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Loss of ERE Binding Activity by Estrogen Receptor-α Alters Basal and Estrogen-Stimulated Bone-Related Gene Expression by Osteoblastic Cells

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Abstract Estrogen receptor (ER)-α can signal either via estrogen response element (ERE)-mediated pathways or via alternate pathways involving protein–protein or membrane signaling. We previously demonstrated that, as compared to wild type (WT) controls, mice expressing a mutant ER-α lacking the ability to bind EREs (non-classical estrogen receptor knock-in (NERKI)) display significant impairments in the skeletal response to estrogen. To elucidate the mechanism(s) underlying these in vivo deficits, we generated U2OS cells stably expressing either WT ER-α or the NERKI receptor. Compared to cells transfected with the control vector, stable expression of ER-α, even in the absence of E2, resulted in an increase in mRNA levels for alkaline phosphatase (AP, by 400%, $P < 0.01$) and a decrease in mRNA levels for insulin growth factor-I (IGF-I) (by 65%, $P < 0.001$), with no effects on collagen I (col I) or osteocalcin (OCN) mRNA levels. By contrast, stable expression of the NERKI receptor resulted in the suppression of mRNA levels for AP, col I, OCN, and IGF-I (by 62, 89, 60, and 70%, $P < 0.001$). While E2 increased mRNA levels of AP, OCN, col I, and IGF-I in ER-α cells, E2 effects in the NERKI cells on AP and OCN mRNA levels were attenuated, with a trend for E2 to inhibit col I mRNA levels. In addition, E2 had no effects on IGF-I mRNA levels in NERKI cells. Collectively, these findings indicate that ERE signaling plays a significant role in mediating effects of estrogen on osteoblastic differentiation markers and on IGF-I mRNA levels. J. Cell. Biochem. 103: 896–907, 2008.

Key words: osteoblasts; estrogen receptor; NERKI; signaling pathways

Estrogen regulates a large number of physiological processes and alters the expression of various target genes [Norman and Litwack, 1987]. Estrogen target genes can be classified into two categories: those that contain estrogen response elements (EREs) and those that do not contain EREs in their regulatory regions. For the regulation of the first category of genes, the liganded estrogen receptor (ER) binds directly to the ERE followed by recruitment of coactivators or corepressors, along with the members of general transcription apparatus [McKenna et al., 1999]; this is often referred to as the "classical pathway" of ER action. For the regulation of the second category of genes, the liganded receptor does not interact directly with the DNA in the promoter of the target genes; rather it interacts with other transcription factors bound to their respective response elements in these genes, or functions via putative membrane-bound ERs which results in the activation of MAP kinase signaling pathways [Kushner et al., 2000; Fitzpatrick et al., 2002]. This is often referred to as the "non-classical" pathway of ER action. To begin to understand the relative contributions of the classical versus non-classical ER-α signaling pathways in various tissues, Jakacka and colleagues introduced two-point mutations (E207A/G208A) in the DNA-binding domain of the mouse ER-α [Jakacka et al., 2001]. This mutant receptor, while transcriptionally inactive on a consensus ERE element, demonstrated significant transactivation

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from an AP-1 reporter, thus establishing the ability of this receptor to selectively activate the non-classical signaling pathway. This group subsequently generated and characterized the reproductive phenotype of mice in which one allele of the endogenous ER-α was replaced by the mutant ER-α (non-classical ER knock-in (NERKI) mice) [Jakacka et al., 2002].

In addition to its central role in the reproductive system, estrogen also plays a significant role in bone metabolism in both sexes [Riggs et al., 2002]. Both ER isoforms (ER-α and ER-β) are expressed in bone [Spelsberg et al., 1999], although current evidence indicates that, of the two ERs, ER-α is likely the dominant ER regulating bone metabolism, at least in mice [Sims et al., 2002]. In studies using female mice in whom the only functional ER was the NERKI receptor (ER-α-/NERKI), we found that, in contrast to their wild type (WT) littermates, these mice gained cortical bone following ovariectomy (OVX), and estrogen therapy suppressed this increase in a dose-dependent manner [Syed et al., 2005]. In addition, female ER-α-/NERKI mice had an attenuated response to estrogen in trabecular bone as compared to WT mice [Syed et al., 2005]. We also recently demonstrated that male ER-α-/NERKI mice had both trabecular and cortical osteopenia and reduced bone formation rates [Syed et al., 2007]. Since osteoblasts possess ERs [Eriksen et al., 1988; Arts et al., 1997; Bodine et al., 1998; Rickard et al., 1999], the observed paradoxical effects of OVX and estrogen on cortical bone and attenuated response to estrogen in trabecular bone in the ER-α-/NERKI mice could be due to an alteration of the balance between the classical and the non-classical signaling pathways of ER-α in osteoblastic cells. Thus, we have sought to develop cell-based models to distinguish the effects of classical and non-classical signaling pathways in these cells. Here, we report the development and characterization of cell clones generated by stably transfecting the U2OS osteoblast-like osteosarcoma cell line, which lacks any endogenous ER-α or ER-β [Monroe et al., 2003] with either WT or NERKI (E207A/G208A) ER-α cDNA expression constructs.

METHODS

Reagents

Mouse WT ER-α cDNA in the pMT2 vector was obtained from Dr. Malcolm Parker (Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK). The cDNA was released from the pMT2 vector by digesting with EcoRI followed by cloning into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Mouse mutant ER-α (E207A/G208A) cDNA (henceforth will be designated as NERKI cDNA) in the pcDNA3.1 vector was obtained from the laboratory of Dr. J. Larry Jameson, Northwestern University Medical School, Chicago, IL. Generation of the ERE (2X)-TK-luciferase vector (expressing firefly luciferase in response to estrogen) has previously been described by our group [Harris et al., 1995]. The pRL-TK vector (expressing Ranilla luciferase) was purchased from Promega (Madison, WI). The pAP-1 (7X)-Luc plasmid was obtained from Stratagene (La Jolla, CA). The ER antagonist ICI 182,780 was purchased from TOCRIS (Ballwin, MO). 17β-estradiol (E2) was purchased from Sigma (St. Louis, MO) and lipofectamine reagent was purchased from Invitrogen. The rabbit polyclonal anti-mouse ER-α antibody (MC20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL anti-rabbit IgG, horseradish peroxidase-linked whole antibody from donkey and the ECL Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture

Human U2OS osteosarcoma cells were maintained in phenol-red free Dulbecco's Modified Eagle's medium (DMEM/F12) containing 10% (v/v) fetal bovine serum (FBS) supplemented with 1× antibiotic (Penicillin and Streptomycin)/antimycotic (Amphotericin B) (Invitrogen). The U2OS stable transfectants containing either pcDNA3.1, ER-α, or NERKI were cultured in the same media supplemented with 0.7 mg/ml of geneticin. For the purpose of E2 or ICI treatment, cells were placed in 5% triple-stripped FBS medium for 96 h. To deplete the medium of all residual sex steroids, charcoal-stripped FBS from HyClone (Logan, UT) was extracted twice with dextran-coated charcoal (Sigma) followed by sterile filtration. At the end of the incubation, cells were harvested and replated into 6-well plates at a density of 3×10^5 cells/well. Cells were allowed to attach and treated with ICI or E2 at different concentrations for 48 h in fresh 5% triple-stripped FBS medium. Finally, cells were collected in RLT

lysis buffer (QIAGEN Inc., Valencia, CA) and stored at −80°C until RNA and cDNA samples were prepared.

