Journal of Cellular Biochemistry 89:152–164 (2003)

Estrogen Receptor-β Modulates Synthesis of Bone Matrix Proteins in Human Osteoblast-Like MG63 Cells

Lihuan Cao, Rongfa Bu, Jennifer I. Oakley, Sara E. Kalla, and Harry C. Blair*

Departments of Pathology and Cell Biology & Physiology, University of Pittsburgh School of Medicine, and Veteran's Affairs Medical Center, Pittsburgh, PA 15243

Abstract Estrogens have complex effects on the skeleton, including regulation of modeling and maintenance of bone mass, which vary with cell type and developmental stage. Osteoblasts are key regulators of skeletal matrix synthesis and degradation. However, whether osteocytes, osteoblasts or earlier progenitors mediate estrogen effects, and the importance of estrogen receptors (ERs) α and β, remain unclear. To address estrogen response in human cells closely related to secretory osteoblasts, we studied MG63 cells with ERα or ERβ reduced to low levels by stable transfection of antisense plasmids. Collagen and alkaline phosphatase expression increased with estrogen in wild-type and ERα-suppressed cells, but not in ERβ-suppressed cells. Matrix secretion occurs as osteoblasts cease dividing, and, in keeping with this, cell proliferation was reduced by estrogen except in ERβ-antisense cells. No effects of estrogen on wild type or ER-suppressed cells were seen in expression of BMP 2, the BMP antagonist noggin, or Indian hedgehog, products that regulate differentiation of osteoblasts. In contrast to expectations that estrogen would modulate bone degradation, RANKL, CSF-1, and osteoprotegerin did not respond measurably to estrogen, regardless of ER status. In keeping with this result, estrogen response was not observed in assays of osteoclast development from CD14 cells supported by wild-type or ER-silenced MG63 cells. Since estrogens are major regulators of bone degradation in vivo, estrogen effects on osteoclasts may depend on interaction with stimuli present in bone but absent in the model studied. cDNA hybridization showed that additional estrogen-binding proteins including ERRα and BCAR3 were expressed by MG63, but estrogen effects in ERβ-silenced cells were small, so these proteins are either minor regulators in MG63 cells, or act in concert with stimuli in addition to estrogen. We conclude that, in the MG63 cell line, estrogen increases synthesis of matrix proteins via ERβ, and that, in the absence of additional stimuli, these cells are not major mediators of estrogen effects on osteoclast differentiation. Further, ERα is probably much more important in earlier stages of skeletal development, such as growth plate response, than in osteoblasts. J. Cell. Biochem. 89: 152–164, 2003.

Key words: osteoclast; osteoporosis; estrogen-related receptor; CSF-1; RANKL; BMP-2

Sex hormones play important roles in the development and maintenance of the skeleton. There are sex-specific differences in skeletal shape, and important changes in skeletal maintenance occur when estrogenic or androgenic hormones are lost [Rizzoli et al., 2001]. Changes in sex steroids correlate with major changes in hypophyseal hormones including inhibin [Gaddy-Kurten et al., 2002], follicle stimulating hormone and luteinizing hormone, and it is unclear to what extent effects on the skeleton depend on central mechanisms. However, large amounts of bone are lost after the female menopause, and this is reversed by estrogens [Compston, 2001]. This, and in vitro work showing direct estrogen response in bone cells [Robinson et al., 1997], highlight the importance of estrogen response mechanisms in bone.

Many estrogen effects are mediated by the well-characterized estrogen receptors (ERs) α and β. ERα is the classical sex-related receptor; ERβ is widely distributed in epithelial and mesenchymal tissues. Other estrogen-binding proteins include another receptor of the steroid receptor superfamily, the estrogen-related receptor ERRα, and several estrogen-binding

Grant sponsor: National Institutes of Health awards; Grant numbers: AG12951, AR47700; Grant sponsor: Department of Veteran's affairs.

*Correspondence to: Dr. Harry C. Blair, 705 Scaife Hall, University of Pittsburgh, Pittsburgh, PA 15261.
E-mail: hcblair@imap.pitt.edu

Received 1 November 2002; Accepted 6 January 2003
DOI 10.1002/jcb.10486

proteins that are not transcription factors but which affect cell metabolism by other mechanisms. The role for these additional receptors is unclear but initial reports suggest that ERRα regulates bone formation [Bonnelye et al., 2001], and that some estrogenic effects do not depend on transcription-factors [Brubaker and Gay, 1999].

Several types of transgenic mice with changes in ERs have been studied [reviewed in McCauley, 2001]. The loss of ERα in mice is associated with shortening of the long bones, suggesting a growth-plate effect, and decreased osteocalcin. This growth-plate effects of ERα would be expected under the hypothesis that the classical sex-related receptor is in major part responsible for differential growth to produce sexual dimorphism in the developing skeleton. ERα also regulates osteoprotegerin and RANK-ligand in orchiectomized male mice [Lindberg et al., 2001], suggesting that ERα regulates osteoclast differentiation under some circumstances. Male ER-β deficient mice do not have a bone phenotype, while female ER-β deficient mice have increased trabecular bone density, suggesting that ERβ has a role in bone matrix synthesis. On the other hand, when both ERα and β are suppressed, changes in skeletal density are insignificant [Ogawa et al., 2000], although ovariectomy still causes bone loss, pointing to a possible role for receptors other than ERα and β. Thus, functions of ERα and β in bone remain uncertain. Additionally, rodents maintain epiphyseal bone growth throughout live, complicating comparison with the mature human skeleton, which has closed epiphyses.

Cellular studies of estrogen effects in bone have not indicated a consistent or predominant role for a specific estrogen receptor. ERβ is increased during osteoblast differentiation [Arts et al., 1997; Onoe et al., 1997], although the important osteoblast promoter Cbfa1 is produced in response to both ERα and β [Tou et al., 2001]. Extensive transfection and inhibitor studies in human fetal osteoblasts suggested that both ERα and β regulate expression of proteins in nontransformed fetal osteoblasts at varying differentiation states [Waters et al., 2001]. Estrogen effects are also reported in several studies of macrophages and osteoclasts, and under some circumstances estrogens modify osteoclastic activity, but the mechanisms remain controversial. Some of these studies used cells with very high concentrations of ER introduced by expression plasmids, and it is unclear whether cells with typical ER concentrations respond similarly.

We studied the MG63 osteosarcoma, a human cell line used widely for studies of hormone effects, closely related to secretory, matrix-producing osteoblasts. Both ERα and ERβ mediate transcriptional responses in MG63 [Lu and Giguere, 2001; Lambertini et al., 2002], although the functional importance of the receptors is unclear. We compared wild type MG63 cells to cells stably transfected with plasmids producing antisense ERα and ERβ, which were essentially devoid of the antisense target receptors, and compared the responses of the cell lines to estrogen. Cell proliferation, matrix proteins, cytokine production, and support of osteoclast differentiation from CD14 monocytes were studied. In contrast to estrogen effects in whole animals or in cell lines transfected to express very large amounts of ER, major estrogen effects were reduced cell proliferation and increases in matrix-related proteins. Osteoclast-related cytokines or osteoclast differentiation did not vary at physiological estrogen concentrations. These differences between ERα- and ERβ-expressing cells suggest that ERβ is the major receptor modifying matrix secretion, and that estrogen-dependent osteoblast-mediated changes in bone degradation require interactions of ERs with stimuli that are not present in this isolated cell model.

