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Functional Properties of a Conditionally Phenotypic, Estrogen-Responsive, Human Osteoblast Cell Line

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Abstract Osteoblasts are established targets of estrogen action in bone. We screened 66 conditionally immortalized clonal human osteoblast cell lines for estrogen receptors (ERs) using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for ERα mRNA and transactivation of adenovirus–estrogen response element (ERE)-tk-luciferase by 17β-estradiol (17β-E_2) for functional ER protein. One of these cell lines, termed HOB-03-CE6, was chosen for further characterization. The cells, which were conditionally immortalized with a temperature-sensitive SV40 large T antigen, proliferated at the permissive temperature (34°C) but stopped dividing at the nonpermissive temperature (≥39°C). Alkaline phosphatase activity and osteocalcin secretion were upregulated by 1α,25-dihydroxyvitamin D_3 in a dose-dependent manner. The cells also expressed type I collagen and other bone matrix proteins, secreted a variety of growth factors and cytokines, formed mineralized nodules based on alizarin red-S and von Kossa histochemical staining, and responded to dexamethasone, *all-trans* retinoic acid, and transforming growth factor-β1. This cell line expressed 42-fold less ER message than MCF-7 human breast cancer cells, as determined by quantitative RT-PCR. However, adenovirus-ERE-tk-luciferase activity was upregulated three- to fivefold in these cells by 17β-E_2 with an EC_{50} of 64 pM. Furthermore, this upregulation was suppressed by co-treatment with the anti-estrogen ICI-182,780. Cytosolic extracts of these cells specifically bound [^{125}I]-17β-E_2 in a concentration-dependent manner with a B_{max} of 2.7 fmoles/mg protein (~1,200 ERs/cell) and a K_d of 0.2 nM. DNA gel-shift analysis using a [^{32}P]-ERE demonstrated the presence of ERs in nuclear extracts of these cells. Moreover, binding of the extracts to this ERE was blocked by a monoclonal antibody to the human ER DNA-binding domain. We evaluated these cells for 14 of 20 reported endogenous responses to 17β-E_2 in osteoblasts. Although most of these responses appeared to be unaffected by the steroid, 17β-E_2 suppressed parathyroid hormone-induced cAMP production, as well as basal interleukin-6 mRNA expression; conversely, the steroid upregulated the steady-state expression of alkaline phosphatase message in these cells. In summary, we have identified a clonal, conditionally phenotypic, human osteoblast cell line that expresses functional ERs and exhibits endogenous responses to 17β-E_2. This cell line will be a valuable in vitro model for exploring some of the molecular mechanisms of estrogen action in bone. J. Cell. Biochem. 65:368–387.

Key words: immortalized; clonal; alkaline phosphatase; osteocalcin; mineralization; vitamin D_3; dexamethasone; parathyroid hormone; interleukin-6; bone; osteoporosis

Estrogen is an important regulator of skeletal homeostasis [Riggs and Melton, 1992; Dempster and Lindsay, 1993; Turner et al., 1994]. This is demonstrated by the dramatic loss of bone that occurs during postmenopausal osteoporosis [Riggs and Melton, 1992; Dempster and Lindsay, 1993]. Therapeutically, estrogen is classified as an antiresorptive agent, since it suppresses osteoclast differentiation and activity [Riggs and Melton, 1992; Dempster and Lindsay, 1993]. Although some of these effects are probably due to direct action of the steroid hormone on hematopoietic cells and osteoclasts [Turner et al., 1994], estrogen also has indirect effects on resorption through bone marrow stromal cells and osteoblasts [Turner et al., 1994; Manolagas and Jilka, 1995].

Osteoblasts are bone-forming cells and are known to possess functional estrogen receptors (ERs) [Turner et al., 1994]. More than a dozen different in vitro osteoblast models, ranging from primary rodent and human cells to clonal transformed and osteosarcoma-derived mammalian cell lines, have been shown to possess ERs and/or to exhibit endogenous responses to

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17β-estradiol (17β-E_2) [Gray et al., 1987; Komm et al., 1988; Eriksen et al., 1988; Kaplan et al., 1988; Pilbeam et al., 1989; Somjen et al., 1989; Colston et al., 1989; Ernst et al., 1989, 1991; Etienne et al., 1990; Keeting et al., 1992; Suzuki et al., 1993; Mahonen and Maenpaa, 1994]. Compared to cell lines like MCF-7 human breast cancer cells [Kasid et al., 1984], these osteoblast models contain low numbers (60–4,500/cell) of high affinity (K_d = 0.05–1.1 nM) ERs [Komm et al., 1988; Eriksen et al., 1988; Kaplan et al., 1988; Etienne et al., 1990; Benz et al., 1991; Keeting et al., 1992; Masuyama et al., 1992; Migliaccio et al., 1992; Davis et al., 1994]. Estrogen has been reported to have numerous effects on osteoblasts in vitro: these include the regulation of DNA synthesis and cellular proliferation [Gray et al., 1987; Bankson et al., 1989; Ernst et al., 1989; Liel et al., 1992; Masuyama et al., 1992; Ikegami et al., 1994; Majeska et al., 1994; Verhaar et al., 1994]; the stimulation of alkaline phosphatase, creatine kinase, lactate dehydrogenase, γ-glutamyl transferase, and aspartate aminotransferase activity [Gray et al., 1987; Bankson et al., 1989; Somjen et al., 1989; Majeska et al., 1994; Verhaar et al., 1994]; the upregulation of transferrin, transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-I, α1 type (I) procollagen, progesterone receptor, c-*fos*, heat-shock protein, and interleukin-1β (IL-1β) expression [Komm et al., 1988; Eriksen et al., 1988; Bankson et al., 1989; Gray et al., 1989a,b; Ernst and Rodan, 1991; Oursler et al., 1991; Cooper and Uoshima, 1994; Harris et al., 1992; Majeska et al., 1994; Pivirotto et al., 1995]; the suppression of cytokine-induced IL-6 and tumor necrosis factor-α (TNF-α) expression [Girasole et al., 1992; Rickard et al., 1992]; the suppression of ER expression [Davis et al., 1994]; the enhancement of 1α,25-dihydroxyvitamin D_3 (vitamin D_3) action [Liel et al., 1992; Lajeunesse, 1994; Ishibe et al., 1995]; potentiation of bradykinin-stimulated arachidonic acid release [Cissel et al., 1996]; and the regulation of parathyroid hormone (PTH) responsiveness [Ernst et al., 1989; Pilbeam et al., 1989; Fukayama and Tashjian, 1989; Oursler et al., 1991; Eielson et al., 1994; Rao et al., 1994; Kaji et al., 1996; Monroe and Tashjian, 1996]. Although some of these responses have been reported to occur in several osteoblast models, others have only been observed in a single cell line. Moreover, the response of some of these osteoblast models (e.g., normal human osteoblasts) to 17β-E_2 is variable [Girasole et al., 1992; Rickard et al., 1992; Chaudhary et al., 1992; Rifas et al., 1995]. This variation may result from the low numbers of ERs found in these cells or from the observation that only a portion of osteoblasts in culture appear to express the receptor [Ikegami et al., 1993].

Consequently, in order to facilitate the study of estrogen action on the osteoblast, other investigators have developed rodent and human cell lines that overexpress ERα: these include the osteosarcoma-derived cell lines ROS.SMER-14 [Migliaccio et al., 1992], HTB 96-ER [Watts and King, 1994], and SaOS2-ER [Huo et al., 1995], as well as the conditionally immortalized human fetal cell line hFOB-ER9 [Harris et al., 1995; Kassem et al., 1996]. Compared to other osteoblast models, these cell lines have the advantage of stably expressing moderate to high levels of ER (~2,000–4,000/cell). However, one concern about these cell lines is that overexpression creates an artificial environment [Watts and King, 1994]. Furthermore, since the expression of the ER in these cell lines is under the control of a viral promoter, the factors that regulate the expression of the endogenous receptor gene cannot be studied. Therefore, we undertook a different approach to identifying an estrogen-responsive human osteoblast cell line.

We have developed more than 70 conditionally immortalized adult human bone cell lines that appear to contain examples of different stages of osteoblast differentiation [Bodine et al., 1996a,b]. We screened this collection of cell lines for estrogen responsiveness using reverse-transcriptase polymerase chain reaction (RT-PCR) analysis to measure ERα mRNA levels and transactivation of adenovirus-estrogen response element (ERE)-thymidine kinase (tk) promoter-luciferase by 17β-E_2 to detect functional ERs. Using this approach, we have identified an estrogen-responsive conditionally immortalized adult human osteoblast cell line, which we refer to as HOB-03-CE6 (Human OsteoBlast-patient 03-Cortical Explant clone 6). In this report, we describe the initial characterization and usefulness of this cell line.

MATERIALS AND METHODS

Materials

Except where noted, tissue culture reagents were purchased from Gibco BRL (Grand Island, NY), while other reagents and chemicals were

obtained from Sigma (St. Louis, MO) or VWR (Philadelphia, PA). The following HOB cell culture media were used in these studies. Growth medium consisted of phenol red-free D-MEM/F-12 containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) Penicillin-Streptomycin and 2 mM GlutaMAX-1. Experimental medium consisted of phenol red-free D-MEM/F-12 containing 2% (v/v) heat-inactivated charcoal-stripped FBS (HyClone, Logan, UT), 1% (v/v) penicillin–streptomycin, and 2 mM GlutaMAX-1, 50 μg/ml ascorbic acid, and 10 nM menadione sodium bisulfite (vitamin K_3). BSA-medium consisted of phenol red-free D-MEM/F-12 containing 0.25% (w/v) bovine serum albumin (BSA, Pentex crystallized, Miles Laboratories, Kankakee, IL), 1% (v/v) penicillin–streptomycin, 2 mM GlutaMAX-1, 50 μg/ml ascorbic acid, and 10 nM menadione sodium bisulfite.

Development and Maintenance of the Cell Line

Normal cortical bone fragments were obtained from a femoral end of a 77-year-old woman who had undergone knee replacement surgery. Explant bone cell cultures were established by a variation of the procedure of Robey and Termine [1985]. Osteoblasts were immortalized by infection with adenovirus-*ori*-SV40 *tsA* 209 [Lei et al., 1992] and cloned as previously described [Bodine et al., 1996a]. Immunocytochemistry for the T antigen indicated that essentially all of the HOB-03-CE6 cells expressed the *tsA* 209 mutant protein (data not shown). Cultures were maintained in vented T-175 flasks at 34°C using growth medium. Cells were passed at a ratio of ~1:3 twice a week using a solution of 0.05% (w/v) trypsin-0.53 mM ethylenediaminetetraacidic acid (EDTA); the flasks were allowed to become ~80% confluent before passing (~50,000 cells/cm^2). Frozen stocks of cells were maintained at ≤−150°C in growth medium containing 20% (v/v) heat-inactivated FBS and 10% (v/v) dimethylsulfoxide.

HOS-TE85 human osteosarcoma cells and MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). HOS-TE85 cells were cultured in MEM containing 10% (v/v) FBS, 1% (v/v) nonessential amino acids, 1% (v/v) penicillin–streptomycin and 2 mM GlutaMAX-1, while MCF-7 cells were cultured in D-MEM/F-12 containing 10% (v/v) FBS, 1% (v/v) penicillin–streptomycin and 2 mM GlutaMAX-1.

Measurement of Cellular Proliferation

Cells in Growth Medium were seeded at 50,000 cells/well (low-density) into six-well plates (~5,000/cm^2) and incubated for 1 day at 34°C. The medium was changed to experimental medium, and the cells were incubated at either 34°C or 39°C for up to 13 days. The wells in each plate were washed with calcium/magnesium-free phosphate-buffered saline (PBS), trypsinized, and counted with a Coulter Multisizer IIe system (Coulter, Miami, FL) as previously described [Bodine et al., 1996a].

Alkaline Phosphatase and Bone Matrix Protein Assays

Cells in growth medium were seeded at 17,000 cells/well (high-density) into 96 well plates (~50,000/cm^2) and incubated at 34°C overnight. Cells were washed with PBS, BSA-medium was added to each well, and the cells were incubated at 39°C for 24 h. The medium was changed, and the cells were treated with either vehicle (ethanol, 0.1%, v/v) or vitamin D_3 (Calbiochem, La Jolla, CA) for 48 h at 39°C. After treatment, the conditioned media were saved and stored at −80°C, and the cells were washed with PBS and assayed for alkaline phosphatase activity as previously described [Bodine et al., 1996a].