Stable Transfections

The three constructs, pcDNA3.1, ER-α, and NERKI were linearized using Pvu I. One microgram of each linearized construct was utilized for the transfection with the liposome method. U2OS cells were grown to 50–80% confluence in 6-well plates and then treated with a mixture of DNA and lipofectamine reagent in OPTI-MEM I medium. After 5 h of transfection, an equal volume of medium containing 20% (v/v) FBS was added to make a final serum concentration of 10%. After 72 h, the transfected cells were harvested and replated in 150 mm plates in a medium supplemented with 0.7 mg/ml of geneticin and cultured for 10 days when individual colonies were visible. Several neomycin-resistant colonies were isolated using ring cloning and expanded in media containing 1 mg/ml of geneticin. The colonies were tested using Western blot analysis for the presence of ER-α at the mRNA and protein levels.

Preparation of Whole Cell Extracts

After harvesting, the stably transfected cells were extracted with a lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 1% (v/v) NP40, 1% (v/v) sodium deoxycholate, and 0.1% (v/v) SDS) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The extracts were sonicated for 5 s followed by centrifugation at 14,000 rpm in microcentrifuge for 5 min. The protein concentration of the supernatant was measured using the D_C protein assay kit (Bio-Rad, Richmond, CA).

Western Blot Analysis

Equal amounts of protein from the whole cell extracts were denatured at 95°C for 5 min, resolved on a 10% SDS–polyacrylamide gel, and electrotransferred onto an Immbilon-P membrane (Millipore Corporation, Bedford, MA). The membrane was then sequentially incubated in blocking reagent (20 mM Tris-buffered saline (TBS) (pH 7.6) containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk powder), in blocking reagent containing rabbit polyclonal anti-ER-α antibody (MC20, raised against a peptide mapping to the carboxy terminus of the mouse ER-α), in blocking reagent containing the secondary antibody, and anti-rabbit IgG coupled with horseradish peroxidase. The membrane was washed three times each time between the steps with TBS containing 0.1% Tween 20. ER-α proteins were visualized by chemiluminescence using the ECL kit following the manufacturer's protocol.

Transient Transfection and Luciferase Assay

Stably transfected cell lines, cultured for 4 days in phenol-red free estrogen depleted medium (5% (v/v) triple-stripped FBS) were plated in 12-well plates and grown to above 50% confluence. The cells were then treated with a mixture of DNA (500 ng of either ERE-TK-Luc or AP-1-Luc reporter plasmids and 50 ng of pRL-TK plasmid) and FuGENE 6 transfection reagent (Roche Diagnostics) in DMEM media without serum using the manufacturer's protocol. After 5 h, the transfection reagent was removed, and each well was washed with DMEM and then treated with either ethanol vehicle, 10 nM E2, 100 nM ICI, or a combination of E2 and ICI in DMEM containing 0.1% (v/v) BSA. After 48 h, each well was washed with 1× PBS and the cells were extracted with 1× Passive Lysis Buffer (Promega). Thirty microliter of the extract was assayed using the Dual Luciferase Reporter System (Promega) with a Turner TD-20E luminometer (Sunnyvale, CA). Promoter activity was quantified as a ratio of firefly luciferase to Renilla luciferase.

MTS Proliferation Assay

Cell proliferation was studied using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega). U2OS pcDNA, ER-α, and NERKI cells were plated for 96 h in triple-stripped FBS DMEM/F12 medium. The cells were then replated for 24 h in the same media in 96-well plates, and then treated either with vehicle (ethanol) or 10^{-8} M E2 for 72 h. At the end of the incubation, MTS tetrazolium compound was added and cells were incubated for 1 h at 37°C. Dual wave absorbance (at 490 and 650 nm) was measured using the Spectra Max Microplate Reader (Molecular Devices, Sunnyvale, CA). Intensity of absorbance was proportional to the number of live cells. The initial (Day 0) level of proliferation, which was measured simultaneously in all studied cell lines 4 h after cells were plated, was subtracted to account for the possible variations in initial cell density.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from stably transfected cells using the RNeasy Mini Kit (QIAGEN Inc.), according to the manufacturer's protocol. Two microgram of total RNA was then used for the cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

Polymerase Chain Reaction (PCR)

Conventional RT-PCR was used to initially assess the presence of ER in the cell lines developed and to assess the expression of specific genes. A 25 μl reaction mix for PCR contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each sense and antisense primers, 2 μl of cDNA, and 0.5U of Taq DNA polymerase (Roche Diagnostics). The template was initially denatured at 94°C for 3 min. The PCR was performed for 35 cycles with annealing at 55°C for 1 min, elongation at 72°C for 1 min, and denaturation at 94°C for 1 min. The following primers were used for PCR:

- ER-α, sense primer: 5′-GGCAAAGAGAGTGCCAGGCTTTG-3′, antisense primer: 5′-CAGAAACGTGTACACTCCGGAATT-3′;
- hTBP (TATA-box binding protein); sense primer: 5′-CCCCATGACTCCCATGACCC-3′, antisense primer: 5′-CGTGGTTCGTGGCTCTCTTATC-3′;
- hPR (progesterone receptor), sense primer: 5′-GATCTATGCAGGACATGACAACA-3′, antisense primer: 5′-ACACCATTAAGCTCATCCAAGAA-3′;
- hpS2, sense primer: 5′-CCCAGACAGAGACGTGTACAG-3′, antisense primer: 5′-TATTAGGATAGAAGCACCAGGG-3′.

