osteoclasts may be more sensitive to E2 than osteoblasts because they lack induction of the ERE-BP intracellular negative feedback.

Estrogen plays a prominent role in balancing bone resorption and formation by suppressing the osteoclastogenic stimulus in women and men. The loss of estrogen with menopause or ovariectomy is the major determinant of accelerated bone loss in women. E2, not testosterone, is the major sex steroid correlated with bone loss in older men. Similarly, men born with aromatase deficiency or inactivating mutations of ERα suffer from severe osteoporosis and fractures, reversed only by administration of estradiol. Thus factors that regulate the sensitivity to estrogen could influence the timing or severity of menopause, extent of bone loss, and risk of fractures. Modulation of the sensitivity to estrogen has been described previously through heterogeneous distribution of ER isoforms in bone, ER polymorphisms, and an E2-stimulated nongenomic activation of MAPK. We propose that ERE-BP functions as an additional modulator of E2 sensitivity and contributes to the regulation of osteoclastogenesis and bone resorption.

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Authors’ roles: Dr. Chen contributed to experimental design and carried out experiments. Dr. Gilbert provided technical expertise and cell culture. Dr. Lu provided constructs and technical expertise in molecular studies. Drs. Liu and You carried out the histology. Dr. Weitzmann was involved in manuscript discussion and provided critical materials for experiments. Drs. Nanes and Adams hypothesized the role of ERE-BP in bone, designed experiments, interpreted data, and wrote the manuscript.
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Fig. 1.
Endogenous ERE-BP expression in bone cells. (A) Immunohistochemistry staining of ERE-BP in bone sections. Paraffin-embedded murine bone tissue sections were deparaffined according to standard procedures. Immunohistochemistry was performed using a Vector ABC kit (see “Methods”). Positive ERE-BP staining was seen in nuclei of osteoclasts (OCs), osteoblasts (OBs), chondrocytes, and osteocytes. (B–D) Gene expression analysis by real-time PCR and Western blot. The mRNA levels of RANKL, ERE-BP, and OPG in osteoclast precursors (RAW cells) and osteoblasts (ST–2 and MC3T3 cells) by vehicle (C) or stimulated with 10 nM estrogen (E$_2$) were quantitated by real-time PCR (mean ± SD; a = control; b = p < 0.05 versus a and Western blots (lower panel of B–D).
Fig. 2.
ERE-BP overexpression results in increased RANKL. (A, B) ST–2–lentivirus-transduced control and ERE-BP cells were treated with 10 nM of vitamin D₃ for 24 hours. The expression of ERE-BP and RANKL was detected by real-time PCR (mean ± SD; a = control; b = p < 0.05 versus a). (C) RANKL expression is positively correlated with 1,25(OH)₂D₃ dose in ST–2-lentivirus-transduced control and ERE-BP cell lines, as measured by real-time PCR. The relative expression level of RANKL and OPG measured for each dose of 1,25(OH)₂D₃ was calculated as fold increase versus 10⁻¹¹ M. (D, left panel) RANKL and ERE-BP proteins detected by Western blot. Band density of RANKL was measured using NIH Image software (right panel).
Fig. 3.
ERE-BP increases osteoclast differentiation by increasing osteoclastogenic factors. (A) TRACP staining for osteoclasts. ST–2 mouse stromal osteoblast precursor cells transduced with a control lentiviral vector or lentiviral vector bearing ERE-BP were cocultured with primary monocytes from mouse spleen in α-MEM + 10% FBS + 10 nM 1,25(OH)_{2}D_{3} + 100 µg/m of ascorbic acid for 10 days. Cells were stained for TRACP activity. (B) TRACP+ OCs (red;> 3 nuclei) were counted using a microscope. Data are mean ± SD of results from three determinations (a = control; b = p < 0.05 versus a). (C–F) Gene expression in cocultures was determined by real-time PCR. (mean ± SD; a = control; b = p <0.05 versus a).
Fig. 4.
Ratio of OPG/RANKL is decreased and calcium level is increased in the ERE–BP coculture system. (A–C) Gene detection. ST–2-lentivirus vector or ST–2-ERE-BP cell lines were cocultured with primary monocytes from mouse bone marrow in α-MEM + 10% FBS + 10 nM 1,25(OH)2D3 + 100 µg/mL of ascorbic acid for 10 days. The mRNA levels of OPG, RANKL, and OPG/RANKL were measured by real-time PCR (mean ± SD; a = control; b = p < 0.05 versus a). (D) Calcium detection. Cells were plated onto bone slices in a 96-well plate. Cell supernatants were collected on day 10. Quantitative measurement of bone resorption was performed by Calcifluor assay (see “Methods”).
Fig. 5.
TRACP⁺ osteoclast numbers are inhibited by adding OPG-Fc and anti-mRANKL antibody in the ERE-BP coculture system. ST2-ERE-BP cell line was cocultured with primary monocytes from mouse bone marrow as in Fig. 4 but with or without 500 to 1500 ng/mL of recombinant mouse OPG-Fc or 0.5 µg – 2 µg/mL of anti-mRANKL antibody for 6 days. (A) TRACP staining was done on day 6. (B) TRACP⁺ osteoclast numbers were counted (6 wells/group). (C) TRACP mRNA was measured on day 6 in the coculture system. Experiments were repeated two times (mean ± SD; a versus b = p < 0.05).
ERE-BP acts directly on osteoclast differentiation. (A) RAW 264.7 cells (control or ERE-BP-lentivirus-transduced) were plated in α-MEM + 10% FBS + 50 ng/mL of RANKL in 12-well plates. After 7 days, mRNA was measured by real-time PCR (mean ± SD; a = control; b = p < 0.05 versus a). (D) Cells were layered onto bone slices in 96-well plates with osteoclastogenesis medium (see “Methods”), and supernatants were collected on day 7 for calcium assay (mean ± SD; a = control; b = p < 0.05 versus a).
ERE-BP stimulates RANKL, increases RANKL-induced NF-κB, and inhibits E2-responsive transcription. ST–2 cells (A, C–E) or RAW cells (B) were transiently transfected with the indicated luciferase reporters and expression plasmids. (A) −7000 to + 111 region of murine RANKL promoter. (B) NF-κB reporter (BD Biosciences). (C) ERE promoter. (D) ERE promoter. (E) −7000 to + 111 region of murine RANKL promoter. Cells were treated with or without 10 nM of estradiol (E2) as indicated. Luciferase activity was assessed 24 hours later and normalized using Renilla luciferase activity. Data are the mean of triplicate determinations of luciferase activity in three independent experiments. ANOVA was used with multiple comparisons by the method of Tukey (mean ± SD; bars with different letters are different from each other, p < 0.05).