Bone matrix proteins were measured in the conditioned media using commercially available enzyme-linked immunosorbant assays (ELISA) as previously described [Bodine et al., 1996a].

Histochemistry

Cells in growth medium were seeded at low density into six-well plates and incubated at 34°C until confluent (~4 days). Cultures were then incubated in experimental medium at 39°C in the absence or presence of dexamethasone (Dex) and β-glycerol phosphate (β-GP) for up to 11 days. Cell layers were washed on ice with cold PBS, fixed with 2% (w/v) paraformaldehyde, and rinsed with 0.1 M cacodylate buffer. Fixed cell layers were incubated for 30 min at 37°C with 20 mg/ml napthol AS-Mx phosphatase disodium salt and 40 mg/ml Fast Red TR salt in a pH 8.4 Tris buffer as described by Lowry et al. [1954].

The formation of in vitro mineralized nodules were determined either by alizarin red-S histochemical staining [Bodine et al., 1996a] or by von Kossa (3%, w/v, $AgNO_3$) histochemical staining [Clark, 1982].

RNA Analysis

Northern hybridizations. Cells in growth medium were seeded at low-density into 100-mm dishes and incubated at 34°C until confluent (~4 days). Cultures were next incubated in growth medium at 39°C for 1 week and then incubated in experimental medium at 39°C for 24 h. Cells were treated in BSA-medium with vehicle (0.1%, v/v, ethanol), Dex, vitamin D_3 (kindly provided by Dr. M. Uskokovic, Hoffmann-La Roche, Nutley, NJ), TGF-β1 (R & D Systems, Minneapolis, MN), *all-trans*-retinoic acid, or 17β-E_2 for 24 h at 39°C. Total RNA was isolated, and 10 µg of each sample was processed for Northern hybridizations as previously described [Shalhoub et al., 1992]. The following DNA probes were used: human type I procollagen, human IL-6 (Genetics Institute, Cambridge, MA), human bone/liver/kidney-type alkaline phosphatase [Weiss et al., 1986], human histone H4 [Pauli et al., 1989], and human TGF-β1 [Derynck et al., 1985]. Autoradiograms were quantified by scanning densitometry using a UVP Gel Documentation System (San Gabriel, CA), and the results were expressed in arbitrary densitometric units after normalization to 18S ribosomal RNA from the ethidium bromide-stained gel.

RT-PCR. Confluent flasks of cells (~60,000 cells/cm^2) were incubated for 48 h in growth medium at 39°C. Flasks were rinsed in PBS, and total cellular RNA was isolated using TRIzol according to the manufacturer's instructions (Gibco BRL). After extraction, RNA was precipitated by recommended procedures and dissolved in diethylpyrocarbonate (DEPC)-treated water. Samples were treated with 1 U/µg of RNase-free DNase (Boehringer Mannheim, Indianapolis, IN), and RNA was phenol/chloroform extracted, ethanol precipitated, dissolved in DEPC-water, and quantified by ultraviolet (UV) spectrophotometry. Reverse transcription reactions were performed at 42°C with 0.5 µg of RNA in 1× PCR Buffer (Gibco BRL) containing 5 mM $MgCl_2$, 2.5 µM ER-specific reverse primer (5′-CCAGCAGCATGTCGAAGATC-3′), 0.5 µM cyclophilin (CYCLO)-specific reverse primer (5′-GGAAGGTGAAAGAAGGACTGAGC-3′), 1 mM deoxyribonucleotide triphosphates (dNTPs), 20 units of RNasin (Promega, Madison, WI), and 200 units of Superscript II Reverse Transcriptase (RT) (Gibco BRL). After heat inactivation, PCR was initiated by adding a master mix containing ERα-specific forward primer (5′-GGAGACATGAGAGCTGCCAAC-3′) and CYCLO-specific forward primer (5′-TTCCAGGATTCATGTGCCAGGGTG-3′) directly to the RT reaction. Final concentration of reagents in the PCR reaction was as follows: 0.5 µM each ER-specific primer, 0.1 µM each CYCLO primer, 1× PCR Buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 0.5 units of *Taq* DNA Polymerase (Gibco BRL). Two-step PCR was carried out in a Stratagene Robocycler (La Jolla, CA) for 25 cycles, using an annealing temperature of 64°C. Samples were fractionated on 1.5% (w/v) agarose gels and transferred to Hybond-N^+ (Amersham, Arlington Heights, IL) by alkali Southern blotting in 0.4 N NaOH, 0.6 M NaCl. Blots were prehybridized at 42°C in Rapid-Hyb buffer (Amersham). Internal oligonucleotide probes specific for the ERα (5′-TGAACCAGCTCCCTGTCTGCCAGGTTGGT-3′) and CYCLO (5′-TCCATCTACGGAGAGAAATTTGAGGATGAG-3′) cDNA fragments were end-labeled with [^{32}P]-γ-ATP (3000 Ci/mmol, New England Nuclear, Boston, MA) using polynucleotide Kinase (Gibco BRL). Probes were added to the prehybridization solution at 3.0×10^6 counts per min (cpm)/ml and incubated at 42°C. Blots were washed twice in 2× NaCl/sodium citrate (SSC), 0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature and then twice in 0.2× SSC, 0.1% SDS at 42°C. Blots were exposed to X-ray film and were quantified using a Molecular Dynamics PhosphoImager SI (Sunnyvale, CA).

The relative amount of ERα message in MCF-7 and HOB-03-CE6 cells was determined by competitive quantitative PCR [Hayward-Lester et al., 1996]. A standard RNA competitor was synthesized which shared primers with wild-type ERα but was 86 bp shorter. The primer used in the RT reaction was 5′-CAGCATGTCGAAGATC-3′, while the forward primer was 5′-GGAGACATGAGAGCTGCCAACC-3′. These primers yielded 350-bp and 436-bp products of standard and wild-type ERα, respectively. The RT reaction was performed for 45 min at 42°C in 50 mM Tris–HCl, pH 8.3, containing 75 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM dNTPs, 30 pmoles reverse primer, and 200 units Superscript II RT. After heat inactivation, 35 cycles of PCR were performed in 26 mM Tris–HCl, pH 8.4, containing 55 mM KCl, 1.35 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM dNTPs, 30 pmoles forward primer, and 2.5 units *Taq* DNA using an annealing temperature of 64°C. PCR products were separated by high-performance liquid chromatography (HPLC) on a Ranin Dynamax System (Woburn, MA) using a DNASep

column (Sarasep, Santa Clara, CA); the products were eluted at 50°C with 0.1 M triethylammonium acetate (Fluka, Ronkonkoma, NY) and 25% (v/v) acetonitrile. The elution of products were quantified by absorbance at 254 nm.

Steroid-Binding Assay

Cells in growth medium were seeded at 1×10^7 cells/dish (high-density) into 15–20 150-mm dishes (~55,000/cm^2) and incubated at 34°C overnight. The medium was changed, and the cells were incubated at 39°C for 48 h. Each dish was rinsed with warm PBS and cold lysis buffer (10 mM Tris–HCl, pH 7.5, containing 1 mM EDTA, 1 mM DTT). The cells were scraped from the dishes, and a lysate was prepared using a hand-held homogenizer (Dremel Moto-Tool®, model 395, Racine, WI). The lysate was centrifuged at 100,000*g* for 45 min, and aliquots of cytosol were added to varying concentrations of [^{125}I]-17β-E_2 (2,200Ci/mmol, New England Nuclear) in the presence or absence of 1,000-fold molar excess diethylstilbestrol. After incubation for 2 h at room temperature, an equal volume of cold 5% (w/v) dextran-coated charcoal was added, and the mixture was centrifuged at 850*g*. Aliquots of the supernatant were removed, placed into a scintillation vial, and counted in a Beckman LS 6500 (Fullerton, CA) using Ready Protein plus cocktail (Beckman). The counting efficiency for ^{125}I was estimated to be 80%. The protein concentration of the cytosol was determined by the method of Bradford [1976] with reagent purchased from BioRad and BSA as the standard.

DNA Gel-Shift Analysis

Cells in Growth Medium were seeded at 1×10^7 cells/dish into 10–15 150-mm dishes and incubated at 34°C overnight. The medium was changed and the cells were incubated at 39°C for 48 h. Dishes were rinsed with cold PBS and Hypotonic Buffer [20 mM Hepes, pH 7.9, containing 20 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT]. Cells were lysed with cold Lysis Buffer [Hypotonic Buffer containing 63 pM okadaic acid, 50 ng/ml aprotinin, 50 ng/ml leupeptin, 50 ng/ml pepstatin, 2.5 mM phenylmethylsulfonylfloride, and 0.2% (v/v) NP-40], scraped from the dish, and nuclei were pelleted by centrifugation at 16,000*g*. Nuclei were extracted with cold High-Salt Buffer [Lysis Buffer containing 20% (v/v) glycerol and 420 mM NaCl] and centrifuged again. The protein concentration of the extract was determined by the method of Bradford [1976]. The extract was stored at −80°C. The 45-base pair (bp) synthetic vitellogenin A2 promoter [Klein-Hitpass et al., 1988] and the 45-bp nonspecific DNA sequence (which had the ERE mutated) were radiolabeled by the Klenow enzyme (Random Prime kit, Gibco BRL) using [^{32}P]-α-dCTP to a specific activity of ~3,500 cpm/ng. The DNA sequences, which contained 2-bp overhangs, were as follows; ERE: 5′-CCAAAGTCAGGTCACAGTGACCTGATCAAAGTTAATGTAACCTCA-3′; nonspecific: 5′-CCAAAGTCTTCGTGCAGCATTAGTATCATAGTTACTGTACCCTCA-3′. Varying concentrations of the nuclear extract were incubated with ~11 ng of the [^{32}P]-ERE in binding buffer [20 mM Hepes, pH 7.9, containing 80 mM KCl, 10% glycerol (v/v), 2 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM DTT, and 0.2 mg/ml poly dI:dC] for 60 min on ice. The specificity of the binding reaction was determined by co-incubation with 50- to 100-fold molar excess radioinert ERE or nonspecific DNA. Nuclear extracts were also preincubated for 60 min at room temperature with a monoclonal antibody to the DNA-binding domain of the human ER (MAI-310, Affinity BioReagent, Golden, CO) or with a nonspecific I_gG (Pierce, Rockford, IL). Samples were electrophoresed on 5% (w/v) nondenaturing polyacrylamide gels, which were then fixed in 10% (v/v) glacial acetic acid/20% (v/v) methanol, dried, and exposed to X-ray film (−80°C).

Luciferase Assay

Cells in growth medium were seeded at 17,000 cells/well into 96-well plates and incubated at 34°C overnight. Cells were infected for 2 h at 37°C with a 1:5 dilution of adenovirus-ERE-tk-luciferase (~200 plaque-forming units per cell) in experimental medium. This virus contains two copies of the *Xenopus laevis* vitellogenin A2 ERE (5′-GGTCACAGTGACC-3′) linked to the thymidine kinase promoter (−110 to +10) and the luciferase gene. This construct was inserted in place of the E1a adenoviral gene. After infection, the wells were washed with PBS, and the cells were treated with experimental medium containing either vehicle (ethanol, 0.1%, v/v) or 17β-E_2 in the absence or presence of 1,000-fold molar excess ICI-182,780 (Zeneca, Wilmington, DE) for 48 h at 39°C. Essentially all the cells were infected under these conditions. After treatment, the wells were washed with PBS,

and the cells were lysed with cell culture lysis reagent (Promega). Cell lysates were transferred to a 96-well luminometer plate (Dynatech, Chantilly, VA), and luciferase activity was measured in a MicroLumat LB 96 P luminometer (EG & G Berthold, Postfach, Germany) using luciferase substrate (Promega). Data were transformed by logarithms, and the Huber M-estimator was used to downweight the outlying transformed observations. EC_{50} calculations were determined using the JMP software (SAS Institute, Cary, NC).

Measurement of Intracellular Cyclic AMP

Cells in growth medium were seeded at 100,000 cells/well (high-density) into 24-well plates (~55,000/cm^2) and incubated at 34°C overnight. Cells were washed with PBS, BSA-medium, containing either vehicle (ethanol, 0.1%, v/v) or 17β-E_2 was added to each well, and the cells were incubated at 39°C for 48 h. The medium was changed, and the cells were pretreated with 0.5 mM isobutylmethylxanthine (IBMX) for 5 min at 37°C in the absence or presence of 17β-E_2. The cells were treated at 37°C in the presence of IBMX with either vehicle (ethanol, 0.1%, v/v), PTH [fragment 1–34], prostaglandin E_2 (PGE_2), or forskolin for 10 min at 37°C in the absence or presence of 17β-E_2. Cells were washed with cold PBS, and intracellular cyclic adenosine monophosphate (cAMP) was extracted and measured as previously described [Bodine et al., 1996a].