Real-Time PCR

In order to compare the level of the expression of various endogenous genes between the different clones or between the different treatment groups, real-time PCR was performed using the iCycler (Bio-Rad Laboratories, Inc., Hercules, CA). A 25 μl reaction mix contained 1× Iq™ SYBR Green Supermix (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP (dATP,dCTP,dGTP,dTTP), iTaq DNA polymerase (0.625 U), 3 mM $MgCl_2$, SYBR Green I, 10 mM fluorescein) (Bio-Rad), the appropriately diluted cDNA as template and 7.5 pmole of each sense and antisense primers. After initial denaturation for 3 min at 95°C, the amplification of each mRNA was carried out 45 cycles with annealing at 62°C for 10 s, extension at 72°C for 20 s, and denaturation at 95°C for 5 s. The following human gene-specific primers were used:

- TBP, sense primer: 5′-AGTTGTACAGAAGTTGGGTTTTC-3′, antisense primer: 5′-AACAATTCTGGGTTTGATCATTC-3′;
- alkaline phosphatase (AP), sense primer: 5′-GGCCCTCTCCAAGACGTACAA-3′, antisense primer: 5′-CAATGCCCACAGATTTCCCAGC-3′;
- osteocalcin (OCN), sense primer: 5′-GTCCAGCAAAGGTGCAGCCTT-3′, antisense primer: 5′-TG-GTCAGCCAACTCGTCACAGT-3′;
- collagen Iα2 (col I), sense primer: 5′ GAGAGGCTGGTACTGCTGGA 3′, antisense primer: 5′-GACCAGGTTCACCCACAGCA-3′;
- insulin growth factor I (IGF-I), sense primer: 5′-TGATTTCTTGAAGGTGAAGATGC-3′, antisense primer: 5′-CCTGTGGGCTTGTTGAAATAAAAG-3′.

The copy number of the amplified product was calculated from a standard curve (generated for each run), obtained by plotting known input concentrations of the specific product (prepared previously) at log dilutions to the PCR threshold cycle. Each treatment group had six replicates, each of which were assayed in triplicates.

Statistical Analysis

All data are presented as mean values ± SEM. Multiple comparison analysis (2-way ANOVA) was used to assess effects of genotypes and treatments. Individual comparisons between genotypes among treatments were analyzed via unpaired *t*-tests. Differences were considered statistically significant for a *P*-value of less than 0.05.

RESULTS

Development of WT and Mutant Mouse ER-α Expressing Cell Lines

The U2OS cell line was stably transfected either with the pcDNA3.1 vector, WT, or NERKI ER-α expression constructs. Since the vector contained a neomycin resistance gene, several colonies were isolated based on neomycin resistance. The presence of the ER was determined using both PCR and Western blot analysis. Several positive clones for WT ER-α, NERKI, and for vector alone were detected. Figure 1A shows the receptor expression profile

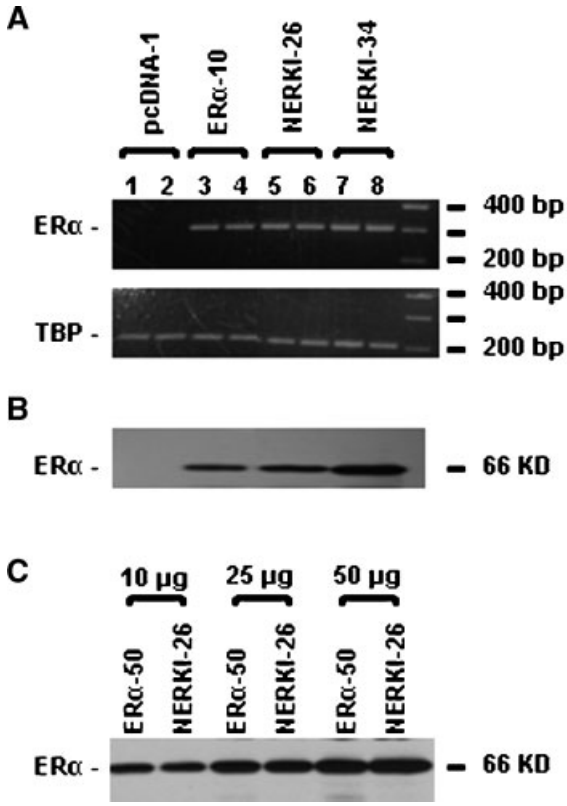

Fig. 1. Detection of the expression of estrogen receptor in the stably transfected clones. The expression of the stably transfected cDNAs were examined (**A**) at the mRNA level by RT-PCR using cDNAs prepared from total RNAs isolated from pcDNA-1, ER-α-10, NERKI-26, and NERKI-34 cells, and (**B**) at the protein level by Western blot using 50 µg of total protein prepared from all the aforementioned clones using the methods and antibodies as described in the Methods section. Since in some of the studies a separate clone, ER-α-50, was used, **panel** (**C**) shows a comparison of ER-α levels in ER-α-50 and NERKI-26 cells. Shown is a representative picture from two independent experiments.

of one clone for the vector alone (pcDNA-1), one clone for the WT (ER-α-10), and two clones for the NERKI (NERKI-26 and NERKI-34) receptors. The absence of expression of endogenous ER-α by U2OS cells was confirmed, since PCR analysis failed to amplify a band when cDNA from the vector only clone (pcDNA-1) was used as the template. However, a 302 bp band, corresponding to ER-α, was amplified when cDNAs from the clones ER-α-10, NERKI-26, and NERKI-34 were used as the templates for the RT-PCR. Western blot analysis was then performed with the total protein isolated from all the clones tested using an anti-ER-α antibody. The results in Figure 1B show that a 66 kDa protein was expressed in clones ER-α-10, NERKI-26, and NERKI-34, but not in the clone pcDNA-1. Of the two NERKI clones, the level of expression of the 66-kDa protein in the NERKI-26 clone was similar to that in the ER-α-10 clone. While we initially used the ER-α-10 clone, in some of the studies another ER-α clone (ER-α-50) generated later was also used, and Figure 1C shows another Western blot wherein the level of expression of the ER in ER-α-50 and NERKI-26 was also similar.