MATERIALS AND METHODS

Antibodies and Recombinant Proteins

Anti-human ER-α mouse monoclonal and ER-β goat polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-osteoprotegerin and anti-collagen I were from Sigma. Antibodies to bone morphogenic proteins 2 and 4, RANK-ligand, and recombinant human CSF-1 were from RDI (Flanders, NJ). Human recombinant RANKL was the extracellular portion, from PeproTech, Inc., (Rocky Hill, NJ).

Cell Culture and Transfected Cells

Human MG63 osteosarcoma cells (American Type Culture Collection, Bethesda, MD) were used at cell passage 60–70 and were grown in Eagle's minimum essential medium, α-modification, with 10% fetal bovine serum,

10 U/ml penicillin, and 10 μg/ml streptomycin. For assays of estrogen dependence, media were charcoal stripped to eliminate exogenous estrogens and phenol red. ER segments, ~500 bp, were isolated by polymerase chain reaction using RNA from MG63 cells by phenol/guanidine isothiocyanate extraction (RNAzol B, Tel-Test, Friendswood, TX). PCR used cDNA from reverse transcription of 2 μg total RNA. Primers for ERs were from GenBank M12674 and AB006589 for ERs α and β, respectively, with Xho 1 and Bam H1 restriction sites added to the 5′ ends of the sense and antisense primers to allow convenient cloning. For ERα, AAACTCGAGATGACCATGACCCTCCAC and AAAGGATCCAGCGAGTCTCCTTGGCA amplified nucleotides 293–844; for ERβ AAACTCGAGATGGATATAAAAAACTCA and AAAGGATCCTGTAGCATCCCTCTTTGA amplified nucleotides 1275–1716. In each case, the ATG following the restriction site in the first (sense) primer corresponds to the translation start site. After restriction of the plasmid and PCR fragments, the ER segments were ligated into pcDNA3.1(+) (Invitrogen, San Diego, CA) with antisense orientation specified by the restriction sites selected. Insert expression is driven by a CMV viral promoter, and the expressed RNA is polyadenylated. The plasmids were grown in *E. coli*, verified by sequencing, and transfected into MG63 by lipofection (FUGENE 6, Roche, Indianapolis, IN) with selection using G418 200 μg/ml. Clonal lines were isolated on filters soaked with trypsin/EDTA. These were grown and examined for ER expression.

Western Blots

Cells were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 0.14 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Insoluble material was pelleted at 10,000*g* for 10 min. Protein lysates were dissolved in sample buffer with dithiothreitol, separated on SDS–polyacrylamide, and transferred to polyvinylidene difluoride membranes. Membranes were reacted for 1 h at room temperature with PBS containing 0.1% Tween 20 and 5% nonfat dry milk to block nonspecific binding, and incubated overnight at 4°C with the appropriate antibody. Primary antibodies were used at 1 μg/ml (monoclonal) or 1:2,000–1:3,000 dilution (polyclonal). For ERs, primary antibodies were coupled to secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, using horseradish peroxidase-coupled anti-mouse IgG at 1:5,000 or horseradish peroxidase-coupled anti-goat IgG at 1:2,000, with three washes between steps. Detection used oxidation of luminol by the peroxidase (ECL, Amersham, Arlington Heights, IL) to produce chemiluminescence, recorded on film. For abundant proteins, alkaline phosphatase conjugated second antibodies were used at 1:5,000. Alkaline phosphatase was detected using incubation in 1 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.2 mM nitro blue tetrazolium. For collagen expression, cell culture supernatants were assayed.

Protein and Enzyme Assays

Protein concentration was measured by reduction of copper in an alkaline environment with bicinchoninic acid to detect the product [Smith et al., 1985], reading the protein concentration relative to albumin standards as absorbance at 662 nM (BCA Protein Assay, Pierce, Rockford, IL). Alkaline phosphatase was demonstrated in situ using napthol AS-MX phosphate substrate and fast blue to show the product as a blue precipitate, with reactions run in 50 mM carbonate at pH 8.6 to select alkaline phosphatase activity. To measure alkaline phosphatase activity in solution, cell layers were washed three times with 50 mM Tris, 150 mM NaCl, pH 7.4, and collected in 1 ml of 50 mM Tris, 0.1% Triton-X 100, pH 7.4. The alkaline phosphatase activity was then measured using *p*-nitrophenyl phosphate substrate 3 mM, in 200 mM Tris-HCl buffer, pH 10 measuring absorption at 405 nm to determine *p*-nitrophenol, with results expressed as nMol/mg protein. Tartrate-resistant acid phosphatase, an osteoclast marker, was measured using naphthol AS-BI phosphate substrate and fast garnet GBC to show the product as a red precipitate, in 0.67 M tartrate, pH 5.6, to select for the tartrate-resistant enzyme. Computer image analysis (Fovea Pro, Gainseville, FL) was used to count labeled cells.

mRNA-Based Assays

mRNA was isolated on oligo-dT spin columns (RNeasy, QIAGEN, Santa Clarita, CA). RNAs were evaluated after reverse transcription by hybridization to cDNA on slides [Sapolsky and

Lipshutz, 1996] (Affymetrix, Santa Clara, CA). For these determinations 10 μg of mRNA was used to make double stranded cDNA. From this, biotin labeled cRNA was made, and hybridized to an array of ds cDNAs on glass for 3′ regions of 6,000 coding regions of proteins, the amount of labeled biotin is measured in 16 replicates for each target. PCR assays were used for mRNAs for selected proteins. cDNA was made from 1 μg of mRNA, and 30 cycles of PCR with specified primers was performed, using 1 min each at 56, 72, and 94°C each cycle, which pilot experiments showed were in the approximately linear amplification ranges for the targets. Primers: Human Indian hedgehog GenBank XM050846 sense CTTGTCAGCCGTGAGGCC (347–364) antisense CTGTGAAAGAGTCTCAGGG (796–778). Human noggin GenBank U31202 sense AAGGCTTGGACCCTGCGA (262–279) antisense GCGGAAGAAAGGCACACA (628–611). Human, bone morphogenetic protein 4 GenBank BC020546 sense CTTCAGTCTGGGGAGGAG (478–495) antisense GATGAGGTGCCCAGGCAC (801–784). Human bone morphogenetic protein 2 GenBank NM001200 sense AGGCACTCAGGTCAGCCG (576–593) antisense CACGGGGGAATTTCGAGTT (884–870).

Human Osteoclasts

Monocytes were collected from human peripheral blood. White cells from heparinized blood were recovered on ficoll and purified using antibody binding to isolate CD14 white blood cells (MACS magnetic bead separation, Miltenyi Biotec, Auburn, CA). Resulting cells were plated at 1×10^4 per cm^2 in Dubelco's modified minimal essential medium with 10% heat inactivated fetal bovine serum with MG63 cells as described [Blair et al., 2000], or in media supplemented with CSF-1 and RANK-ligand as specified in Results. Human procedures were approved by the Institutional Review Board.