Statistical Analysis

Data were analyzed for statistical significance ($P < 0.05$) by the Behren's–Fisher t-test or by one-way analysis of variance (ANOVA) using the Dunnett's test.

RESULTS

Screening for an Estrogen-Responsive Cell Line

Figure 1 outlines the protocol we used to screen the HOB cell lines for functional ERs. Total RNA was isolated from confluent cultures of 66 HOB clones after incubation in growth medium for 2 days at the nonpermissive temperature (39°C). This RNA was then evaluated for ERα mRNA by RT-PCR. More than 90% of the cell lines expressed ER message, although the levels of this mRNA varied among the clones. Cell clones that expressed moderate to high levels of ER mRNA by RT-PCR (~30%) were analyzed for the presence of functional receptors based on the ability to transactivate adenovirus-ERE-tk-luciferase with 17β-E_2. Based on this criterion, only five of the HOB cell lines exhibited a statistically significant (≥1.5-fold) increase in luciferase activity after 48 h of steroid treatment at 39°C (data not shown). One of these clones, the HOB-03-CE6 cells, was chosen for further characterization.

Osteoblastic Properties of the HOB-03-CE6 Cell Line

Figures 2–4 depict some of the osteoblastic characteristics of the HOB-03-CE6 cell line. As shown in Figure 2A, the cells proliferated in experimental medium at the permissive temperature (34°C) but stopped dividing at 39°C. The cells also secreted moderate levels of type I

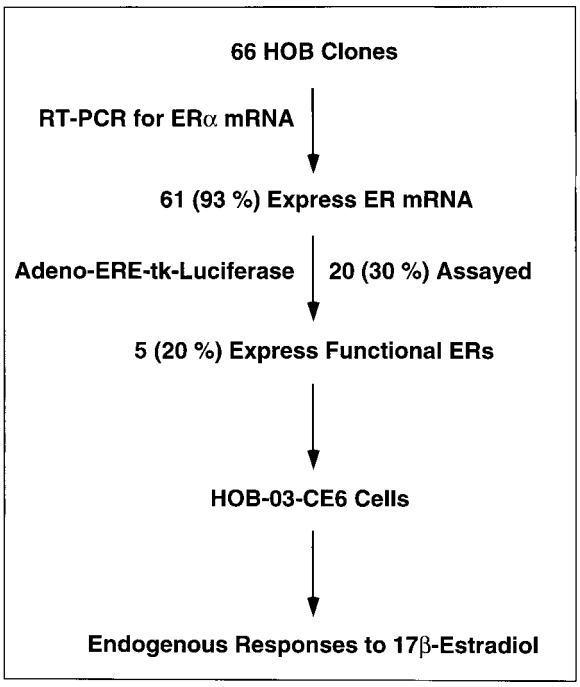


Fig. 1. Screening paradigm for the identification of estrogen-responsive human osteoblast cell lines. RNA was isolated from 66 human osteoblast (HOB) clones after 2 days in growth medium at 39°C and evaluated for estrogen receptor (ER)-α mRNA by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. This analysis indicated that >90% of the cell lines expressed ER message. Clones that expressed moderate to high levels of ER mRNA (~30%) were analyzed for the presence of functional receptors based on the transactivation of the adenovirus-estrogen response element-thymidine kinase promoter (adeno-ERE-tk)-luciferase vector by 17β-E_2. Only 20% of these cell lines exhibited a 1.5-fold or greater increase in luciferase activity after steroid treatment. One of these clones, the HOB-03-CE6 cells, were then evaluated for endogenous responses to 17β-E_2.

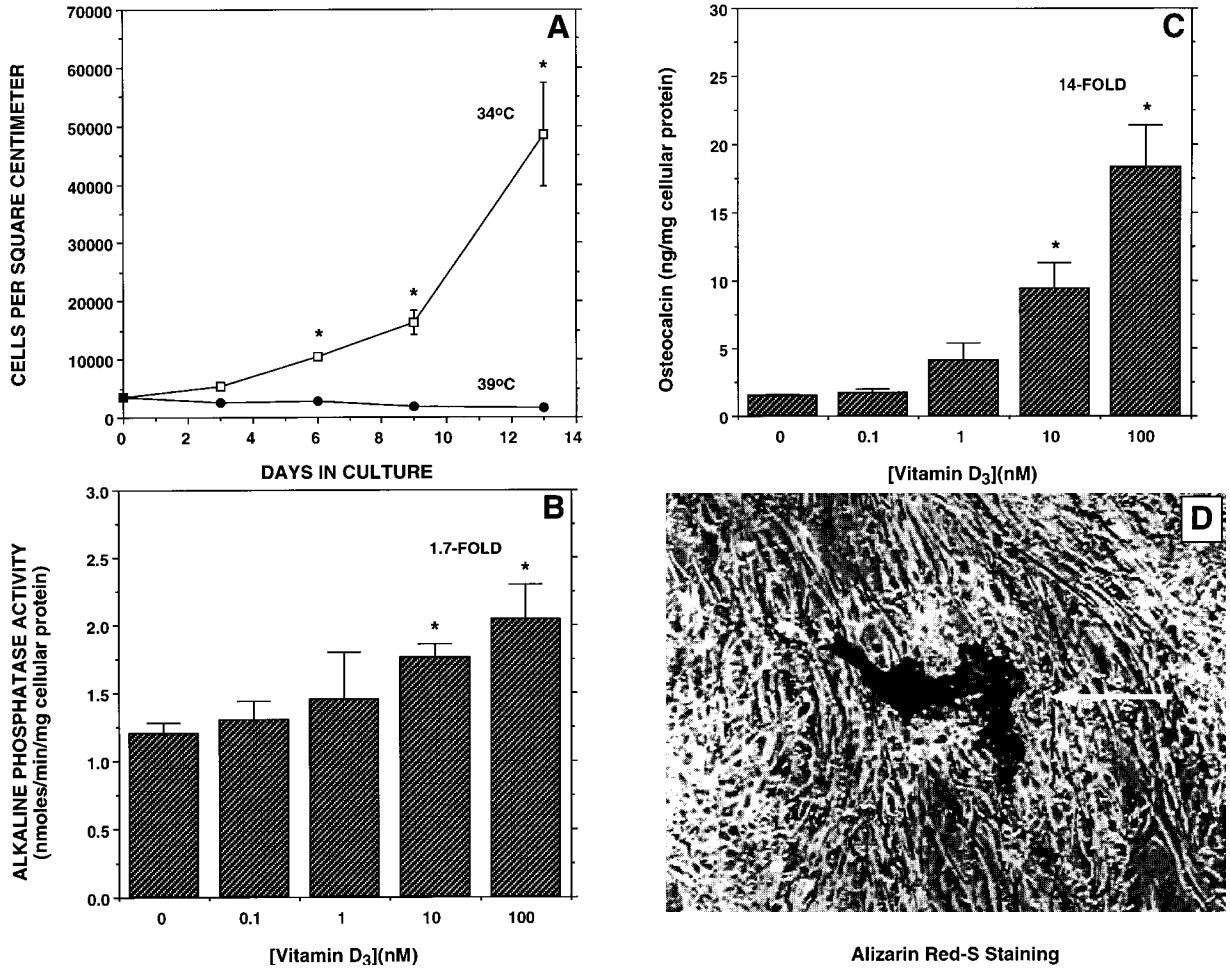


Fig. 2. Osteoblastic characteristics of the HOB-03-CE6 cells. Cellular proliferation in experimental medium at 34°C and 39°C (**A**), vitamin D_3-regulated alkaline phosphatase activity (**B**), and osteocalcin secretion (**C**) after 48 h in BSA-medium at 39°C, and mineralized nodule formation after 5 days in experimental medium containing 5 mM β-GP at 39°C (**D**). Procedures were performed, as described in Materials and Methods. **A–C:** Results are presented as the mean ±SD, n = 6–8; *$P <$ 0.01–0.001 from the no-treatment controls (Dunnett's ANOVA test). The results are representative of two similar experiments.

procollagen C-peptide after 48 h in BSA-medium at 39°C (3.5 ± 1.1 μg/mg cellular protein, n = 8). In addition, the cells expressed moderate levels alkaline phosphatase activity under the same conditions (Fig. 2B). Moreover, vitamin D_3 upregulated enzyme activity 1.7-fold in a dose-dependent manner. Vitamin D_3 also enhanced the secretion of osteocalcin from the cells 14-fold in a dose-dependent manner (Fig. 2C). Finally, the cells formed mineralized nodules after 5 days at 39°C in experimental medium containing 5 mM β-GP (Fig. 2D). However, we observed that mineral deposition in the cultures was sparse under these basal conditions and addressed whether the addition of the glucocorticoid, Dex, to the cultures would enhance mineralization; this phenomenon has

Fig. 3. Morphologic and histochemical examination of dexamethasone-treated HOB-03-CE6 cells. Cells were seeded at low density and cultured in growth medium at 34°C until confluent. The cells were then treated as follows: **A,B:** Toluidine blue staining for control (**A**) and 100 nM Dex/10 mM β-GP-treated cultures (**B**) 3 days after hormone treatment in Experimental Medium at 39°C. **C,D:** Toluidine blue staining of control (**C**) and Dex/β-GP-treated cultures (**D**) 11 days after hormone treatment at 39°C. **E,F:** Alkaline phosphatase histochemical staining of control (**E**) and Dex/β-GP-treated cultures (**F**) 10 days after hormone treatment at 39°C. **G,H:** Dex/β-GP-treated cultures we stained for calcium phosphate by von Kossa (**G**) or for calcium accumulation by Alizarin red-S (**H**) 10 days after hormone treatment at 39°C, as described in Materials and Methods for details.

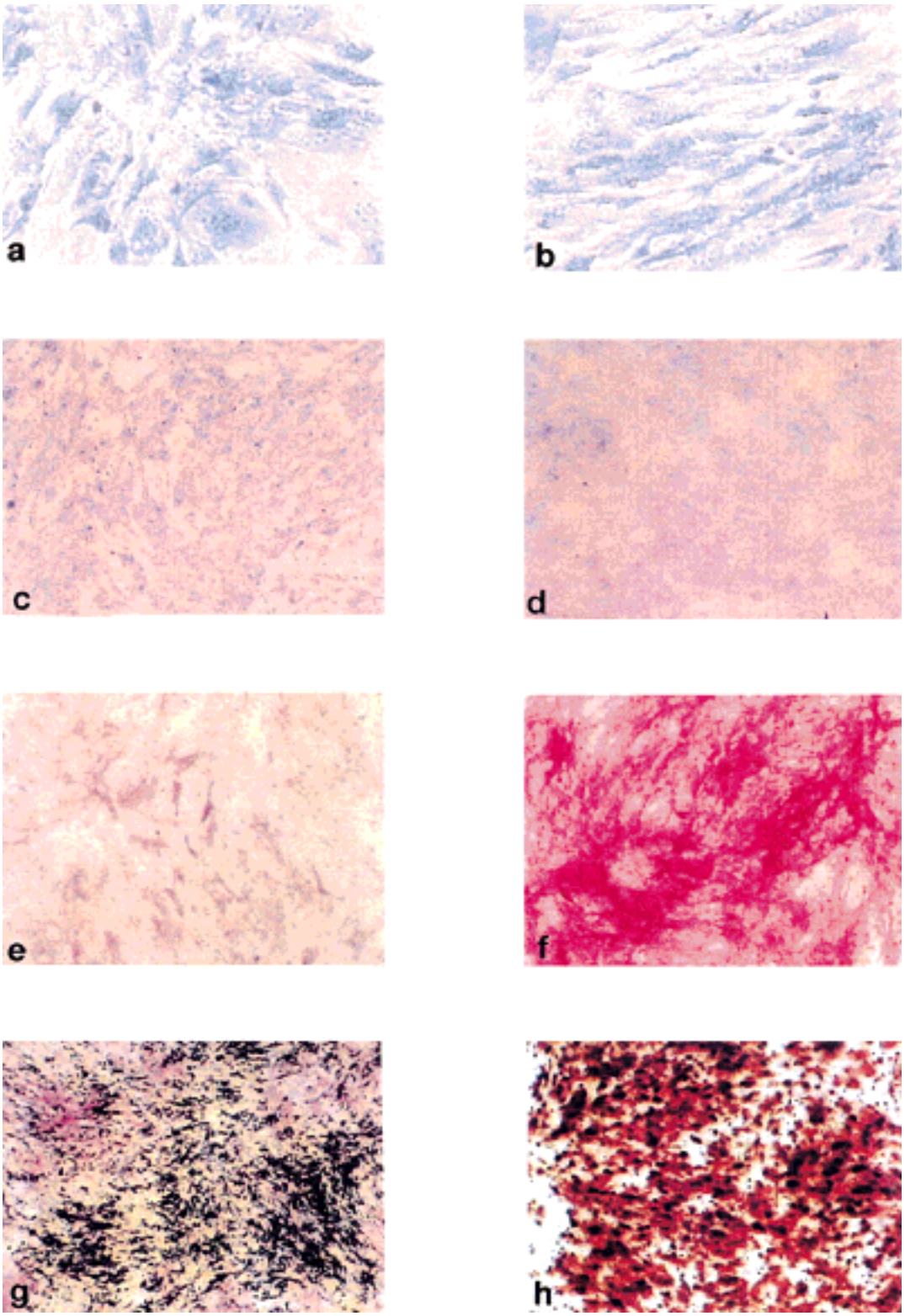


Figure 3.

been reported for rat calvaria-derived osteoblasts [Bellows et al., 1985; Shalhoub et al., 1992] as well as rat-derived [Leboy et al., 1991] and human-derived [Cheng et al., 1996] bone marrow stromal cell cultures.