Characterization of the Stably Transfected Cell Lines

The positive clones were initially tested in transient reporter gene assays using a luciferase plasmid containing either an ERE response element (ERE-TK-Luc) or AP-1 response elements (AP-1-Luc). Since previous studies [Jakacka et al., 2001; Wang et al., 2004] reported that the anti-estrogen, ICI 182,780 itself activates the AP-1 reporter in human embryonic kidney or breast cancer cells transfected with either the WT or NERKI receptor, we examined effects of both E2 and ICI 182,780 in these cells. Figure 2A shows the results when using the ERE reporter construct. As is evident, there was almost no detectable basal luciferase activity in the vector only clone (pcDNA-1) when transfected with the reporter plasmid and treated with vehicle, and the activity did not change when the cells were treated with either E2 or ICI. There was, however, detectable basal luciferase activity in the clone ER-α-10 when treated with vehicle, which was significantly upregulated (7-fold) in response to E2 treatment, but ICI 182,780 had no effect. As expected, the two clones containing the NERKI receptor failed to upregulate luciferase activity in response to either E2 or ICI 182,780.

Figure 2B shows the comparable results with the AP-1 reporter construct. While there was some detectable basal luciferase activity in the presence of vehicle in all the clones tested, the basal activity of the AP-1-responsive reporter was increased in both of the NERKI clones, with NERKI-34, which expressed higher levels of the mutant ER-α (Fig. 1), having a 5.6-fold greater level of basal luciferase activity than the ER-α-10 clone. E2 had a modest stimulatory effect (1.8-fold) on the AP-1 reporter in the ER-α-10 clone. By contrast, E2 treatment of the NERKI clones resulted in a marked suppression of AP-1 reporter activity. ICI 182,780 treatment had no effect on AP-1 reporter activity in the ER-α-10 cells, but did upregulate AP-1 reporter activity in both of the NERKI clones.

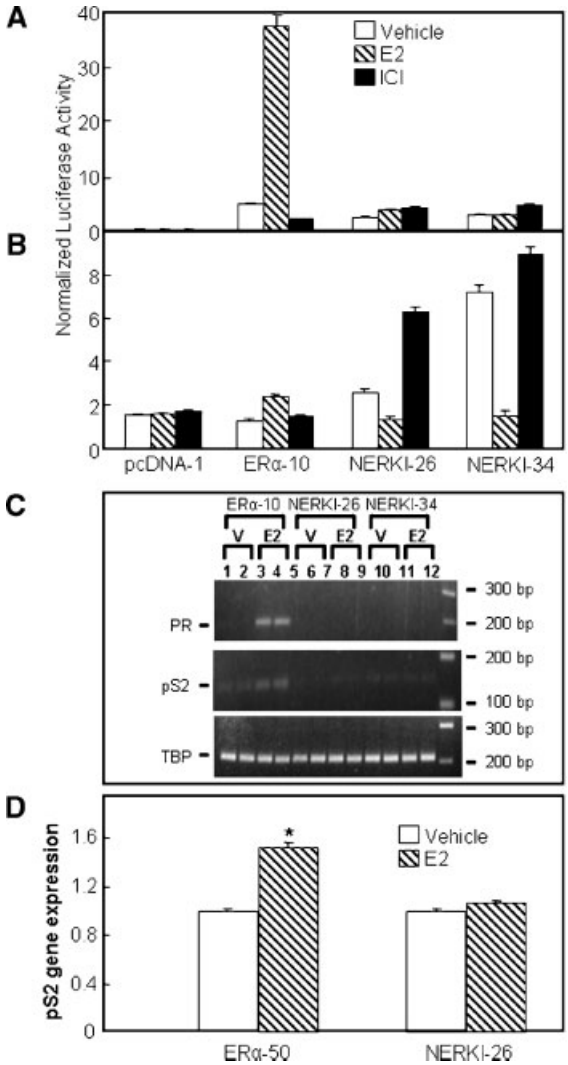

Fig. 2. Demonstration of the classical and non-classical pathways in the stably transfected clones. Different clones were transiently transfected with reporter plasmids. **A**: ERE-TK-Luc for the demonstration of the classical pathway. **B**: AP-1-Luc for the demonstration of non-classical signaling followed by treatment with different ligands. Luciferase activity was measured as described in the Methods section. **C**, **D**: Examination of endogenous gene expression to assess ER-α signaling. The ER-α-10, ER-α-50, NERKI-26, and NERKI-34 clones were cultured in a medium containing triple-stripped serum for 4 days followed by 48 h in DMEM containing 0.1% BSA and then treated with either vehicle or E2 for 48 h in the same medium. cDNAs prepared from the total RNA isolated from these treated clones were used as templates for the amplification of endogenous genes by RT-PCR (C) or quantitative, real-time RT-PCR (D). There were six replicates for each treatment. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ compared to the respective vehicle treatment.

In addition to the transient reporter gene assays, we also examined the expression of two E2-responsive endogenous genes with known EREs in their promoters using cDNAs prepared from total RNA isolated from ER-α-10, NERKI-26, and NERKI-34 clones after treatment with vehicle or E2. Figure 2C demonstrates that the progesterone receptor (PR) mRNA was induced in the presence of E2 only in the ER-α-10 clone, but not in the two NERKI clones. Although there was some basal level of expression of the pS2 gene in all the clones tested, its expression was upregulated by E2 only in the clone ER-α-10, but not in the NERKI clones. To confirm these findings, quantitative, real-time RT-PCR was also performed using the ER-α-50 and NERKI-26 cell clones. As shown in Figure 2D, E2 treatment upregulated (by 1.5-fold ($P = 0.002$)) pS2 gene expression in the cells with WT receptor but failed to do so in NERKI clone.

Effects of E2 on Cell Proliferation

Cell proliferation after 72 h of E2 treatment was measured based on the ability of viable cells to reduce the MTS dye to the insoluble blue formazan product that can be measured colorimetrically. All four previously described cell lines were treated either with vehicle or E2. As shown in Figure 3, proliferation of the pcDNA control vector cell line was not altered by E2 treatment. However, the ER-α-10 and both NERKI clones showed a modest, but statistically significant, decrease in proliferation following E2 treatment. Of note, the basal level of proliferation (defined here as in the absence of

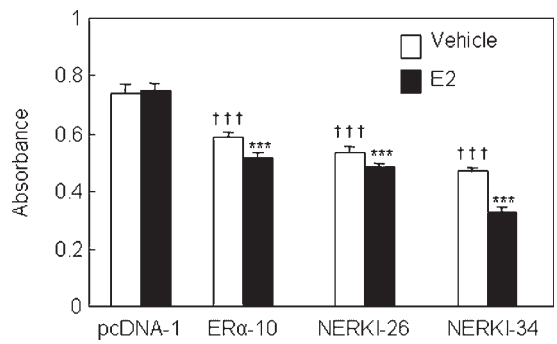

Fig. 3. Effects of E2 treatment on proliferation of U2OS pcDNA-1, ER-α-10, and NERKI cell lines. pcDNA-1, ER-α-10, NERKI-26, and -34 clones were cultured in a medium containing triple-stripped serum for 4 days followed by 24 h in the same type of media and then treated with either vehicle or E2 for 48 h. Proliferation activity was assessed by dual-wave colorimetric spectrophotometry and described as a function of change in absorbance that depends on the number of viable cells in culture. Data are mean ± SE from 10 replicates for each treatment and are representative of three independent experiments. $^{***}P < 0.001$ compared to the corresponding vehicle group; $^{\dagger\dagger\dagger}P < 0.001$ compared to pcDNA cells treated with vehicle.

exogenous estrogen) was significantly lower in the ER-α-10 and both NERKI clones compared to the control (pcDNA-1) clone.