RESULTS

Plasmids expressing 450 bp antisense regions of human ERα and ERβ were constructed, verified by PCR and sequencing, and transfected into MG63 cells grown in G418 to select cells containing the desired plasmids. Several clones were isolated and studied by Western analysis with enhanced chemiluminescence detection. Wild type MG63 and MCF-7 cells, a breast cancer line, were used as positive controls. Most isolates of ERα or ERβ MG63 cells expressed no detectable ERs when the antisense plasmid was present, and two such isolates were used for further work (Fig. 1). Low level expression is often underestimated by Western blot, so residual ERα and ERβ were also studied by PCR, in cells grown 14 days without G418, which (Fig. 1B) showed ~90% suppression of mRNA of either estrogen receptor in the antisense cells, although some of this may represent untranslated sense–antisense dimers (see Discussion). Controls amplifying actin cDNA were done to insure quality of reverse transcripts compared (not illustrated; semi-quantitative PCR of proteins that did not vary between cell types are shown in Fig. 4, below). Over periods exceeding two weeks, antisense cells were grown in G418 to prevent plasmid loss.

All assays of estrogen effects compared cells in charcoal stripped media with cells in the same media supplemented with 17-β-estradiol as indicated in Materials and Methods. Estrogen decreased proliferation in control and ERα antisense cells when assessed after 14 days (Fig. 2A). The ERβ-antisense line had ~30% lower basal proliferation; this was attributed to clonal selection or a secondary effect of the plasmid. In initial studies five ERα and ERβ antisense clones were grown, and in both antisense types some clones showed slower growth. Experiments similar to those shown in Figure 2 were performed comparing charcoal stripped media to media with estradiol added at 1 nM–1μM in log increments. At 1 μM estradiol, poor survival was noted, suggesting toxic effects. Otherwise, all assays with estradiol added, relative to charcoal stripped media, gave similar results at 1–100 nM estradiol, and 10 nM was used in most subsequent experiments to characterize estrogen effects. Differences in alkaline phosphatase activity were apparent on direct observation of the cells within one week in 10 nM estrogen (Fig. 2B).

Estrogen increased synthesis of collagen and alkaline phosphatase in MG63 cell by approximately a factor of two (Fig. 3A,B). Type I collagen was determined by Western blot, and alkaline phosphatase was assayed in the cell lysates. These proteins, the major bone matrix protein and a protein required for mineralization, increased in response to estrogen in ERα antisense cells as well. On the other hand, in

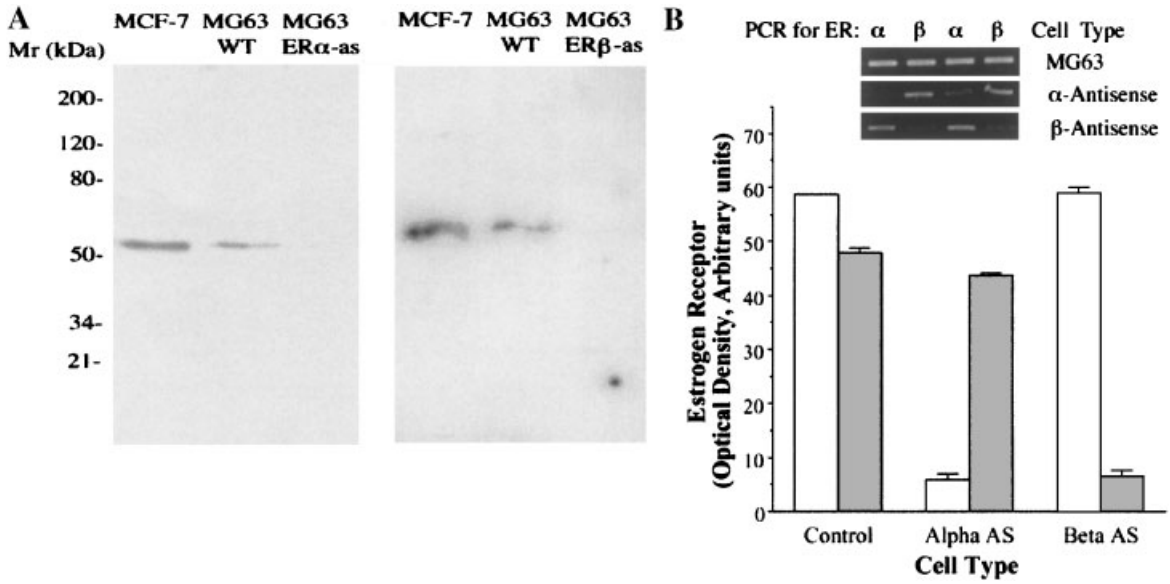

Fig. 1. ERα and ERβ antisense MG63 cells express very low levels of the respective ERs. **A**: Cell lysates were probed for the α (left) and β (right) receptor isoforms with antibodies, visualized by enhanced chemiluminescence. Wild type MG63 expressed both ERα and β. In antisense cells the receptors generally were not detectable. MCF-7, a breast cancer cell line that expresses both receptors, is as a positive control. **B**: Signals in control and transfected cells from ERα (open bars) and ERβ (shaded bars) RNAs by PCR in cells grown for 14 days in media without G418. Under these conditions, in antisense cells the receptors were ~10% of the matched RNA in control cells. Inset shows semi-quantitative PCR; actin controls did not vary significantly between samples (not shown). Densitometry from one of two experiments with similar results, n = 2 ± range.

ERβ antisense cells showed blunted response that was not statistically different from untreated cells. Despite the readily measurable effects on matrix proteins, measurements of total cellular protein using [^{3}H]-leucine did not show overall changes in protein synthesis. This may reflect in part that cell number is about 15% higher in the untreated MG63 cells at two weeks (Fig. 2A), although in any case differences in overall protein synthesis were small, even on a per cell basis.

Beyond the markers of osteoblast maturation and matrix synthesis, effects of estradiol on several proteins related to osteoblast differentiation and osteoblast–osteoclast interaction were studied. In most cases little effect of estrogen was seen in MG63 cells or either ER-antisense transfected cell line (Fig. 4). Specifically, 10–100 nM estrogen did not have significant effects on the synthesis of the osteoblast-differentiation related proteins BMP 2 or 4, the BMP antagonist noggin, or on Indian hedgehog, an important signal in the maturation of the growth plate. Assays for BMP 2 and 4 were performed both by Western analysis and PCR with no consistent differences between ER cell types; selected Western and PCR results are shown in Figure 4. To assure that the assays were capable of detecting differences, controls included PTH stimulated cells. PTH changed expression of many proteins as expected, for example increasing IHH and decreasing Noggin (Fig. 5A).