As shown in Figure 3, we monitored the effects of Dex on cell morphology and mineralization of the extracellular matrix (ECM), both morphologically and biochemically. For these experiments, HOB-03-CE6 cells were plated at low density and allowed to proliferate in growth medium at 34°C until confluent; this enabled the cells to synthesize an extensive ECM. Upon reaching confluence, the cells were treated at 39°C with 100 nM Dex and 10 mM β-GP in experimental medium. Dex had an effect on cellular morphology within 3 days of treatment resulting in an elongated and aligned appearance throughout the cultures (Fig. 3B) when compared to the more randomly organized cuboidal cells of the untreated cultures (Fig. 3A). By day 11 of Dex/β-GP exposure, a copious amount of ECM was revealed by toluidine blue staining, which masked the clarity of individual cells (Fig. 3D) when compared to control cells (Fig. 3C). The inclusion of Dex in the culture medium enhanced cellular alkaline phosphatase activity four- to fivefold, while β-GP slightly elevated enzymatic activity over control values (data not shown). Histochemical detection of alkaline phosphatase activity in Dex/β-GP-treated cells revealed that the upregulation of the enzyme occurred uniformly throughout the cultures (Fig. 3F), when compared to control cells (Fig. 3E). These elevated levels of enzymatic activity appeared to be linked to the ability of the ECM to mineralize. When compared to the sparse mineral deposits observed in Figure 2D, Dex/β-GP-treated cultures had a uniform deposition of hydroxyapatite needles as revealed by the von Kossa silver stain (Fig. 3G). Moreover, alizarin red-S staining showed a uniform distribution of calcium deposits throughout the cultures (Fig. 3H).

Figure 4 depicts the responses of the HOB-03-CE6 cells, as measured by Northern hybridization, to 100 nM Dex, 10 nM vitamin D_3, 100 nM *all-trans* retinoic acid, and 100 pM TGF-β1 after 24 h of treatment at 39°C. For these experiments, the cells were also plated at low density and allowed to proliferate in growth medium at 34°C until confluent; the cultures were then incubated at 39°C for 1 week before hormone treatment in BSA-medium. The expression of histone H4 mRNA was very low in control cultures and was not significantly affected by any of the treatments. This observation was consistent with the lack of cellular proliferation at the nonpermissive temperature (Fig. 2A). By contrast, type I procollagen gene expression was dramatically suppressed by retinoic acid. The response of the cells to these hormones was also evident by monitoring the steady-state levels of alkaline phosphatase and IL-6 messages. Both Dex and TGF-β1 elevated alkaline phosphatase mRNA levels two- to threefold, while IL-6 mRNA was downregulated by Dex and retinoic acid. Vitamin D_3 upregulated alkaline phosphatase message levels ~1.5-fold, which was consistent with the observed increase in enzymatic activity (Fig. 2B). Table I summarizes these and other characteristics of the cell line.

HOB-03-CE6 Cells Express Functional ERs

As noted in Figure 1, the levels of ERα mRNA varied among the HOB clones. An example of this variation is shown in Figure 5, which depicts a Southern blot of RT-PCR products from several HOB-03 clones. The HOB-03-CE6 cells expressed relatively high levels of ER mRNA by RT-PCR, although this level was considerably less than that expressed by the rat uterus. By contrast, the HOB-03-C2 cells expressed low levels of ER message, and we could not detect ER mRNA in a clone of HOS-TE85 human osteosarcoma cells, even though this cell line has been reported to express low levels of functional ERs [Komm et al., 1988; Etienne et al., 1990; Ikegami et al., 1993, 1994].

Quantitative RT-PCR was used to determine the relative amount of ERα mRNA present in the HOB-03-CE6 cells. As shown in Figure 6, the HOB-03-CE6 cells contain 42 times less ER message per milligram of total RNA than MCF-7 human breast cancer cells, which are known to express high levels of receptors [Kasid et al., 1984]. Although the data were linear with slopes of −1.0 [Raeymaekers, 1993], the number of ER messages per cell could not be accurately determined, since the standard was not validated against wild-type RNA [Hayward-Lester et al., 1995].

In order to quantify the level ER protein in the HOB-03-CE6 cells, [^{125}I]-17β-E_2 binding assays were performed with cytosolic extracts from confluent cultures that had been incu-

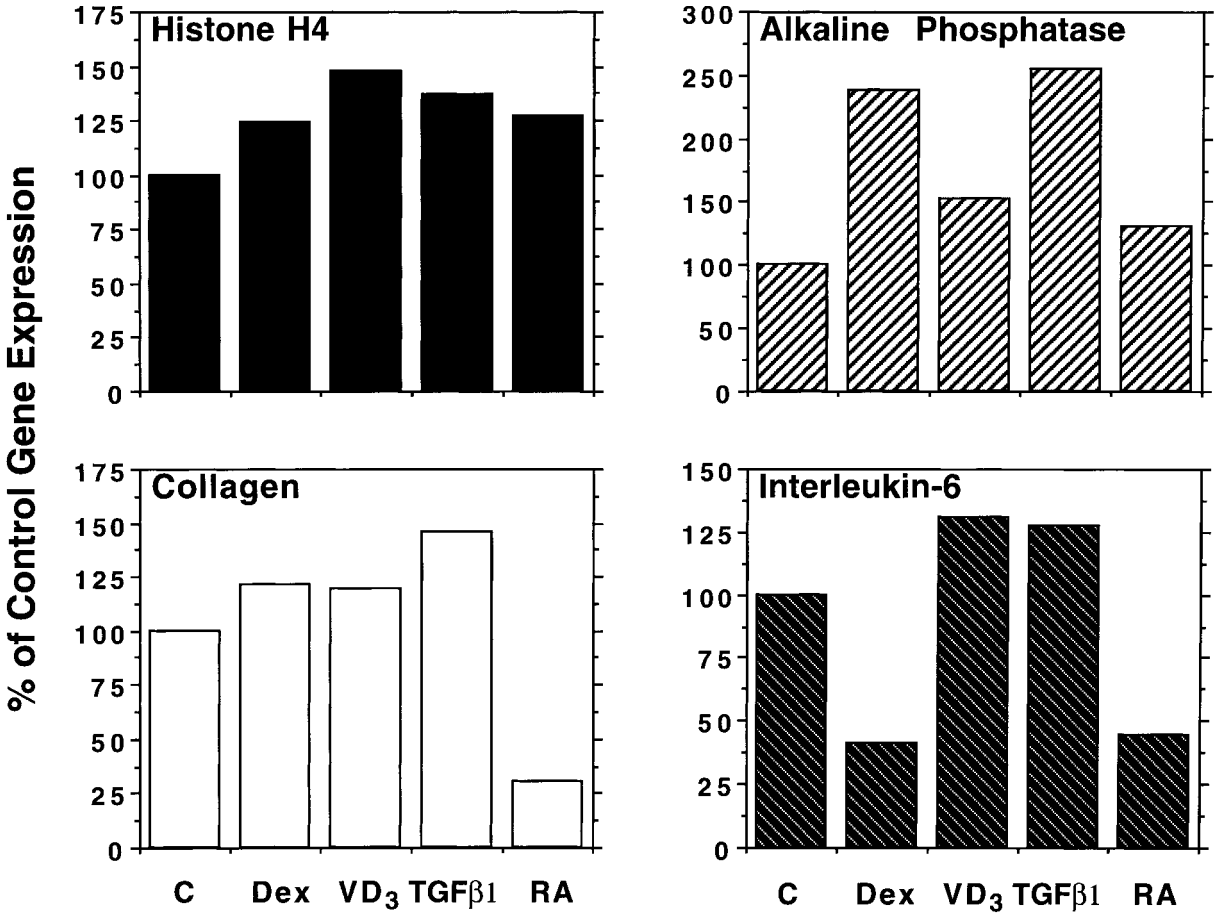


Fig. 4. Hormone and growth factor regulation of gene expression by the HOB-03-CE6 cell line. Cells were cultured in growth medium until confluent at 34°C, incubated for 7 days in experimental medium at 39°C and then treated for 24 h in BSA-medium at 39°C in the absence (Control, C) or presence of 100 nM dexamethasone (Dex), 10 nM vitamin D_3 (VD_3), 100 pM TGF-β1, or 100 nM *all-trans* retinoic acid (RA). Total RNA was isolated and analyzed by Northern hybridization for histone H4, type I procollagen (collagen), alkaline phosphatase, or interleukin-6, as described in Materials and Methods. The results were quantified by scanning densitometry and normalized to 18S ribosomal RNA; data are expressed as the percentage relative to the control.

bated at 39°C for 2 days. As shown in Figure 7A, these cytosols contained specific binding sites for the ligand. A Scatchard analysis of these data demonstrated the presence of a single class of cytosolic steroid binding sites [Scatchard, 1949] (Fig. 7B). The B_{max} for [^{125}I]-17β-E_2 was 2.73 fmoles per mg of cellular protein, while the K_d for ligand was 0.22 nM. Since an HOB-03-CE6 cell contains about 768 ± 43 pg of protein, each of these cells expresses approximately 1,265 ERs. This number of ERs per cell was within the range reported for normal human osteoblasts [Eriksen et al., 1988; Benz et al., 1991] and was consistent with the quantitative RT-PCR comparison to MCF-7 cells that contain ~50,000–150,000 receptors per cell [Kasid et al., 1984]. In addition, the K_d for steroid was consistent with that of the ER reported inother osteoblast-like cells [Komm et al., 1988; Kaplan et al., 1988; Etienne et al., 1990; Benz et al., 1991; Masuyama et al., 1992; Davis et al., 1994].