Effects of Wild Type ER-α or the NERKI Receptor on Basal Expression of Endogenous Bone-Related Genes in the Absence of Estrogen

To determine the effects of the NERKI receptor on the expression of estrogen-regulated bone-related genes, we examined the expression of the endogenous genes, AP, OCN, and col I, which are osteoblast differentiation markers [Monroe et al., 2003], and also IGF-I, which plays a critical role in both skeletal modeling and remodeling [Yakar et al., 2002]. For these studies, we chose the NERKI-26 clone and initiated comparative studies with the ER-α-10 clones, which expressed similar levels of the ER as the NERKI-26 clone (Fig. 1). However, since the ER-α-10 clones appeared to lose responsiveness to E2 due to downregulation of expression of the ER (data not shown), we selected another developed clone with very similar ER-α expression at the mRNA and protein levels (ER-α-50, Fig. 1C) as ER-α-10 for the additional studies. Figure 4 displays the basal level of gene expression in control (pcDNA-1-transfected), ER-α-50, and NERKI-26 cells following culture for 7 days in media free of estrogen (triple-stripped FBS). As is evident, even in the absence of estrogen, mRNA levels of AP were increased by 5-fold in ER-α-50 cells compared to the control cells (Fig. 4A), whereas mRNA levels for col I (Fig. 4B) and OCN (Fig. 4C) remained unchanged. By contrast, AP, col I, and OCN mRNA levels were markedly reduced, by 62, 89, and 60%, respectively, in the NERKI-26 compared to control cells (pcDNA-1), and by 93, 90, and 55%, respectively, in the NERKI-26 compared to ER-α-50 cells. Additionally, compared to the control (pcDNA-1) cells, the NERKI-26 cells showed a comparable reduction in IGF-I mRNA levels (70%) as was present in the ER-α-50 cells (65%) (Fig. 4D).

Effects of E2 on Bone-Related Gene Expression in ER-α and NERKI Cell Lines

Figure 5 shows the dose response of E2 treatment for 48 h on mRNA levels of bone-related genes in ER-α-50 and NERKI-26 cells. In the ER-α-50 cells, E2 dose dependently increased mRNA levels of AP (Fig. 5A) and OCN (Fig. 5B), with a more variable increase in

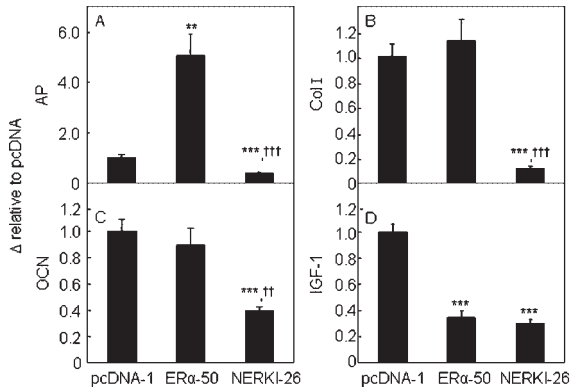

Fig. 4. Evaluation of basal level of endogenous gene expression (**A**) AP, (**B**) col I, (**C**) OCN, and (**D**) IGF-1 in U2OS pcDNA-1, ER-α-50, and NERKI-26 cell lines (normalized to TBP and to the level of expression of the gene/TBP ratio in pcDNA-1 cells). pcDNA-1, ER-α-50, and NERKI-26 clones were cultured in a medium containing triple-stripped serum for 7 days. cDNA was prepared and assayed in triplicates for each sample for an RT-PCR reaction. All samples were run simultaneously for each gene of interest to determine the difference in the level of mRNA expression. Data are mean ± SE for six replicates for each treatment and are representative of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control vector pcDNA-1 cell line; $^{\dagger\dagger}P < 0.005$ and $^{\dagger\dagger\dagger}P < 0.001$ compared to the ER-α-50 clone.

mRNA levels for col I (Fig. 5C). The response of these genes in the NERKI-26 cells appeared to be more complex, with low doses of E2 (0.01 nM) suppressing AP mRNA levels while higher doses increased AP levels, although the fold increase over vehicle at the highest dose of E2 (10 nM) was only about half of that seen in the ER-α-50 cells at that dose (Fig. 5A). There was a similar, but non-significant, bi-phasic trend for OCN mRNA levels in response to E2 in the NERKI-26 cells (Fig. 5B). Col I mRNA levels, which were already suppressed under basal conditions in the NERKI-26 cells compared to control (pcDNA-1) cells (Fig. 5B), were suppressed even further by E2 treatment (Fig. 5C), although due to the variability of the assay, the changes following E2 treatment did not achieve statistical significance. The results for the formal 2-way ANOVA analysis of these effects are indicated in the legend to Figure 5.

In order to assess the specificity of the effects on gene expression, we performed studies using the ER antagonist, ICI 182,780 and also assessed E2 effects on IGF-I mRNA levels in the ER-α-50 and NERKI-26 cells. Figure 6 shows that E2 at doses of 0.01 and 10 nM increased mRNA levels for AP (Fig. 6A), OCN (Fig. 6B), col I (Fig. 6C), and IGF-I (Fig. 6D). For