Although estrogen has been shown to influence osteoclast differentiation in rodents (see Discussion), estrogen did not measurably change expression of the osteoclast-inducing signals CSF-1, RANKL, or osteoprotegerin in the MG63 cells tested, with or without antisense ER plasmids (Fig. 4A,B). Because this result is in contrast with studies showing estrogen response of osteoclast-related cytokines in other types of bone cells and in whole animal models, this point was studied further by comparing osteoclast differentiation from CD14 cells in co-cultures of the MG63 cells expressing varying estrogen receptors. In keeping with the invariance of RANKL and CSF-1, estrogen did not affect measurably osteoclast differentiation in co-cultures of osteoclast precursors and any of the MG63 cell lines (Fig. 6A). Further, estrogen did not affect osteoclast formation in cultures of CD14 monocytic cells with RANKL and CSF-1, in the absence of supporting stromal cells (Fig. 6A,B). This experiment was run at estradiol concentrations from 1 to 100 nM, and four

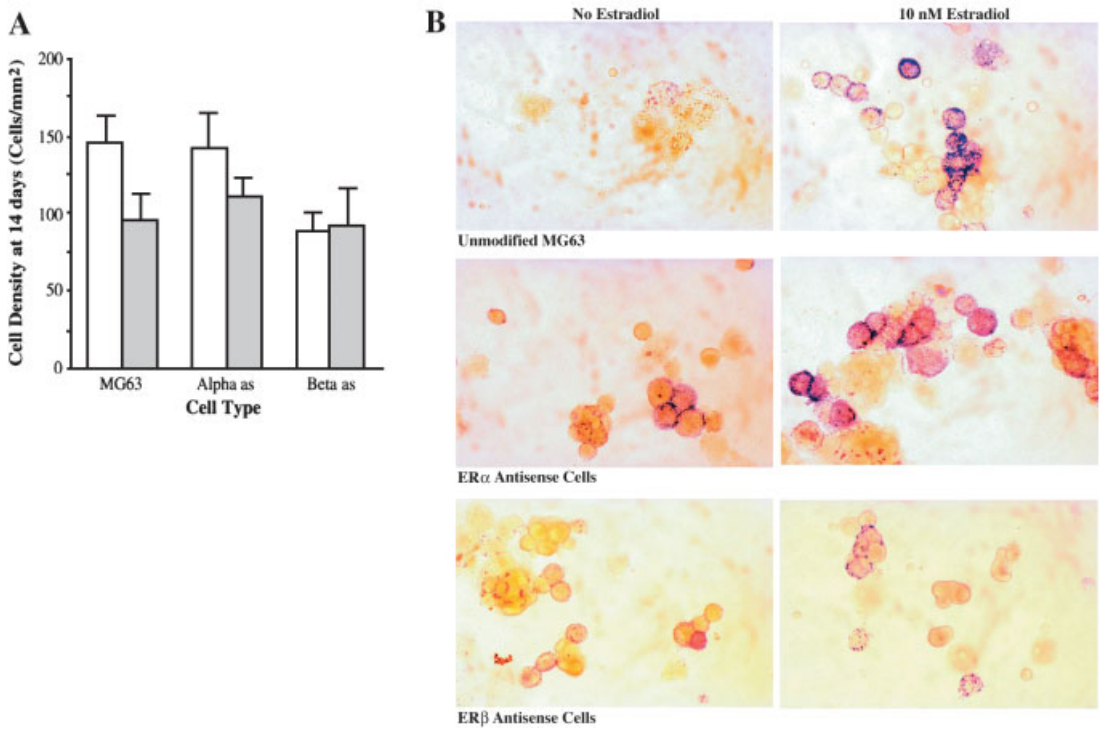

Fig. 2. Effect of estradiol on cell number and alkaline phosphatase expression. **A**: Cell division was retarded by estrogen in control MG63 cells ($P < 0.05$) and cells with ERα antisense were not statistically different from controls, but no difference ± estrogen was observed in ERβ antisense cells. The lower basal proliferation of ERβ antisense cells was attributed to plasmid or clonal selection differences (see text). Cell counts at day 14, n = 4 mean ± SD. Matched cultures of MG63 cells on glass at day 14 in DMEM with 10% FCS, without (open bars) or with (shaded bars) 10 nM estradiol, in charcoal stripped media. **B**: Differences in alkaline phosphatase activity in cells at day 7 culture labeled in situ. On glass, the cells grew in small nodules. In unmodified or ERα antisense cells, estrogen increased alkaline phosphatase activity (dark blue clusters of cells, **right top** and **middle panels** relative to left), but did not affect alkaline phosphatase in the ERβ antisense cells (**bottom pair**).

experiments using different CD14 isolates were run with similar results.

Wild type and ERα or ERβ antisense cell lines were also studied by gene-chip (Affymetrix) analysis to determine whether mechanisms that mediate estrogen response other than the two major ERs may exist. Cells for these tests were grown in basal medium (10% serum, not charcoal stripped), containing ~1 nM estradiol. These showed that mRNAs for ERRα (GenBank HUMHERRA, estrogen receptor-related protein) and BRCA3 (Genbank U92715, *Homo sapiens* breast cancer antiestrogen resistance 3 protein), which mediates estrogen and tamoxifen-related effects in other cells, were expressed at significant levels in the MG63 cells. Figure 7 shows the results for the four most highly expressed estrogen-related genes other than ERα or ERβ in the MG63 cells tested, which included ERRα and BCAR3. Expression of all of these mRNAs were similar in control, ERα and ERβ antisense cells (±30% of control MG63 expression levels as log average, n = 16).

DISCUSSION

The studies demonstrate, in a defined human osteoblastic cell line, that ERβ is the major estrogen receptor responsible for changes in osteoblast proliferation matrix synthesis. These changes in matrix secretion are not directly related to induction of cytokines that cause osteoclast formation. The MG63 osteosarcoma is a well-characterized cell line closely related to osteoblasts at the bone matrix secretion stage. We chose this for analysis of estrogen effects on bone to allow the effects of ERs α and β on bone formation and degradation to be studied at one stage of differentiation and in isolation from other factors. Work in whole animals and mesenchymal stem cells has shown that estrogen effects on bone are complex, and implicates several cytokines and multiple estrogen