DNA-gel shift experiments were performed to confirm the presence of ERs in nuclear extracts from confluent cultures of cells that had been incubated at 39°C for 2 days. Figure 8 depicts an autoradiogram of a DNA gel-shift experiment using a 45-bp [^{32}P]-DNA fragment containing part of the *Xenopus laevis* vitellogenin A2 promoter. This DNA fragment contained the 15-bp ERE as well as 28 bp of flanking sequence. Nuclear extracts from the HOB-03-CE6 cells bound and retarded the migration of the [^{32}P]-ERE in a protein concentration-dependent manner (lanes 1–4). This binding

TABLE I. Properties of the HOB-03-CE6 Cell Line*

Factor	Description
Patient	77-year-old woman
Bone sample	Cortical bone from a femoral end
Cell culture	Explant culture of bone chips
T-antigen expression	Express *tsA* 209 T antigen at both 34°C and 39°C
Osteoblastic characteristics at 39°C	Do not proliferate
	Express collagen type I and III, osteocalcin, osteonectin, osteopontin, and TGF-β3 mRNAs
	Express type I collagen and CD44 proteins
	Express alkaline phosphatase and creatine kinase activities
	Secrete type I procollagen C-peptide and osteocalcin
	Secrete IGF-I, IGF-BP3, TGF-β1 and 2, IL-6, IL-1β, MCP-1, and PGE_2
	Respond to vitamin D_3, PTH, glucocorticoids, retinoids, TGF-β1, IL-1, TNF-α, and 17β-estradiol
	Form mineralized extracellular matrix
	Express functional estrogen receptors

IGF, insulin-like growth factor; IGF-BP, IGF-binding protein; TGF, transforming growth factor; IL, interleukin; MCP, monocyte chemoattractant protein; PGE, prostaglandin; PTH, parathyroid hormone; TNF, tumor necrosis factor.
*Proliferation was determined by cell counting; mRNA was measured by Northern hybridization or RT-PCR; proteins were quantified by Western blot, ELISA, or enzymatic activity; mineralized matrix was detected by alizarin red-S or von Kossa histochemical staining.

was specific, since 50- and 100-fold excess radioinert ERE successfully competed for the [^{32}P]-ERE (lanes 5 and 6), while a 45-bp radioinert nonspecific DNA fragment (which had the vitellogenin A2 promoter ERE mutated) did not compete (lanes 7 and 8). Finally, a monoclonal antibody to the human ER DNA-binding domain blocked the binding of the [^{32}P]-ERE to the nuclear extract (lanes 9 and 10), but a nonspecific antibody did not prevent this interaction (lanes 11 and 12). These data show that the observed DNA gel shifts were due to the interaction of the endogenous ER with the [^{32}P]-ERE.

Finally, the presence of functional ERs was demonstrated by the ability to transactivate adenovirus-ERE-tk-luciferase with 17β-E_2. As shown in Figure 9, 17β-E_2 upregulated ERE-tk-luciferase activity 4.9-fold in a dose-dependent manner when confluent cultures of cells were incubated in experimental medium for 48 h at 39°C. The range of luciferase induction from 12 separate experiments was 4.1 ± 1.3-fold, and functional ERs could be detected in this cell line up to 30 passages in culture. The EC_{50} of 17β-E_2 for the transactivation of ERE-tk-luciferase was 64 ± 16 pM (n = 12), and this activation was suppressed by co-incubation with 1,000-fold excess ICI-182,780, a specific steroidal anti-estrogen [Wakeling and Bowler, 1992]. However, non-estrogenic steroids like progesterone, dihydrotestosterone, and Dex did not have a significant agonistic effect on enzymatic activity (data not shown). When similar experiments were performed with MCF-7 cells, 1 nM 17β-E_2 upregulated adenovirus-ERE-tk-luciferase activity 18- to 20-fold (data not shown), which was again consistent with the relatively high level of ER mRNA and protein in these cells.

Endogenous Cellular Responses to 17β-Estradiol

As cited in the introduction, approximately 20 endogenous responses to 17β-E_2 have been reported in a dozen different osteoblast models. We evaluated the HOB-03-CE6 cells for 14 of these published responses to 17β-E_2, but for most of these the steroid did not have a significant effect or had only a slight effect (data not shown).

One of the effects of 17β-E_2 that we did observe in the cells was the suppression of PTH-stimulated cAMP production. As shown in Figure 10A, treatment of confluent cultures of cells in BSA-medium with 0.1–100 nM human PTH (fragment 1–34) for 10 min at 37°C stimulated a dose-dependent increase in intracellular cAMP production (2.7-fold with 10 nM PTH). This indicated that the cells contain functional PTH receptors, which is a characteristic of the osteoblast [Gehron Robey and Termine, 1985]. However, when the cells were pretreated with 10 nM 17β-E_2 for 48 h at 39°C, the ability of PTH to stimulate cAMP production was significantly reduced (1.4-fold with 10 nM PTH). This suppression appeared to be selective, since the stimulation of cAMP production by 100 nM PGE_2 (Fig. 10B) or 1 mM forskolin (Fig. 10C) was not significantly affected by the steroid.

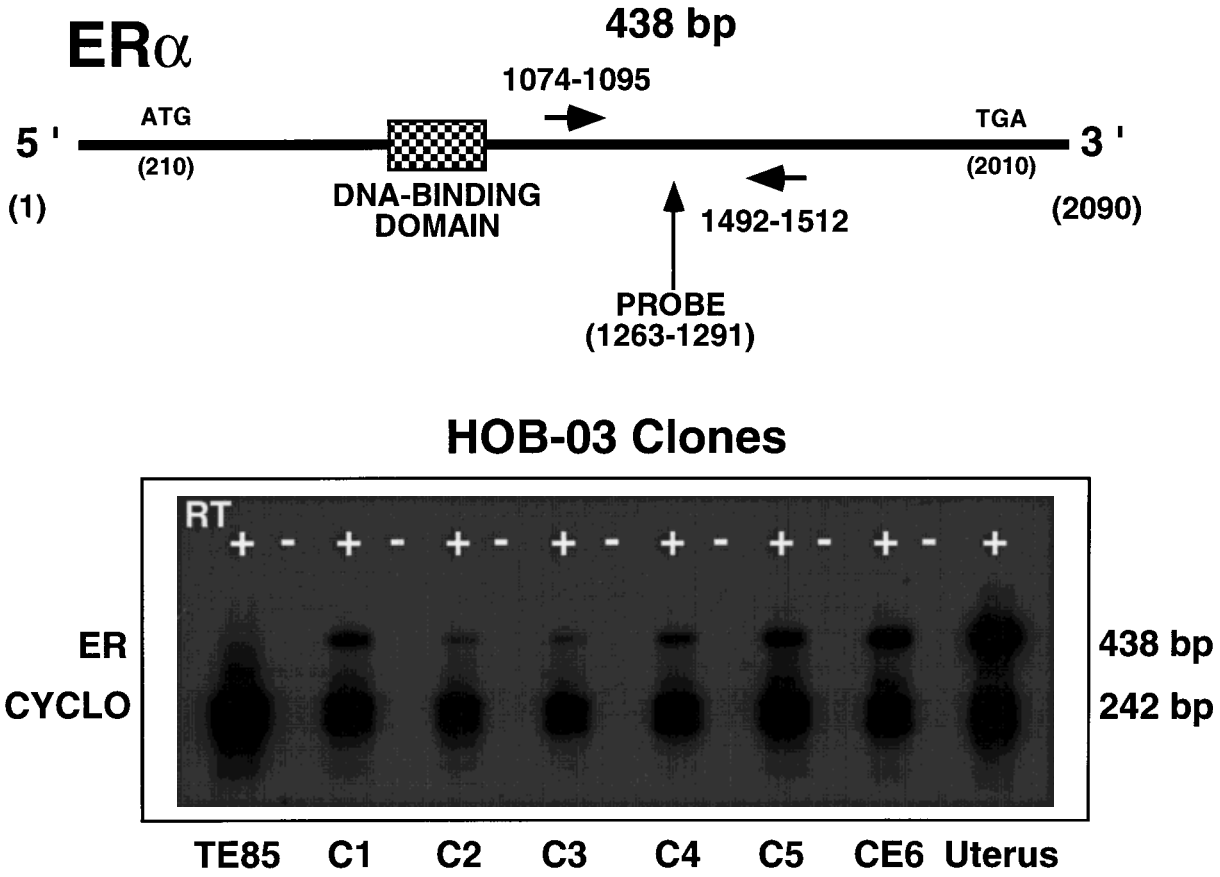


Fig. 5. RT-PCR analysis of HOB RNA for estrogen receptor-α message. Confluent flasks of HOB-03 clones (C1–C5 and CE6) were incubated for 2 days in growth medium at 39°C, and total RNA was isolated from the cells for RT-PCR, as described in Materials and Methods. RT-PCR products were electrophoresed on agarose gels, and Southern hybridizations were performed with [^{32}P]-labeled DNA probes for the 438-bp estrogen receptor (ER)-α cDNA fragment or the 242-bp cyclophilin (CYCLO) cDNA fragment. HOS-TE85 human osteosarcoma cell RNA (TE85) and rat uterine (Uterus) RNA were also analyzed. RT, reverse transcriptase; +, RT included in the RT-PCR reaction; −, RT left out of the reaction. The results are representative of two or more experiments.

We also examined the effects of 17β-E_2 on gene expression in these cells. Parameters reflecting cellular proliferation (histone H4), ECM production (type I procollagen), mineralization (alkaline phosphatase), and bone resorption (IL-6) were analyzed by Northern hybridization in response to 1–100 nM 17β-E_2. For these experiments, the cells were cultured as described in Figure 4 and then incubated with steroid for 24 hr in BSA-medium. As shown in Figure 11, 17β-E_2 had a slight stimulatory effect on the steady-state levels of histone H4 and collagen mRNAs. By contrast, 1 nM 17β-E_2 suppressed IL-6 expression >50%, although higher concentrations of steroid were less effective. Of those shown in Figure 11, the best response of 17β-E_2 in these cells was a dose-dependent 2.3-fold upregulation of alkaline phosphatase mRNA.

DISCUSSION

Although the expression of ERs by osteoblasts has been known for almost a decade [Komm et al., 1988; Eriksen et al., 1988; Kaplan et al., 1988], the in vitro models used to study estrogen action in these cells are potentially deficient for research on human diseases such as postmenopausal osteoporosis. For example, primary and transformed rodent cells have been used to examine the effects of estrogen on osteoblasts [Gray et al., 1987; Komm et al., 1988; Somjen et al., 1989; Ernst et al., 1989, 1991], but there are concerns about species differences between rodents and humans. Some human osteosarcoma-derived osteoblast-like cell lines appear to express low numbers of ERs [Komm et al., 1988; Etienne et al., 1990]; however, these cells do not display a normal phenotype

[Clover and Gowen, 1994] and exhibit a deregulated proliferation/differentiation relationship [Stein and Lian, 1993]. Likewise, an SV40 large T-antigen transformed human osteoblastic cell line (HOBIT) has been shown to be estrogen responsive [Keeting et al., 1992], but these cells also exhibit a deregulated proliferation/differentiation relationship, since the wild-type T antigen is constitutively active. Explant cultures of normal human osteoblasts possess functional ERs [Eriksen et al., 1988; Kaplan et al., 1988; Benz et al., 1991]. Although these cells are thought to display a normal phenotype [Gehron Robey and Termine, 1985], they are also nonclonal, nonimmortal, proliferate slowly, and express variable levels of ERs [Eriksen et al., 1988]. Normal human osteoblasts also exhibit variable responses to 17β-E_2 (e.g., regulation of IL-6 expression) [Girasole et al., 1992; Rickard et al., 1992; Chaudhary et al., 1992; Rifas et al., 1995]. Consequently, these properties limit the use of primary cells for detailed biochemical and molecular studies of estrogen action. Finally, as noted before, osteoblastic cell lines that overexpress the ER have also been developed [Migliaccio et al., 1992; Watts and King, 1994; Huo et al., 1995; Harris et al., 1995], but a concern about these cells is that a stably expressed receptor under the control of a viral promoter may not function the same as the endogenous ER [Watts and King, 1994].