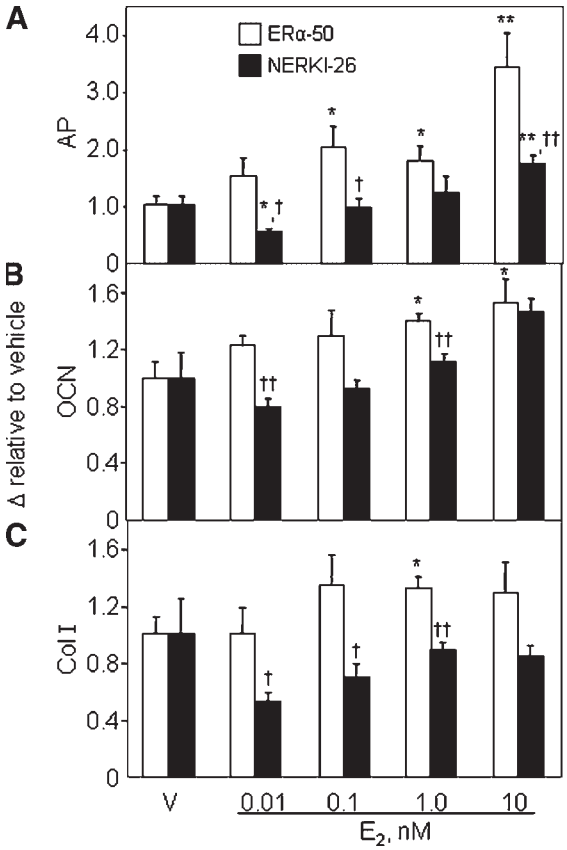

Fig. 5. Evaluation of endogenous gene expression (**A**) AP, (**B**) OCN, and (**C**) col I in U2OS ER-α-50 and NERKI-26 cell lines following E2 treatment. ER-α-50 and NERKI-26 clones were cultured in a medium containing 5% triple-stripped serum for 4 days followed by 24 h in the same type of media and then treated with vehicle (ethanol) or different concentrations of E2 for 48 h. cDNA was extracted and assayed in triplicate for each sample for gene analysis with RT-PCR reaction. All samples from individual cell lines were run simultaneously for each gene of interest to determine the difference in the level of mRNA expression, and the levels for each gene are normalized to TBP and to the level of expression of the gene/TBP ratio in vehicle-treated cells. Using a 2-way ANOVA, we found a significant effect of genotype for all three genes ($P < 0.0001$ for all); an E2 treatment effect for AP and OCN ($P < 0.001$ for both), but not for col I, and a genotype × treatment interaction for AP ($P = 0.03$), a borderline genotype × treatment interaction for OCN ($P = 0.08$), but not for col I. Each value represents mean (± SE) of six replicates for each treatment. $^{*}P < 0.05$ and $^{**}P < 0.005$ compared to the respective vehicle treatment; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.005$ compared to the ER-α-50 clone.

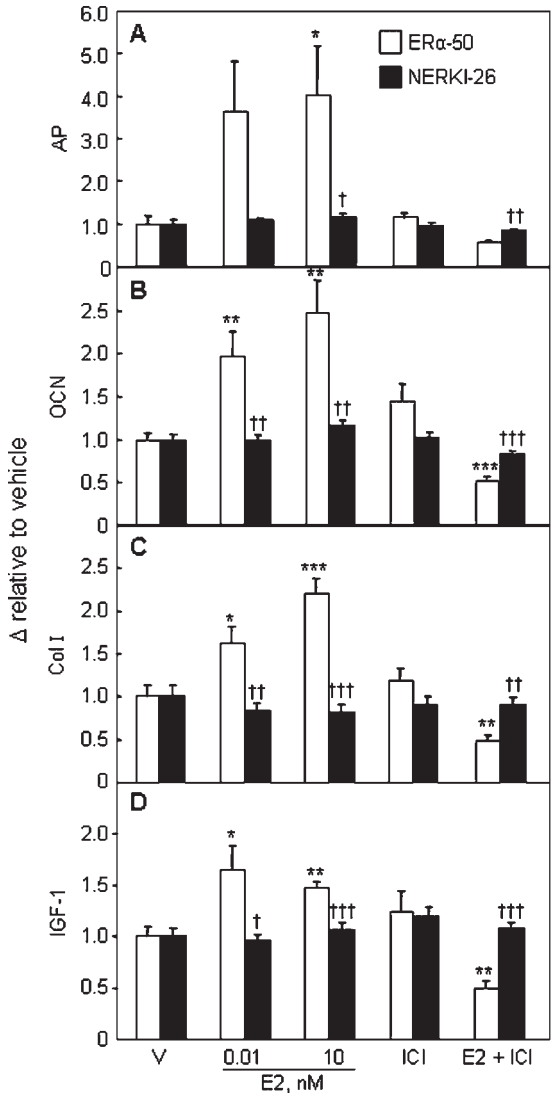

Fig. 6. Effects of E2 and ICI 182,780 on mRNA levels for (**A**) alkaline phosphatase (AP), (**B**) osteocalcin (OCN), (**C**) collagen 1α2 (col I), and (**D**) IGF-I in ER-α-50 and NERKI-26 cells. Cells were cultured in a medium containing triple-stripped serum for 4 days followed by 24 h in the same media and then treated with vehicle (ethanol) or different concentrations of E2, ICI, and ICI + E2 for 48 h. cDNA was extracted and assayed in triplicates for each sample for gene analysis with RT-PCR. All samples from individual cell lines were run simultaneously for each gene of interest to determine the difference in the level of mRNA expression, and the levels for each gene are normalized to TBP and to the level of expression of the gene/TBP ratio in vehicle-treated cells. Data are mean ± SE for six replicates for each treatment and are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$ compared to the respective vehicle treatment; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, and $^{\dagger\dagger\dagger}P < 0.005$ compared to the ER-α-50 clone.

the ICI 182,780 antagonism, we chose an E2 dose of 10 nM and a 10-fold excess of ICI 182,780. ICI alone had no effects, but was able to prevent the effects of E2 on increases in mRNA levels in these genes.

Figure 6 also shows the comparable study with the NERKI-26 cells. In this experiment, the 0.01 nM dose of E2 did not have the suppressive effect on AP mRNA levels as detected previously, but there were small, non-significant increases in mRNA levels for AP (Fig. 6A) and OCN (Fig. 6B) at the 10 nM dose,

with also a trend for suppression of col I (Fig. 6C) mRNA levels with both doses. In contrast to the ER-α-50 cells, neither dose of E2 had any effects on mRNA levels of IGF-I in the NERKI-26 cells (Fig. 6D). For the ICI 182,780 antagonism study, we were particularly interested in assessing the apparent inhibitory effects of the low dose of E2 (0.01 nM) on bone-related gene expression noted in Figure 5. Thus, we used a dose of 0.01 nM E2 and a 10-fold excess of ICI, but neither E2 alone, ICI alone, nor the combination, had any effect on the genes assessed in the NERKI-26 cells.