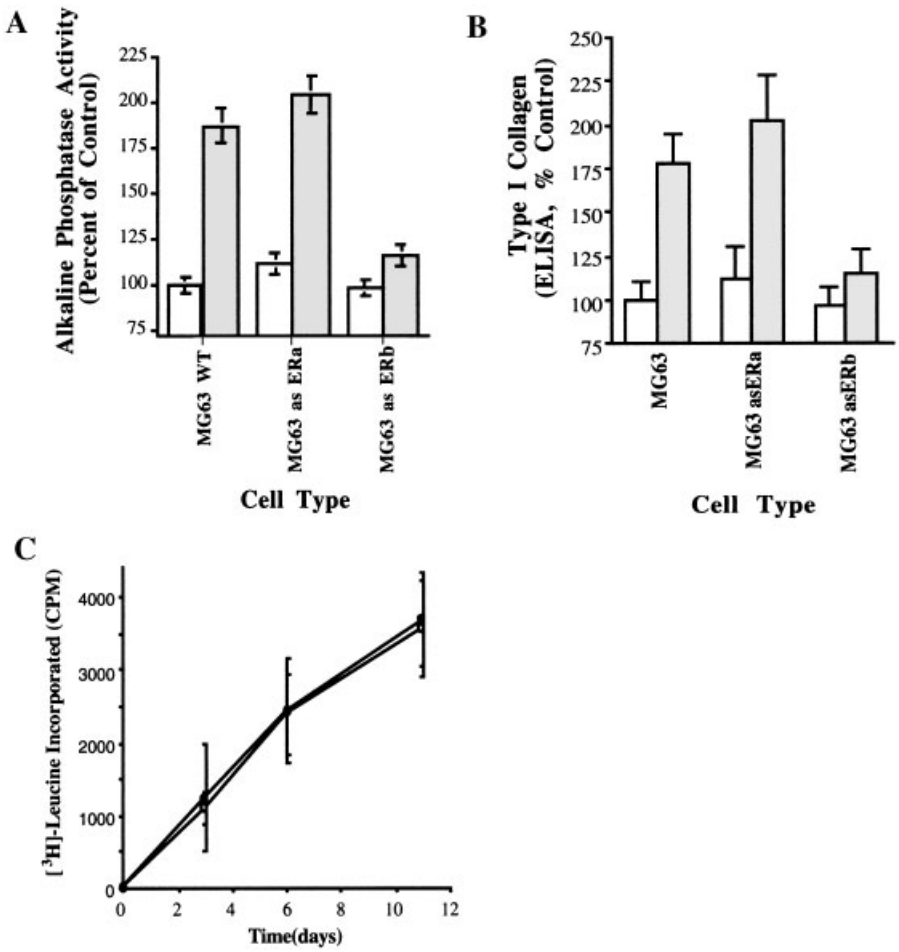

Fig. 3. Effect of estrogen on alkaline phosphatase activity and collagen expression in MG63 cell lines with varying ER phenotype. **A**: Alkaline phosphatase activity increased in response to estrogen (shaded bars) in control cells and ERα antisense cells. Response in ERβ antisense cells was not statistically different ± estradiol; differences in control and ERα antisense cells are significant ($P < 0.01$). N = 4, mean ± SEM. **B**: Type I collagen determined by Western blot, showing response to estrogen (shaded bars). The pattern was similar to that of alkaline phosphatase expression; differences in control and ERα antisense cells are significant ($P < 0.01$) but the small differences in ERβ antisense cells are not significant. N = 4, mean ± SEM. **C**: [^{3}H]-leucine incorporation into labeled protein in MG63 cultures with and without estradiol. Estradiol 10 nM (closed symbols) did not affect protein synthesis relative to cultures in charcoal stripped medium (open symbols). Mean of six determinations ±SD.

receptors (ERα, ERβ, and ERRα) in estrogen responses of bone [Bonnelye et al., 2001; Waters et al., 2001]. Both ERα and β have major effects on osteoblasts during differentiation, but ERα effects were not observed here, and in the absence of ERβ, estrogen effects on matrix synthesis were small. While estrogen-treated osteocytes regulate osteoclasts via TGF-β [Heino et al., 2002], no effects on osteoclast formation were seen in the MG63 cells. The more complex effects observed in other studies are likely to reflect responses of bone cells at other stages of differentiation, or interactions of estrogen with other cell types and with stimuli that are excluded in the ER-modified MG63 cell model.

Normal osteoblasts are one stage of a continuum of development from mesenchymal stem cells to osteocytes. The human osteosarcoma line MG63 models a specific stage of osteoblast development closely related to the secretory osteoblast. While transformed models such as MG63 are quite useful, some responses of normal osteoblasts may not be modeled by the MG63 cells, and responses of cells at other stages of osteoblast development cannot be predicted from these results.

Stable transfection of plasmids producing antisense RNA for ERα or β produced cells with very low levels of the target proteins (Fig. 1). The antisense RNA is expected to complex with the mRNA for its target and result in its

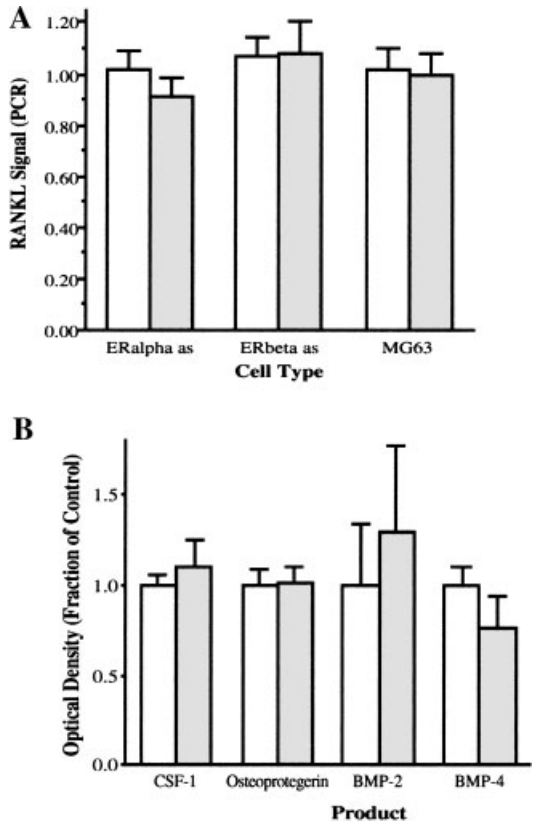

Fig. 4. Effect of 10 nM estrogen (closed bars in each panel) on osteoblast-produced cytokines related to osteoblast–osteoclast signalling and mesenchymal stem cell maturation. Measurements by semi-quantitative PCR or Western blot using RNA or protein extracts from cultures similar to those shown in Figure 2. **A**: PCR analysis of RANK-ligand production showed no significant effect of estrogen in MG63 or either estrogen receptor antisense construct. **B**: Results of estrogen effects on CSF-1 (PCR), BMP-2 (Western blot), BMP-4 (PCR), and osteoprotegerin (PCR) were similar to those for analysis of RANKL production. There were no consistent or significant differences between MG63 and the antisense lines; results are summarized as difference with or without estrogen averaged for MG63 and both antisense lines ($n = 3, \pm SD$). BMP-2 was studied with both Western blot and PCR with similar results (see Fig. 5). Although normalized for comparison, BMP-4 was a minor product relative to BMP-2.

destruction by endonucleases [Jen and Gewirtz, 2000]. Although antisense gene silencing by this or several related methods is widely employed, it remains possible that low levels of residual estrogen receptors, up to 10% of control levels (Fig. 1B) may influence our results. However, earlier studies strongly suggest that low concentrations of estrogen receptors are incapable of supporting estrogen-dependent changes in phenotype [Davis et al., 1994], and in many other gene silencing studies similar levels of suppression have been effective in changing phenotype.

Clear differences were seen between the unmodified and antisense MG63 cell lines. Specifically, we found that 17 β-estradiol at 10 nM increased collagen and alkaline phosphatase expression. This is consistent with observations in mouse mesenchymal stem cells [Zhou et al., 2001], mineralized bone nodule formation in marrow cultures [Qu et al., 1999], and initial estrogen response in osteoblasts [Plant and Tobias, 2001]. The estradiol effect was greatly decreased in ERβ transfected cells, a key finding (Figs. 2 and 3). However, in these cells, closely related to the mature, secretory osteoblast, ERα effects were not observed. In other reports, additional ER effects occur, probably because, in nontransformed cells, ER responses vary with cellular maturity [Robinson et al., 1997].