Therefore, since the need still exists for an improved in vitro model to examine estrogen action on human bone cells, we set out to identify a conditionally transformed osteoblastic cell line that naturally expressed measurable levels of functional ERs. Our goal was to obtain clonal

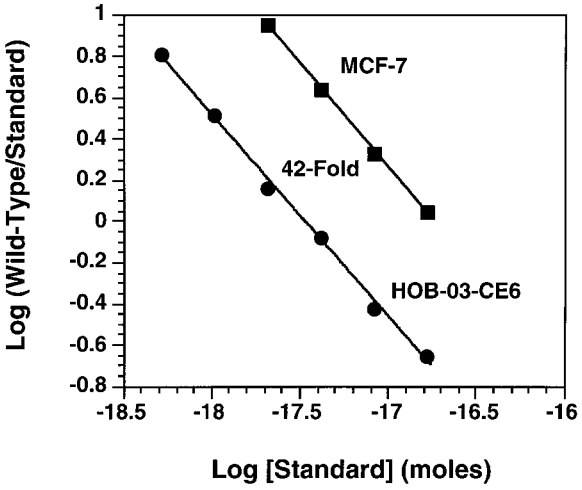
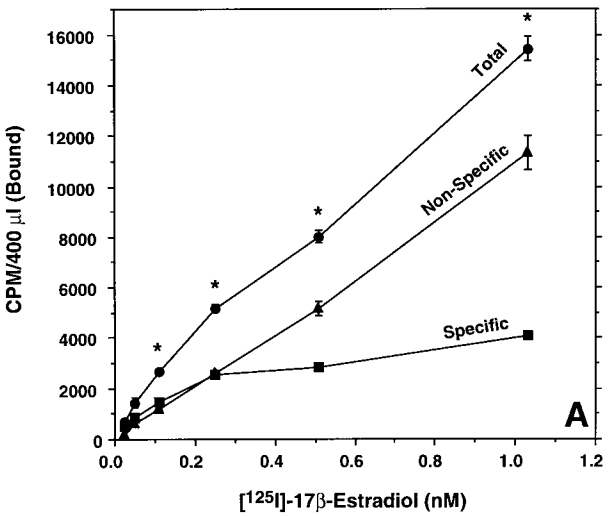


Fig. 6. Quantitative RT-PCR analysis of HOB-03-CE6 cell and MCF-7 cell RNA for estrogen receptor-α message. Confluent dishes of HOB-03-CE6 cells were incubated for 2 days in growth medium at 39°C, and total RNA was isolated from the cells for quantitative RT-PCR, as described in Materials and Methods. RT-PCR products were separated and quantified by HPLC. For comparison, RNA from confluent dishes of MCF-7 human breast cancer cells was also analyzed. The equations for the lines were as follows: MCF-7, $y = -1.00 \times -16.75$ ($R^2 = -0.999$); HOB-03-CE6, $y = -0.98 \times -17.17$ ($R^2 = -0.997$). These lines meet the theoretical expectations (i.e., slopes of −1.00), as established by Raeymaekers [1993].



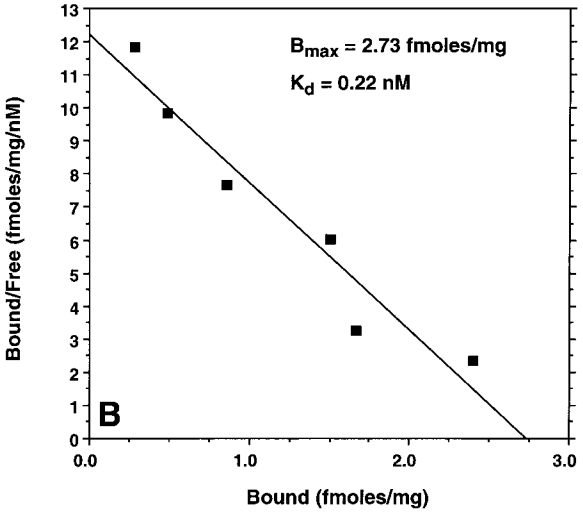


Fig. 7. [^{125}I]-17β-Estradiol binding by HOB-03-CE6 cytosol. **A:** Cytosol was prepared from confluent dishes of cells that were incubated for 2 days in growth medium at 39°C, and [^{125}I]-17β-E_2 binding was performed in the absence (total) or presence (nonspecific) of 1,000-fold excess diethylstilbesterol, as described in Materials and Methods. The results are presented as the mean ± SD, n = 3; *$P < 0.02$–0.001 from the corresponding nonspecific binding controls (Behren's–Fisher's t-test). Specific binding was observed in four separate experiments. **B:** Scatchard analysis of the data presented in panel A ($R^2 = -0.934$). The B_{max} from these data was 2.73 fmoles/mg (~1,200 receptors/cell), while the K_d was 0.22 nM.

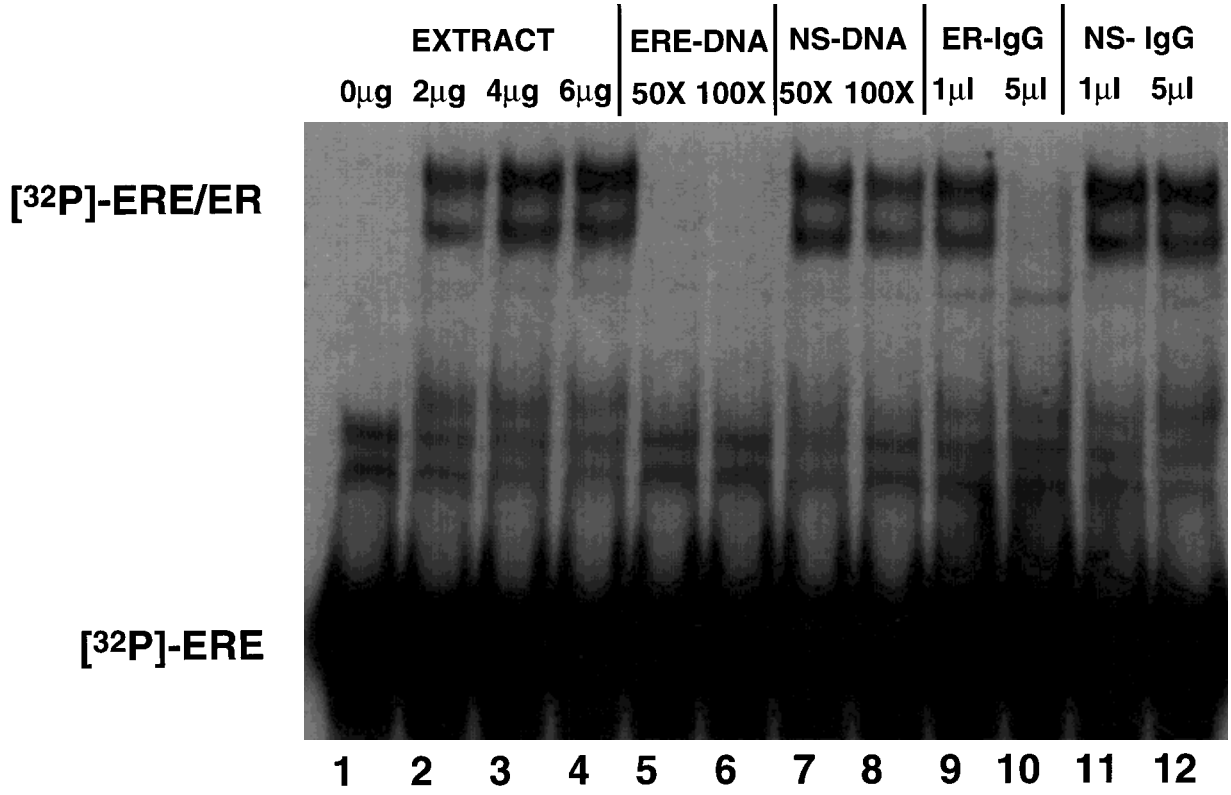


Fig. 8. DNA-gel shift analysis of HOB-03-CE6 nuclear extracts. Nuclear extracts were prepared from confluent dishes of cells that were incubated for 2 days in growth medium at 39°C, and DNA-gel shifts were performed with [^{32}P]-ERE (estrogen-responsive DNA element), as described in Materials and Methods. *Lanes 1–4,* increasing concentrations (0–6 μg) of nuclear extract + [^{32}P]-ERE; *lanes 5–8:* 4 μg of extract + [^{32}P]-ERE + excess (50- or 100-fold) radioinert ERE or nonspecific (NS)-DNA; *lanes 9–12:* 4 μg of extract + [^{32}P]-ERE + different concentrations (1 or 5 μl) of ER antibody (ER-IgG) or nonspecific antibody (NS-IgG). The results are representative of two similar experiments.

cells that exhibit a transformed phenotype under specific culture conditions and that will revert to a normal phenotype when these conditions are modified. To accomplish this, we infected normal human osteoblasts with adenovirus-*ori*-SV40 *tsA* 209 [Lei et al., 1992]. This hybrid virus encodes a temperature-sensitive mutant form of the large T antigen and was designed for optimal conditional-transformation of human cells [Chou, 1989]. Cells immortalized with this T antigen express a transformed phenotype at the permissive temperature (34°C) but revert to a nontransformed phenotype at the nonpermissive temperature (≥39°C). We used this procedure previously to develop a human osteoblastic cell line in the maturation stage of differentiation [Bodine et al., 1996a], as well as a human preosteocytic cell line [Bodine et al., 1996b].

After screening more than 60 conditionally immortalized human osteoblast cell lines for ER mRNA and functional ER protein, we identified the HOB-03-CE6 cells as being estrogen responsive. These cells appeared to be mature osteoblasts based on the in vitro developmental scheme described for primary rat calvaria-derived cells [Stein and Lian, 1993] and on results obtained with the HOB-02-C1 cells [Bodine et al., 1996a]. Like the HOB-02-C1 cell line, the HOB-03-CE6 cells did not proliferate at the nonpermissive temperature; expressed moderate levels of alkaline phosphatase activity; secreted moderate to high amounts of type I collagen and osteocalcin; responded to vitamin D_3, Dex, *all-trans* retinoic acid, TGF-β1, PTH, and PGE_2; and formed mineralized nodules. The cells also expressed mRNAs for other bone matrix proteins and secreted several growth factors and cytokines.

When compared to other HOB clones, the HOB-03-CE6 cells expressed relatively high levels of ERα mRNA by RT-PCR and possessed about 1,200 receptors per cell from [^{125}I]-17β-E_2 binding studies. Moreover, these ERs were functional as determined by [^{32}P]-ERE DNA-gel shift analyses and transactivation of adenovirus-

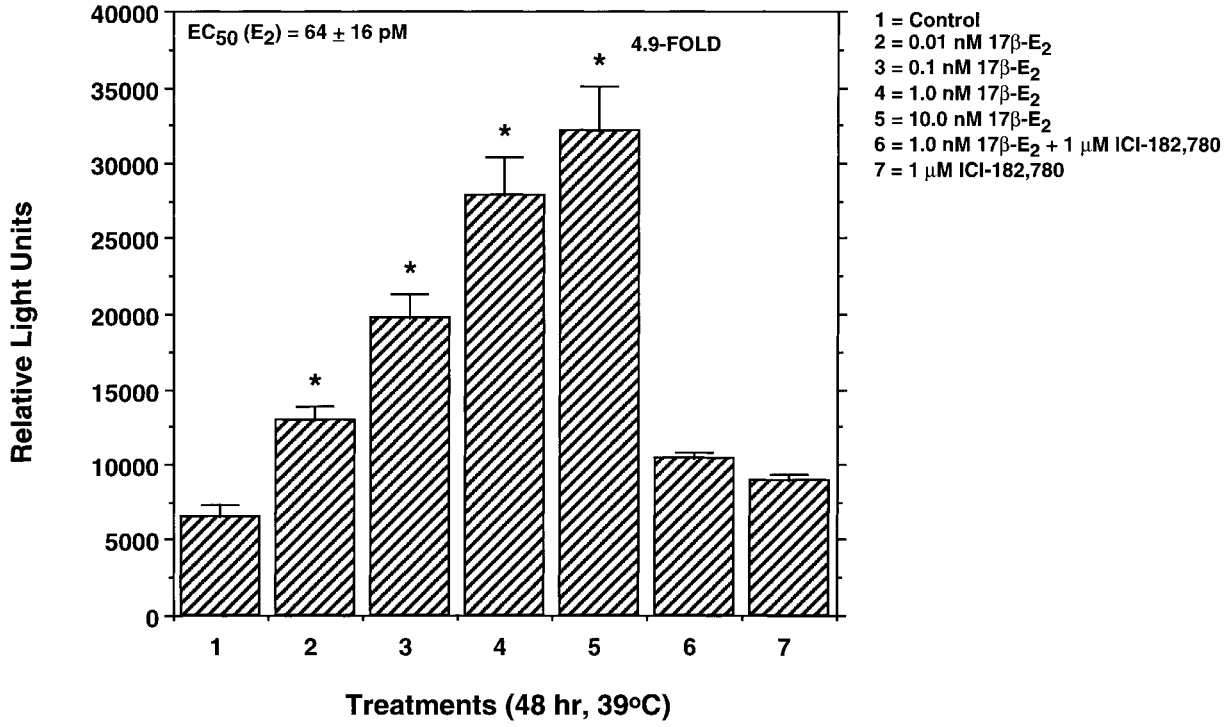


Fig. 9. Trans-activation of adenovirus-ERE-tk-luciferase by 17β-estradiol in HOB-03-CE6 cells. Confluent wells of cells were infected with adenovirus-ERE-tk-luciferase and treated for 48 h at 39°C with experimental medium containing either vehicle (control) or 0.01–10 nM 17β-E_2 in the absence or presence of 1 μM ICI-182,780. After treatment, the cells were assayed for luciferase activity, as described in Materials and Methods. The data are presented as the mean ±SD, n = 8. *$P < 0.01$–0.001 (Dunnett's ANOVA test). The EC_{50} from 12 independent experiments was 64 ± 16 pM.