DISCUSSION

We describe the generation and characterization of human osteoblast-like cells (U2OS) expressing either the WT ER-α or a receptor that cannot bind to classical EREs (NERKI). U2OS cells have been used extensively to characterize the regulation of osteoblastic genes [Rickard et al., 2002; Monroe et al., 2003, 2005] and since they lack any endogenous ER-α or β [Monroe et al., 2003], they are a useful model for examining effects of transfected WT or mutant ERs on endogenous gene expression.

The ER-α- or NERKI-transfected cells expressed the mRNA and full-length protein for the respective ERs. As expected, the WT, but not NERKI, receptor induced transcription of a transiently transfected ERE reporter gene and increased mRNA levels of two endogenous genes known to be regulated by classical EREs, PR and pS2 [Berry et al., 1989; Barkhem et al., 2002; Rickard et al., 2002; Krieg et al., 2004]. Using an AP-1-responsive reporter, we found that basal activity of this promoter, even in the absence of estrogen, was upregulated in the NERKI clones. While E2 treatment modestly increased AP-1 reporter activity in the ER-α-10 cells, E2 markedly suppressed the elevated AP-1 reporter activity in the NERKI cells. By contrast, while ICI 182,780 had no effect by itself in ER-α-10 cells, it significantly upregulated AP-1 reporter activity in the NERKI clones.

In contrast to our findings in osteoblastic cells expressing the WT ER-α, where E2 resulted in some increase in AP-1 reporter activity and ICI 182,780 had no effect, E2 was found to suppress AP-1 reporter activity in 293A and TSA-201 human embryonic kidney cells [Jakacka et al., 2001; Wang et al., 2004] and MCF-7 breast cancer cells [Jakacka et al., 2001], with ICI 182,780 alone increasing AP-1 activity in these cells. These findings suggest that the effects of E2 on AP-1 activity are likely cell specific, probably due to different concentrations of ER coregulators and/or to the participation of the steroid-membrane pathways. Thus, since net AP-1 activity may be determined by direct interactions of the ER with the c-jun/c-fos complex, which has been shown to increase AP-1 activity [Umayahara et al., 1994], as well as inhibitory effects on AP-1 signaling mediated via membrane pathways [Kousteni et al., 2003], the relative balance of these potentially opposing effects on AP-1 signaling may be different in different cell types. Our findings on the effects of estrogen and the estrogen antagonist, ICI 182,780, in osteoblastic cells transfected with the NERKI receptor, however, are similar to what has previously been observed with receptors lacking ERE signaling in embryonic kidney or breast cancer cells [Jakacka et al., 2001; Wang et al., 2004]. Thus, in all systems studied, E2 represses, whereas ICI 182,780 increases, AP-1 reporter activity in cells transfected with the NERKI receptor.

Our findings also indicate that expression of the ER itself in osteoblastic cells, even in the absence of exogenous estrogen, appears to modulate expression of a number of genes in a ligand-independent manner, and that these ligand-independent effects are different for the WT ER-α compared to the NERKI receptor. Thus, relative to non-transfected cells, the introduction of ER-α led to a marked increase in AP and decrease in IGF-I mRNA levels, with no changes in col I or OCN mRNA levels. By contrast, transfection of the NERKI receptor led to a marked suppression of AP, col I, OCN, and IGF-I mRNA levels in these cells. The observed ligand-independent activation of ER-α is not unprecedented, as Ciana et al. [Ciana et al., 2003], using transgenic mice expressing a ERE-luciferase reporter, found that skeletal tissues appeared to have significant ERE-luciferase activity even in the absence of ligand. It is also known that ER-α can be phosphorylated and activated in the absence of estrogen by other agents, including peptide growth factors [Bunone et al., 1996; Cenni and Picard, 1999], and phosphorylation of ER-α increases ER-α–ERE binding in vitro [Denton and Notides, 1992; Weigel and Zhang, 1998]. In addition, Pak et al. [Pak et al., 2006] have reported ligand-independent regulation of the mouse GnRH

promoter activity by ER-β through promoter regions devoid of classical EREs.

Estrogen has been previously found to induce markers of osteoblastic differentiation in U2OS cells stably expressing ER-α and in other systems [Spelsberg et al., 1999; Monroe et al., 2005], and we similarly found that E2 treatment of ER-α-transfected cells led to fairly consistent increases in mRNAs for AP, OCN, and col I. Effects of E2 on stimulating these genes in the NERKI-transfected cells were attenuated (AP and OCN), or even inhibitory (col I), suggesting an important role for classical ERE signaling, or the balance between ERE- and non-ERE pathways, in the effects of estrogen on inducing osteoblast differentiation markers. In addition, while estrogen clearly induced IGF-I mRNA levels in the ER-α-transfected cells, and ICI 182,780 inhibited this induction, estrogen had no effect on IGF-I mRNA levels in the NERKI-transfected cells. These findings are somewhat surprising, since previous studies with the avian IGF-I promoter have demonstrated that estrogen regulates IGF-I mRNA levels via an AP-1 site [Umayahara et al., 1994]. Our findings would suggest that ERE-mediated effects are needed for the induction by estrogen of the IGF-I mRNA in human osteoblastic cells, although whether this involves direct binding of the ER to an ERE in the human IGF-I gene or regulation of the latter by other ERE-regulated proteins remains to be defined. Of note, consistent with our findings in human osteoblastic cells, Hewitt and colleagues have also recently found that while estrogen induces IGF-I mRNA levels in the uteri of WT mice, it fails to do so in the uteri of ER-α knock out [Hewitt et al., 2003, 2005] or ER-α-/NERKI (personal communication) mice, suggesting fundamental differences in the mechanism of estrogen actions on the avian versus human and mouse IGF-I promoters. Collectively, however, the attenuated stimulation (or suppression) of osteoblastic genes and the lack of stimulation of IGF-I mRNA levels in the NERKI cells is consistent with the observed in vivo osteopenic phenotype of the ER-α-/NERKI mice that is also associated with reduced indices of bone formation [Syed et al., 2005, 2007].

Introduction of ER-α (WT or NERKI) itself, in the absence of ligand, reduced proliferation of U2OS cells, and estrogen treatment resulted in a further decrease in proliferation of these cells. The inhibition of proliferation of these cells by estrogen is similar (although smaller in magnitude) to previous findings from our laboratories in cells transfected with a tetracycline-inducible WT ER-α [Monroe et al., 2005]. Nonetheless, these data do indicate that ERE binding is not required for effects of either the unliganded or liganded ER on cell proliferation in this system.