In several experiments, we were unable to demonstrate a measurable effect of estrogen on osteoclast formation from human monocytes, whether osteoclast differentiation was supported by MG63 cell co-culture or by supplementing cultures with CSF-1 and RANKL. Literature on this point is complex. In mouse stromal cell lines, it was reported that 17 β-estradiol stimulates expression of osteoprotegerin via ERα [Saika et al., 2001], which should reduce osteoclast formation. It is also reported that estrogen reduces CSF-1 expression in murine stromal cells [Srivastava et al., 1998], but no comparable effect was seen in the human osteosarcoma cells we studied. Neither an effect of estrogen on osteoclast formation nor a basis for such an effect in CSF-1, RANK-ligand, or osteoprotegerin synthesis was seen (Figs. 4 and 5). These differences could be species related, but more likely reflect that the pluripotent stromal cell lines have additional estrogen responsiveness that are lost in the relatively mature MG63 cell line. ERα is also reported to change osteoprotegerin/RANK-ligand ratios in ovariectomized mice [Lindberg et al., 2001]. We did not see an effect on osteoprotegerin or RANKL in the MG63 cells of any ER type. In the whole animal, such effects may also reflect changes in other mesenchymal cells that produce these proteins. In MG63 cells we could not show a difference in RANK-ligand, CSF-1, or osteoprotegerin related to ERα or β. The estrogen-related increase in osteoprotegerin noted in mouse stromal cells [Saika et al., 2001] suggests, similarly, that estrogen may affect mesenchymal cells at different, mainly earlier,

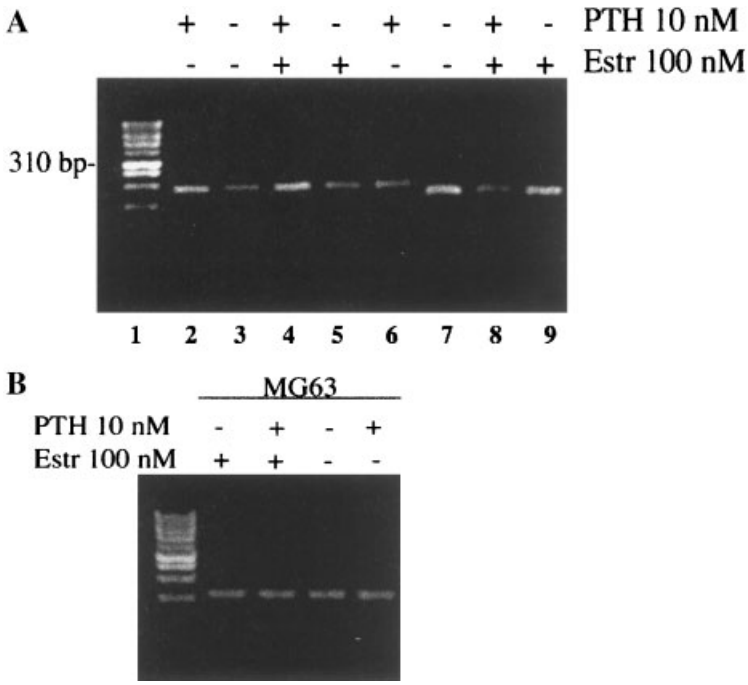

Fig. 5. PTH changed expression of many proteins that were not affected by estrogen. **A**: PTH increased Indian hedgehog (**Lanes 2–5**) and decreased Noggin (**Lanes 6–9**) by PCR, but neither variable changed measurably with estrogen. Analysis in MG63 (unmodified cell line). The differences in density are ~twofold with PTH, but insignificant with estrogen. **B**: In contrast, many important regulatory proteins, for example, BMP-2, did not vary with PTH or estrogen under conditions studied. The result shown also confirms the Western blot densitometry shown in Figure 4B.

stages of differentiation, although species differences between human and mouse make this point speculative. In addition, there may be effect on osteoclast formation mediated by cells beyond the secretory osteoblast phase, the osteocytes. This is supported by work on estrogen-responsive TGF-β secretion by osteocytes [Heino et al., 2002].

ER knockout mice have produced interesting and varied findings. ERα −/− mice have shortened limbs consistent with a growth-plate dependent mechanism [McCauley, 2001]. It is not surprising that such a mechanism exists, since this is needed to provide for sex-specific skeletal shape differences by changes in bone growth rates at cartilage plates. Although we saw no ERα-dependent effects on major bone proteins or bone turnover, the model used, an osteosarcoma cell line modeling mature osteoblasts, would not be expected to be related to changes in factors that influence epiphyseal growth. Indeed, estrogen effects on bone modeling regulators, including Ihh, BMP-2 or -4, and noggin, were insignificant in the MG63 cells examined. These findings are also consistent with the normal development of the skeleton in animals lacking ERs, [McCauley, 2001], and point to the accessory role of the sex steroids in normal skeletal metabolism. In ERβ −/− mice, females were protected against age-related trabecular bone loss [Windahl et al., 2001]. Our findings of ERβ-mediated increases in bone collagen and alkaline phosphatase secretion are in keeping with this. Another type of data supporting important effects of ER β on bone mass is the selective ER modulators that activate ERβ [An et al., 2001] which have effects on bone mass consistent with a primary osteoblastic response [Blair et al., 1996].

An interesting finding in control experiments was that osteoclast formation did not respond to estrogen in CD14 cells cultured with RANKL and CSF-1 (Fig. 6). In mice it is reported that estrogen regulates osteoclast formation via c-jun expression and c-jun N-terminal kinase phosphorylation [Shevde et al., 2000; Srivastava et al., 2001]. This may represent mainly a species difference; human osteoclasts have been reported to lack significant ERs [Collier et al., 1998]. Estrogen also modulates the activity of mature rodent osteoclasts independently of differentiation [Sarma et al., 1998; Parikka et al., 2001], although this may not be relevant in human osteoclasts. Additional studies,