ERE-tk-luciferase by 17β-E_2. Although this cell line contains more than 40-fold fewer ERs than MCF-7 cells [Kasid et al., 1984], we could still detect a three- to fivefold increase in ERE-tk-luciferase activity in response to 17β-E_2. Since this cell line was derived from an explant culture of cortical bone chips, these results imply that cortical as well as cancellous osteoblasts [Eriksen et al., 1988; Kaplan et al., 1988; Benz et al., 1991] are estrogen responsive. In fact, the gradual loss of cortical bone that occurs with age is known to be accelerated in postmenopausal women [Dempster and Lindsay, 1993].

One of the estrogenic responses we observed in the HOB-03-CE6 cells was the suppression of PTH-stimulated cAMP production. The regulation of PTH-responsiveness by 17β-E_2 has also been observed in several other in vitro osteoblast models. Oursler et al. [1991] showed that estradiol and PTH antagonized each other's ability to upregulate TGF-β1 expression in normal human osteoblasts. Estradiol was reported to suppress PTH-stimulated cAMP production in immortalized rat calvaria-derived osteoblasts and primary rat trabecular osteoblasts [Ernst et al., 1989], as well as human SaOS-2 osteosarcoma cells but not in ROS 17/2.8 rat osteosarcoma cells [Fukayama and Tashjian, 1989]. Monroe and Tashjian [1996] recently proposed that the mechanism of this suppression in SaOS-2 cells was to be due to a decrease in membrane-associated adenylate cyclase activity. However, this does not appear to be the mechanism of 17β-E_2-suppressed PTH action in the HOB-03-CE6 cells, since PGE_2- and forskolin-stimulated cAMP production were unaffected by the steroid. Estradiol has also been observed to inhibit PTH-stimulated PGE_2 production and bone resorption in cultured neonatal mouse calvariae [Pilbeam et al., 1989]. More recent work from Kaji et al. [1996] demonstrated that 17β-E_2 antagonized the stimulation of osteoclast formation by PTH in unfractionated mouse bone cell cultures, however the steroid did not block IL-1β, TNF-α, or PGE_2-stimulated osteoclast formation. Furthermore, the mechanism of this suppression appeared to involve an antagonism of the adenylate cyclase pathway and not the protein kinase C pathway. Although 17β-E_2 suppressed the production of

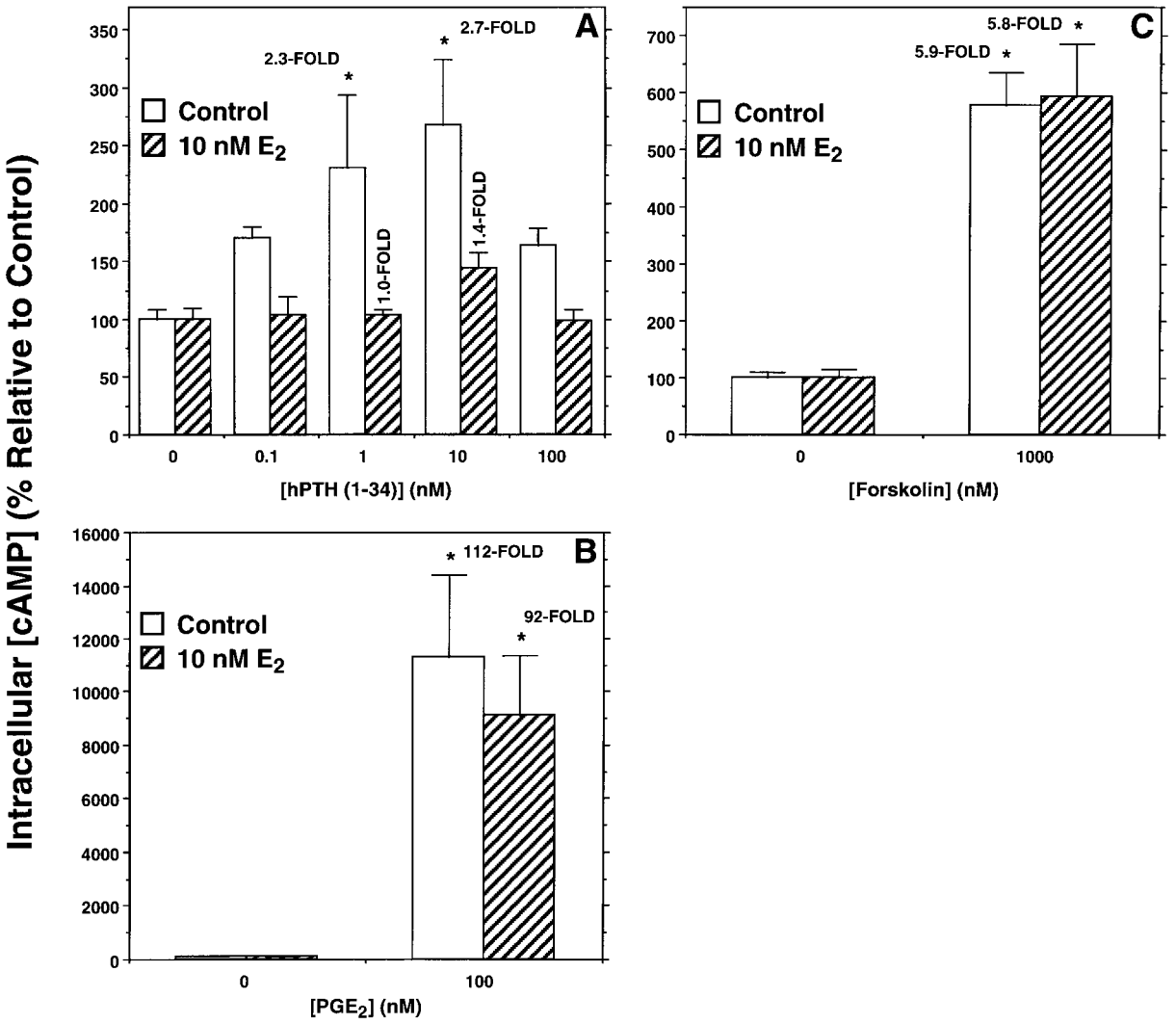


Fig. 10. Suppression of PTH-stimulated cAMP production in the HOB-03-CE6 cells by 17β-E_2. Confluent wells of cells were pre-incubated in BSA-Medium at 39°C in the absence (control) or presence of 10 nM 17β-E_2 for 48 h. The cells were pretreated for 5 min at 37°C with isobutylmethylxanthine (IBMX) and then co-treated with IBMX and 0.1–100 nM human parathyroid hormone (fragment 1–34) [hPTH (1–34)] (**A**), 100 nM prostaglandin E_2 (PGE_2) (**B**), or 1 μM forskolin (**C**) for 10 min at 37°C in the absence or presence of 17β-E_2. Intracellular cAMP was extracted and measured, as described in Materials and Methods. The results are presented as the mean ±SD, n = 4; *$P <$ 0.01–0.001 from the no treatment control (Dunnett's ANOVA test). The results are representative of four similar experiments.

a PTH-inducible osteoclast-differentiating factor(s) by SaOS-2 and MC3T3-E1 cells (immortalized mouse osteoblasts), the steroid also appeared to have a direct effect on PTH-stimulated osteoclast formation from hemopoietic blast cells. While most of the reports concerning the effects of 17β-E_2 on PTH action in osteoblasts involved a suppressive effect, in some instances the steroid also enhanced PTH-responsiveness. For example, in dexamethasone-treated SaOS-2 cells, estradiol and PTH potentiated each other's stimulatory effect on alkaline phosphatase activity [Rao et al., 1994], while in SaOS-2 cells as well as in primary rat and human osteoblasts, 17β-E_2 enhanced the ability of PTH to stimulate fibronectin production [Eielson et al., 1994]. Although these observations appear to be contradictory, the PTH/PTHrP-receptor is coupled to two signal transduction pathways in osteoblasts, and the ER may interact differently with these second messenger systems.

In addition to blocking PTH action, 17β-E_2 downregulated the basal expression of IL-6 mRNA in the HOB-03-CE6 cells. Estradiol has been reported to inhibit the induction of IL-6 expression by IL-1 and TNF-α in some human

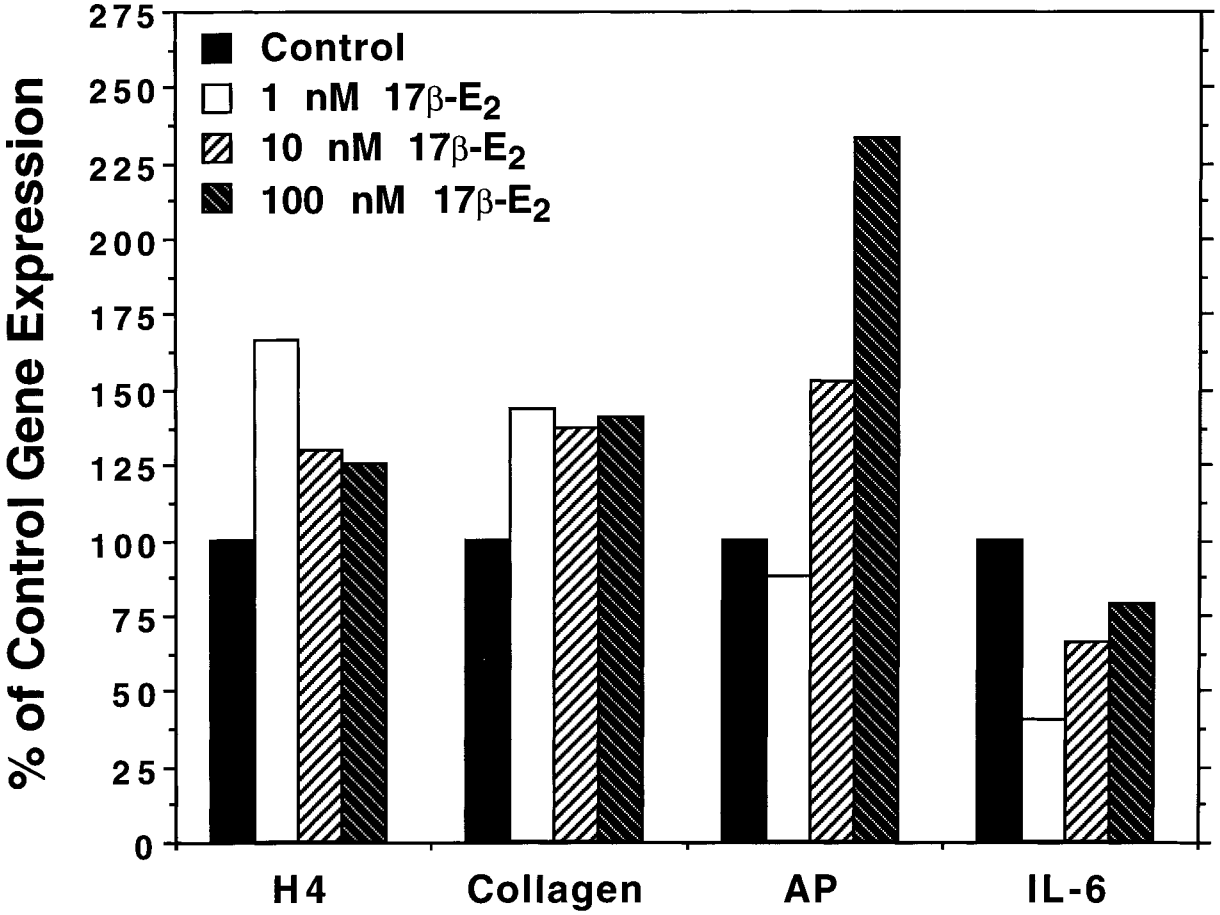


Fig. 11. Estrogen responsiveness of the HOB-03-CE6 cell line. Cells were cultured as described in Figure 4 and then treated for 24 h in BSA-medium at 39°C in the absence (control) or presence of 1–100 nM 17β-E_2. Total RNA was isolated and analyzed by Northern hybridization for histone H4 (H4), type I procollagen (collagen), alkaline phosphatase (AP), or interleukin-6 (IL-6), as described in Materials and Methods. The results were quantified by scanning densitometry and normalized to 18S ribosomal RNA; the data are expressed as the percentage relative to the control.

osteoblastic cell models [Girasole et al., 1992; Stein and Yang, 1995; Kassem et al., 1996], but suppression of basal gene expression is typically not observed. Suppression of IL-6 expression by estradiol is thought to mediate some of the inhibitory effects of the steroid on bone resorption [Manolagas and Jilka, 1995]. On the other hand, 17β-E_2 upregulated the steady-state levels of alkaline phosphatase mRNA in these cells. This is also a frequently reported effect of the steroid on a variety of mammalian osteoblastic cells [Gray et al., 1987; Bankson et al., 1989; Migliaccio et al., 1992; Majeska et al., 1994; Verhaar et al., 1994] and could lead to enhanced bone matrix mineralization.