We do recognize certain limitations of our study. First, while we chose ER-α and NERKI clones expressing similar levels of receptor by Western blot, we did not quantitate the number of ERs in these clones or place them in the context of the number of ERs present in normal osteoblasts. Nonetheless, our findings do reflect the differing effects of E2 in cells expressing roughly equal amounts of the WT or NERKI ER-α. Second, we examined certain bone marker genes in our studies, but not other estrogen-responsive cytokines (e.g., IL-6 and OPG), which we plan to do in subsequent studies. Finally, since we did not examine multiple clones for each endpoint, we cannot exclude the possibility that the site of integration of the ER plasmid impacted our findings.

In summary, we describe the development and characterization of osteoblastic cells stably expressing either the WT or NERKI ER. Our data indicate that both receptors appear to have significant, ligand-independent effects on gene expression in these cells. While classical, ERE-mediated pathways are not needed for inhibition of proliferation of these cells by estrogen, the balance between ERE and non-ERE pathways, appears to be important for the induction of markers of osteoblastic differentiation by these cells. In contrast to previous studies using the avian IGF-I promoter [Umayahara et al., 1994], our data also indicate that ERE signaling (directly or indirectly) is needed for induction of IGF-I mRNA levels by estrogen in osteoblastic cells. Further studies using these cell models may help provide insights into the alterations in the skeletal response to estrogen in the ER-α-/NERKI mice we previously observed [Syed et al., 2005, 2007] and into the role of ERE versus non-ERE pathways in the regulation of osteoblastic differentiation and activity.

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REFERENCES

Arts J, Kuiper GG, Janssen JM, Gustafsson J-A, Lowik CW, Pols HA, Van Leeuwen JP. 1997. Differential expression of estrogen receptors α and β mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067–5070.

Barkhem T, Haldosen L-A, Gustafsson J-A, Nilsson S. 2002. Transcriptional synergism on the pS2 gene promoter between a p160 coactivator and estrogen receptor-α depends on the coactivator subtype, the type of estrogen response element, and the promoter context. Mol Endocrinol 16:2571–2581.

Berry M, Nunez A, Chambon P. 1989. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci USA 86: 1218–1222.

Bodine PVN, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS. 1998. Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048–2057.

Bunone G, Briand P-A, Miksicek RJ, Picard D. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174–2183.

Cenni B, Picard D. 1999. Ligand-independent activation of steroid receptors: New roles for old players. Trends Endocrinol Metab 10:41–46.

Ciana P, Raviscioni M, Mussi P, Vegeto E, Que I, Parker MG, Lowik C, Maggi A. 2003. In vivo imaging of transcriptionally active estrogen receptors. Nat Med 9:82–86.

Denton RR, Notides AC. 1992. Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. J Biol Chem 267:7263–7268.

Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL. 1988. Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84–86.

Fitzpatrick JL, Mize A, Wade CB, Harris JA, Sapiro RA, Dorsa DM. 2002. Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. J Neurochem 82:674–682.

Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL, Spelsberg TC. 1995. Estrogen response in the hFOB 1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. J Cell Biochem 59:193–201.

Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA, Korach KS. 2003. Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. Mol Endocrinol 17: 2070–2083.

Hewitt SC, Collins J, Grissom S, Deroo B, Korach KS. 2005. Global uterine genomics in vivo: Microarray evaluation of the estrogen receptor α-growth factor cross-talk mechanism. Mol Endocrinol 193:657–668.

Jakacka M, Ito M, Weiss J, Chien P-Y, Gehm BD, Jameson JL. 2001. Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. J Biol Chem 276:13615–13621.

Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ, Jameson JL. 2002. An estrogen receptor (ER)alpha deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling in vivo. Mol Endocrinol 16:2188–2201.

Kousteni S, Han L, Chen J-R, Almeida M, Plotkin LI, Bellido T, Manolagas SC. 2003. Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. J Clin Invest 111: 1651–1664.

Krieg AJ, Krieg SA, Ahn BS, Shapiro DJ. 2004. Interplay between estrogen response element sequence and ligands controls in vivo binding of estrogen receptor to regulated genes. Biol Chem 279:5025–5034.

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. 2000. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317.

McKenna NJ, Lanz RB, O'Malley BW. 1999. Nuclear receptor coregulators: Cellular and molecular biology. Endocr Rev 20:321–344.

Monroe DG, Getz BJ, Johnsen SA, Riggs BL, Khosla S, Spelsberg TC. 2003. Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERalpha or ERbeta. J Cell Biochem 90:315–326.

Monroe DG, Secreto FJ, Malayannan S, Getz BJ, Khosla S, Spelsberg TC. 2005. Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. Mol Endocrinol 19:1555–1568.

Norman AW, Litwack G. 1987. Hormones. London: Academic Press, pp. 550–560.

Pak TR, Chung WCJ, Roberts JL, Handa RJ. 2006. Ligand-independent effects of estrogen receptor beta on mouse gonadotropin-releasing hormone promoter activity. Endocrinology 147:1924–1931.

Rickard DJ, Subramaniam M, Spelsberg TC. 1999. The molecular and cellular mechanisms of estrogen action on the skeleton. J Cell Biochem 32/33 (Suppl):123–132.

Rickard DJ, Waters KM, Ruesink TJ, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Riggs BL, Spelsberg TC. 2002. Estrogen receptor isoform-specific induction of progesterone receptors in human osteoblasts. J Bone Miner Res 17:580–592.

Riggs BL, Khosla S, Melton LJ. 2002. Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23:279–302.

Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M, Baron R. 2002. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors beta in bone remodeling in females but not in males. Bone 30:18–25.

Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. 1999. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol 13:819–828.

Syed FA, Modder UIL, Fraser DG, Spelsberg TC, Rosen CJ, Krust A, Chambon P, Jameson JL, Khosla S. 2005. Skeletal effects of estrogen are mediated by opposing actions of classical and nonclassical estrogen receptor pathways. J Bone Miner Res 20:1992–2001.

Syed FA, Fraser DG, Spelsberg TC, Rosen CJ, Krust A, Chambon P, Jameson JL, Khosla S. 2007. Effects of loss of classical ERE signaling on bone in male mice. Endocrinology 148:1902–1910.

Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T. 1994. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433–16441.

Wang MM, Traystman RJ, Hurn PD, Liu T. 2004. Non-classical regulation of estrogen receptor-alpha by ICI182,780. J Steroid Biochem Mol Biol 92:51–62.

Weigel NL, Zhang Y. 1998. Ligand-independent activation of steroid hormone receptors. J Mol Med 76:469–479.

Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu J-L, Ooi GT, Setser J, Frystyk J, Boisclair YR, LeRoith D. 2002. Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest 110:771–781.