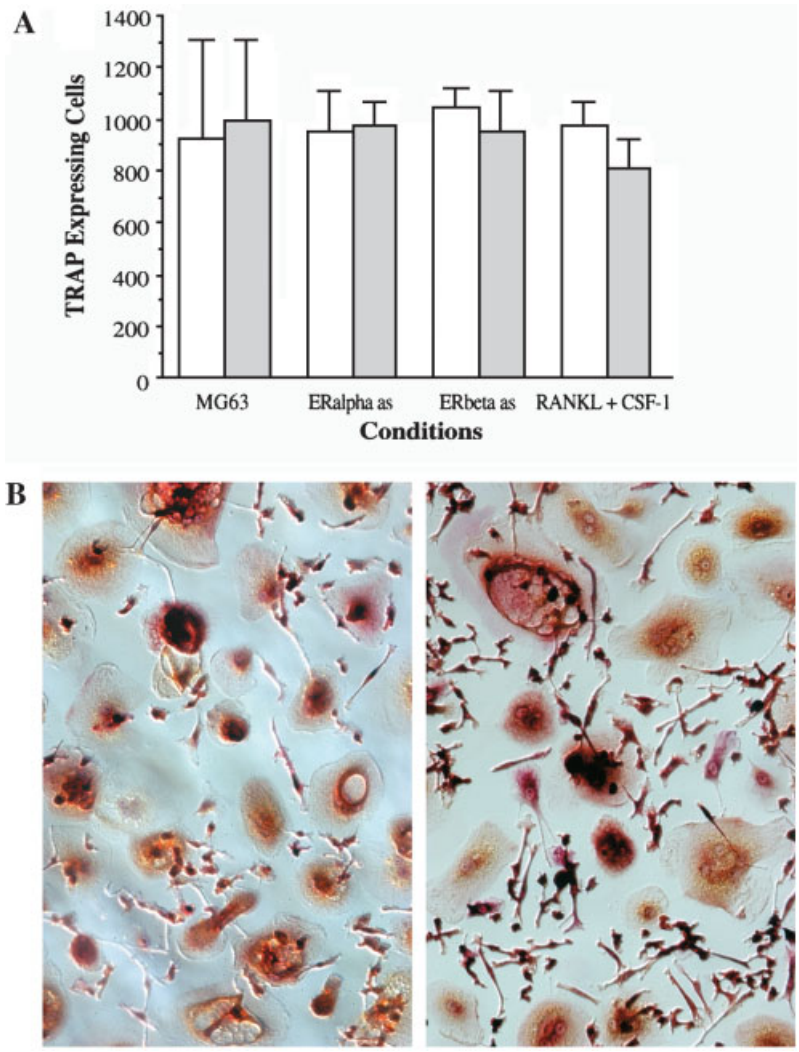

Fig. 6. Differentiation of human osteoclasts in vitro with and without estrogen. **A**: TRAP-expressing cells in osteoclast cultures were made in the presence and absence of 10^{-8} M estradiol, using wild-type MG63 (**left**) and ER-α or β antisense cells, compared to differentiation supported by 20 ng/ml RANKL and 10 ng/ml CSF-1 (**right**). Mean of four determinations ±SD. Results are typical of four similar experiments. **B**: Photomicrographs of osteoclast cultures made from CD14 human monocytes without supporting cells using RANKL (20 ng/ml) and CSF-1 (10 ng/ml), without (**left**) and with (**right**) 10 nM estrogen added to charcoal stripped media. Approximately 50% of the cells are incorporated into giant cells with strong TRAP expression (red). DIC images; TRAP stain was counter-stained with hematoxylin to show nuclei. Fields shown are 335 × 200 μm.

including whether estrogen affects the activity of human osteoclasts to pit bone, will be needed to determine whether the lack of effect on osteoclast formation in vitro is reflected also in activity of human osteoclasts.

While our study implicates ERβ in regulation of matrix production by MG63 cells, it is important to consider that additional effects of estrogens on bone turnover may exist, but may require more complex conditions. There are important interactions of estrogen with PTH [Kanatani et al., 1998] and vitamin D [Schiller et al., 1997]. Some assays of estradiol effects were done with and without PTH, without showing important interactions (Fig. 5). However, interaction with additional factors, such as cell stretch, may be required for effects, such as secretion of TGF-β, which responds to estrogen in osteocytes [Heino et al., 2002]. In addition, apoptosis is a key regulator of bone turnover in vitro, and estrogen is a survival factor for osteocytes and related cells [Tomkinson et al., 1997; Zhou et al., 2001]. Under some conditions, estrogen may promote osteoclast apoptosis [Hughes et al., 1996; Kameda et al., 1997], which may also be mediated by TGF-β.

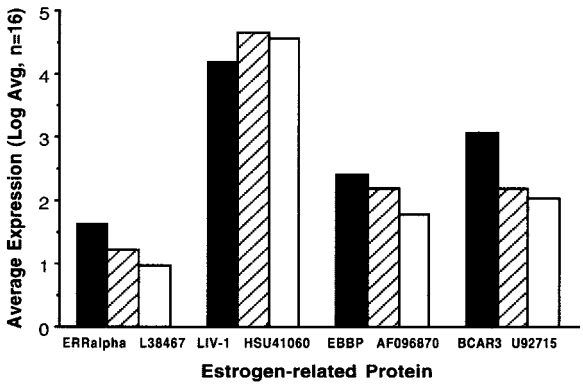

Fig. 7. Highly expressed estrogen-related proteins other than ERα and β were present in similar amounts in ERα- and β-suppresed MG63 cells. These included ERRα and BRCA3, with mRNA-derived signal for each product shown in wild type MG63 cells (solid bars on the left of each group) and cells in which ERα and β were suppressed (middle and right bars of each group). Note that differences in expression were minor, and did not reach significance between groups for any of these mRNAs. Results of mRNA screening using labeled cDNA and Affimetrix gene arrays as log average of fluorescence labeling of 16 determinations (Materials and Methods).

In cells where ER are expressed at very high levels by transduced expression plasmids, ERs have additional effects, such as the effects of ERα observed on cell proliferation [Watts and King, 1994]. Rat osteoblast-like cells, conversely, had limited responsiveness to estrogen when receptor concentration is low [Davis et al., 1994]. In recent work [Rickard et al., 2002], over-expression of ERα or β was capable of inducing progesterone response, although ERα had prominent activity, suggesting that sex-related differences in bone growth may be dependent on ERα, but are not modeled in the MG63 system either due to the mature osteoblast-differentiation state of MG63 cells, or to a relatively low ERα expression.

Gene screening showed that the MG63 cells contain additional estrogen-regulated proteins expressed in relatively high amounts, including ERRα and BCAR3 (Fig. 7). ERRα is expressed in bone development [Bonnelye et al., 1997] and affects bone formation and transcription of osteopontin [Bonnelye et al., 2001]; it is differentially expressed relative to ERα and β in osteoblasts [Bonnelye and Aubin, 2002]. Since ERβ-negative MG63 cells showed only small estrogen response in alkaline phosphatase and collagen secretion, at the differentiation state of these cells either ERR is less important or ERRα requires co-activators not present here. Despite the well-characterized effects of tamoxifen and other estrogen analogs on bone, BCAR3 has no known role in bone physiology. However, as with ERRα, it appears to have no major independent role in regulating the activity of MG63 cells.

Thus, in human MG63 cells, ERβ is the dominant functional receptor in estrogen response in matrix-related protein synthesis. We were unable to demonstrate an effect of estrogen on osteoclast formation from human monocytes. These findings suggest that effects of estrogens on bone loss in the human are mediated largely by cells other than secretory osteoblasts, and may require interactions with additional cells and cytokines. Our results also support a model in which developing osteoblasts, secretory osteoblasts, and osteocytes have distinct and estrogen responses.

ACKNOWLEDGMENTS

We thank Ms Raelene McKeon and Ms Katherine Buddie for technical assistance.

REFERENCES

An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. 2001. Estrogen receptor β-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 276:17808–17814.

Arts J, Kuiper GG, Janssen JM, Gustafsson JA, 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.

Blair HC, Jordan SE, Peterson G, Barnes S. 1996. Variable effects of tyrosine kinase inhibitiors on avian osteoclastic activity and reduction of bone loss in ovariectomized rats. J Cell Biochem 60:629–637.

Blair HC, Sidonio RF, Friedberg RC, Khan NN, Dong S-S. 2000. Proteinase expression during differentiation of human osteoclasts in vitro. J Cell Biochem 78:627–637.

Bonnelye E, Aubin JE. 2002. Differential expression of estrogen receptor-related receptor α and estrogen receptors α and β in osteoblasts in vivo and in vitro. J Bone Miner Res 17:1392–1400.

Bonnelye E, Vanacker JM, Dittmar T, Begue A, Desbiens X, Denhardt DT, Aubin JE, Laudet V, Fournier B. 1997. The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. Mol Endocrinology 11:905–916.

Bonnelye E, Merdad L, Kung V, Aubin JE. 2001. The orphan nuclear estrogen receptor-related receptor-α (ERRα) is expressed throughout osteoblast differentiation and regulates bone formation in vitro. J Cell Biol 153:971–984.

Brubaker KD, Gay CV. 1999. Depolarization of osteoclast plasma membrane potential by 17β-estradiol. J Bone Miner Res 14:1861–1866.

Collier FM, Huang WH, Holloway WR, Hodge JM, Gillespie MT, Daniels LL, Zheng MH, Nicholson GC. 1998. Osteoclasts from human giant cell tumors of bone lack estrogen receptors. Endocrinology 139:1258–1267.

Compston JE. 2001. Sex steroids and bone. Physiol Rev 81: 419–447.

Davis VL, Couse JF, Gray TK, Korach KS. 1994. Correlation between low levels of estrogen receptors and estrogen responsiveness in two rat osteoblast-like cell lines. J Bone Miner Res 9:983–991.

Gaddy-Kurten D, Coker JK, Abe E, Jilka RL, Manolagas SC. 2002. Inhibin suppresses and activin stimulates osteoblastogenesis and osteoclastogenesis in murine bone marrow cultures. Endocrinology 143:74–83.

Heino TJ, Hentunen TA, Vaananen HK. 2002. Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: Enhancement by estrogen. J Cell Biochem 85:185–197.

Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. 1996. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. Nat Med 2:1132–1136.

Jen KY, Gewirtz AM. 2000. Suppression of gene expression by targeted disruption of messenger RNA: Available options and current strategies. Stem Cells 18:307–319.

Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN, Hakeda Y, Kumegawa M. 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. J Exp Med 186:489–495.

Kanatani M, Sugimoto T, Takahashi Y, Kaji H, Kitazawa R, Chihara K. 1998. Estrogen via the estrogen receptor blocks cAMP-mediated parathyroid hormone (PTH)-stimulated osteoclast formation. J Bone Miner Res 13: 854–862.

Lambertini E, Penolazzi L, Aguiari G, del Senno L, Pezzetti F, Sollazzo V, Piva R. 2002. Osteoblastic differentiation induced by transcription factor decoy against estrogen receptor α gene. Biochem Biophys Res Commun 292: 761–770.

Lindberg MK, Erlandsson M, Alatalo SL, Windahl S, Andersson G, Halleen JM, Carlsten H, Gustafsson JA, Ohlsson C. 2001. Estrogen receptor α, but not estrogen receptor β, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice. J Endocrinology 171:425–433.

Lu D, Giguere V. 2001. Requirement of Ras-dependent pathways for activation of the transforming growth factor β3 promoter by estradiol. Endocrinology 142: 751–759.

McCauley LK. 2001. Transgenic mouse models of metabolic bone disease. Curr Opin Rheumatol 13:316–325.

Ogawa S, Fujita M, Ishii Y, Tsurukami H, Hirabayashi M, Ikeda K, Orimo A, Hosoi T, Ueda M, Nakamura T, Ouchi Y, Muramatsu M, Inoue S. 2000. Impaired estrogen sensitivity in bone by inhibiting both estrogen receptor α and β pathways. J Biol Chem 275:21372–21379.

Onoe Y, Miyaura C, Ohta H, Nozawa S, Suda T. 1997. Expression of estrogen receptor β in rat bone. Endocrinology 138:4509–4512.

Parikka V, Lehenkari P, Sassi ML, Halleen J, Risteli J, Harkonen P, Vaananen HK. 2001. Estrogen reduces the depth of resorption pits by disturbing the organic bone matrix degradation activity of mature osteoclasts. Endocrinology 142:5371–5378.

Plant A, Tobias JH. 2001. Characterisation of the temporal sequence of osteoblast gene expression during estrogen-induced osteogenesis in female mice. J Cell Biochem 82: 683–691.

Qu Q, Harkonen PL, Vaananen HK. 1999. Comparative effects of estrogen and antiestrogens on differentiation of osteoblasts in mouse bone marrow culture. J Cell Biochem 73:500–507.

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.

Rizzoli R, Bonjour JP, Ferrari S. 2001. Osteoporosis, genetics and hormones. J Mol Endocrinology 26:79–94.

Robinson JA, Harris SA, Riggs BL, Spelsberg TC. 1997. Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919–2927.

Saika M, Inoue D, Kido S, Matsumoto T. 2001. 17β-estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-α. Endocrinology 142:2205–2212.

Sapolsky RJ, Lipshutz RJ. 1996. Mapping genomic library clones using oligonucleotide arrays. Genomics 33:445–456.

Sarma U, Edwards M, Motoyoshi K, Flanagan AM. 1998. Inhibition of bone resorption by 17β-estradiol in human bone marrow cultures. J Cell Physiol 175:99–108.

Schiller C, Gruber R, Redlich K, Ho GM, Katzgraber F, Willheim M, Pietschmann P, Peterlik M. 1997. 17β-estradiol antagonizes effects of 1α,25-dihydroxyvitamin D3 on interleukin-6 production and osteoclast-like cell formation in mouse bone marrow primary cultures. Endocrinology 138:4567–4571.

Shevde NK, Bendixen AC, Dienger KM, Pike JW. 2000. Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. Proc Natl Acad Sci USA 97: 7829–7834.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Srivastava S, Weitzmann MN, Kimble RB, Rizzo M, Zahner M, Milbrandt J, Ross FP, Pacifici R. 1998. Estrogen blocks *M-CSF* gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. J Clin Invest 102:1850–1859.

Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. 2001. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-κB ligand (RANKL)-induced JNK activation. J Biol Chem 276: 8836–8840.

Tomkinson A, Reeve J, Shaw RW, Noble BS. 1997. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. J Clin Endocrinology Metab 82:3128–3135.

Tou L, Quibria N, Alexander JM. 2001. Regulation of human *cbfa1* gene transcription in osteoblasts by selective estrogen receptor modulators (SERMs). Mol Cell Endocrinology 183:71–79.

Waters KM, Rickard DJ, Riggs BL, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Moore J, Spelsberg TC. 2001. Estrogen regulation of human osteoblast function is determined by the stage of differentiation and the estrogen receptor isoform. J Cell Biochem 83:448–462.

Watts CK, King RJ. 1994. Overexpression of estrogen receptor in HTB 96 human osteosarcoma cells results in estrogen-induced growth inhibition and receptor cross talk. J Bone Miner Res 9:1251–1258.

Windahl SH, Hollberg K, Vidal O, Gustafsson JA, Ohlsson C, Andersson G. 2001. Female estrogen receptor β−/− mice are partially protected against age-related trabecular bone loss. J Bone Miner Res 16: 1388–1398.

Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D. 2001. Estrogen modulates estrogen receptor α and β expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J Cell Biochem 81(S36):144–155.