In summary, transformation of human osteoblasts with a temperature-sensitive adenovirus-SV40 hybrid appears to be a valuable method to develop estrogen-responsive clonal cell lines. Although the presence of functional ERs could not be demonstrated in most of the conditionally immortalized cells, we did identify the HOB-03-CE6 cell line as a reliable in vitro model for the study of estrogen action. Since these cells express measurable levels of ERs, they can be used to explore the regulation of ER expression and activity in a human osteoblast.

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REFERENCES

Bankson DD, Rifai N, Williams ME, Silverman LM, Gray TK (1989): Biochemical effects of 17β-estradiol on UMR106 cells. Bone Miner 6:55–63.

Bellows CG, Aubin JE, Heersche JNM (1987): Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro. Endocrinology 121:1985–1992.

Benz DJ, Haussler MR, Komm BS (1991): Estrogen binding and estrogenic responses in normal human osteoblast-like cells. J Bone Miner Res 6:531–541.

Bodine PVN, TrailSmith M, Komm BS (1996a): Development and characterization of a conditionally transformed adult human osteoblastic cell line. J Bone Miner Res 11:806–819.

Bodine PVN, Vernon SK, Komm BS (1996b): Establishment and hormonal regulation of a conditionally-transformed pre-osteocytic cell line from adult human bone. Endocrinology 137:4592–4604.

Bradford M (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Chaudhary LR, Spelsberg TC, Riggs BL (1992): Production of various cytokines by normal human osteoblast-like cells in response to interleukin-1β and tumor necrosis factor-α: Lack of regulation by 17β-estradiol. Endocrinology 130:2528–2534.

Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli L (1994): Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Chou JY (1989): Differentiated mammalian cell lines immortalized by temperature-sensitive tumor viruses. Mol Endocrinol 3:1511–1514.

Cissel DS, Murty M, Whipkey DL, Blaha JD, Graeber GM, Keeting PE (1996): Estrogen pretreatment increases arachidonic acid release by bradykinin stimulated normal human osteoblast-like cells. J Cell Biochem 60:260–270.

Clark G (1981): Miscellaneous stains. In Clark G (ed): "Staining Procedures." 4th Ed. Baltimore: Williams & Wilkins, pp 187.

Clover J, Gowen M (1994): Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone 15:585–591.

Colston KW, King RJB, Hayward J, Fraser DI, Horton MA, Stevenson JC, Arnett TR (1989): Estrogen receptors and human bone cells: Immunocytochemical studies. J Bone Miner Res 4:625–631.

Cooper LF, Uoshima K (1994): Differential estrogenic regulation of small M_r heat shock protein expression in osteoblasts. J Biol Chem 269:7869–7873.

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.

Dempster DW, Lindsay R (1993): Pathogenesis of osteoporosis. Lancet 341:797–801.

Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assioan RK, Roberts AB, Sporn MB, Goeddel DV (1985): Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 316:701–705.

Eielson C, Kaplan D, Mitnick MA, Paliwal I, Insogna K (1994): Estrogen modulates parathyroid hormone-induced fibronectin production in human and rat osteoblast-like cells. Endocrinology 135:1639–1644.

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.

Ernst M, Rodan GA (1991): Estradiol regulation of insulin-like growth factor-I expression in osteoblastic cells: Evidence for transcriptional control. Mol Endocrinol 5:1081–1089.

Ernst M, Parker MG, Rodan GA (1991): Functional estrogen receptors in osteoblastic cells demonstrated by transfection with a reporter gene containing an estrogen response element. Mol Endocrinol 5:1597–1606.

Ernst M, Heath JK, Rodan GA (1989): Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 125:825–833.

Etienne MC, Fischel JL, Milano G, Formento P, Formento JL, Francoual M, Frenay M, Namer M (1990): Steroid receptors in human osteoblast-like cells. Eur J Cancer 26:807–810.

Fukayama S, Tashjian AH (1989): Direct modulation by estradiol of the response of human bone cells (SaOS-2) to human parathyroid hormone (PTH) and PTH-related protein. Endocrinology 124:397–401.

Gehron Robey P, Termine JD (1985): Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Girasole G, Jilka RL, Passeri G, Boswell S, Boder G, Williams DC, Manolagas SC (1992): 17β-Estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblastic cells in vitro: A potential mechanism for the antiosteoporotic effect of estrogens. J Clin Invest 89:883–891.

Gray TK, Lipes B, Linkhart T, Mohan S, Baylink DJ (1989a): Transforming growth factor beta mediates the estrogen induced inhibition of UMR106 cell growth. Connective Tissue Res 20:23–32.

Gray TK, Mohan S, Linkhart TA, Baylink DJ (1989b): Estradiol stimulates in vitro the secretion of insulin-like growth factors by the clonal osteoblastic cell line, UMR106. Biochem Biophys Res Commun 158:407–412.

Gray TK, Flynn TC, Gray KM, Nabell LM (1987): 17β-Estradiol acts directly on the clonal osteoblastic cell line UMR106. Proc Natl Acad Sci USA 84:6267–6271.

Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL, Spelsberg TC (1995): Estrogen response in the hFOB1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. J Cell Biochem 59:193–201.

Harris SA, Subramaniam M, Riggs BL, Spelsberg TC (1992): Estrogen induces c-*fos* promoter activity in normal human osteoblast-like cells. J Bone Miner Res 7:S94.

Hayward-Lester A, Oefner PJ, Doris PA (1996): Rapid quantification of gene expression by competitive RT-PCR and ion-pair reversed-phase HPLC. BioTechniques 20:250–257.

Hayward-Lester A, Oefner PJ, Sabatini S, Doris PA (1995): Accurate and absolute quantitative measurement of gene expression by single-tube RT-PCR and HPLC. Genome Res 5:494–499.

Huo B, Dossing DA, Dimuzio MT (1995): Generation and characterization of a human osteosarcoma cell line stably transfected with the human estrogen receptor gene. J Bone Miner Res 10:769–781.

Ikegami A, Inoue S, Hosoi T, Kaneki M, Mizuno Y, Akedo Y, Ouchi Y, Orimo H (1994): Cell cycle-dependent expression of estrogen receptor and effect of estrogen on proliferation of synchronized human osteoblast-like osteosarcoma cells. Endocrinology 135:782–789.

Ikegami A, Inoue S, Hosoi T, Mizuno Y, Nakamura T, Ouchi Y, Orimo H (1993): Immunohistochemical detection and northern blot analysis of estrogen receptors in osteoblastic cells. J Bone Miner Res 8:1103–1109.

Ishibe M, Nojima T, Ishibashi T, Koda T, Kaneda K, Rosier RN, Puzas JE (1995): 17β-Estradiol increases the receptor number and modulates the action of 1,25-dihydroxyvitamin D_3 in human osteosarcoma-derived osteoblast-like cells. Calcif Tissue Int 57:430–435.

Kaji H, Sugimoto T, Kanatani M, Nasu M, Chihara K (1996): Estrogen blocks parathyroid hormone (PTH)-stimulated osteoclast-like cell formation by selectively affecting PTH-responsive cyclic adenosine monophosphate pathway. Endocrinology 137:2217–2224.

Kaplan FS, Fallon MD, Boden SD, Schmidt R, Senior M, Haddad JG (1988): Estrogen receptors in bone in a patient with polyostotic fibrous dysplasia (McCune-Albright Syndrome). N Engl J Med 319:421–425.

Kasid A, Strobol JS, Huff K, Greene GL, Lippman ME (1984): A novel nuclear form of estradiol receptor in MCF-7 human breast cancer cells. Science 225:1162–1165.

Kassem M, Harris SA, Spelsberg TC, Riggs BL (1996): Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. J Bone Miner Res 11:193–199.

Keeting PE, Scott RE, Colvard DS, Anderson MA, Oursler MJ, Spelsberg TC, Riggs BL (1992): Development and characterization of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV40 large T antigen. J Bone Miner Res 7:127–136.

Klein-Hitpass L, Ryffel GU, Heitlinger E, Cato ACB (1988): A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with the estrogen receptor. Nucleic Acids Res 16:647–664.

Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M, Greene GL, O'Malley BW, Haussler MR (1988): Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. Science 241:81–84.

Lajeunesse D (1994): Effect of 17β-estradiol on the human osteosarcoma cell line MG-63. Bone Miner 24:1–16.

Leboy PS, Beresford JN, Devlin C, Owen ME (1991): Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol 146:370–378.

Lei K-J, Gluzman Y, Pan C-J, Chou JY (1992): Immortalization of virus-free human placental cells that express tissue-specific functions. Mol Endocrinol 6:703–712.

Liel Y, Kraus S, Levy J, Shany S (1992): Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D receptors in osteoblast-like cells (ROS 17/2.8). Endocrinology 130:2597–2601.

Lowry OH, Roberts NR, Wu M, Hixen WS, Crawford D (1954): The quantitative histochemistry of brain. II. Enzyme measurements. J Biol Chem 207:13–19.

Mahonen A, Maenpaa PH (1994): Steroid hormone modulation of vitamin D receptor levels in human MG-63 osteosarcoma cells. Biochem Biophys Res Commun 205:1179–1186.

Majeska RJ, Ryaby JT, Einhorn TA (1994): Direct modulation of osteoblastic activity with estrogen. J Bone Joint Surg 76:713–721.

Manolagas SC, Jilka RL (1995): Bone marrow, cytokines, and bone remodeling: Emerging insights into the pathophysiology of osteoporosis. N Engl J Med 332:305–311.

Masuyama A, Ouchi Y, Sato T, Hosoi T, Nakamura T, Orimo H (1992): Characteristics of steroid receptors in cultured MC3T3-E1 osteoblastic cells and effect of steroid hormones on cell proliferation. Calcif Tissue Int 51:376–381.

Migliaccio S, Davis VL, Gibson MK, Gray TK, Korach KS (1992): Estrogens modulate the responsiveness of osteoblast-like cells (ROS 17/2.8) stably transfected with estrogen receptor. Endocrinology 130:2617–2624.

Monroe JJ, Tashjian AH (1996): Pre-treatment with 17β-estradiol attenuates basal- and PTH-stimulated membrane adenylate cyclase in human osteoblast-like SaOS-2 cells. Biochem Biophys Res Commun 225:320–325.

Oursler MJ, Cortese C, Keeting P, Anderson MA, Bonde SK, Riggs BL, Spelsberg TC (1991): Modulation of transforming growth factor-β production in normal human osteoblast-like cells by 17β-estradiol and parathyroid hormone. Endocrinology 129:3313–3320.

Pauli U, Chiu JF, DiTullio P, Kroeger P, Shalhoub V, Rowe T, Stein G, Stein J (1989): Specific interactions of histone H1 and a 45 kilodalton nuclear protein with a putative matrix attachment site in the distal promoter region of a cell-regulated human histone gene. J Cell Physiol 139: 320–328.

Pilbeam CC, Klein-Nulend J, Raisz LG (1989): Inhibition by 17β-estradiol of PTH-stimulated resorption and prostaglandin production in cultured neonatal mouse calvariae. Biochem Biophys Res Commun 163:1319–1324.

Pivirotto LA, Cissel DS, Keeting PE (1995): Sex hormones mediate interleukin-1β production by human osteoblastic HOBIT cells. Mol Cell Endocrinol 111:67–74.

Raeymaekers L. (1993): Quantitative PCR: theoretical considerations with practical implications. Anal Biochem 214:582–585.

Rao LG, Wylie JN, Sutherland MSK, Murray TM (1994): 17β-Estradiol and parathyroid hormone potentiate each other's stimulatory effects on alkaline phosphatase activity in SaOS-2 cells in a differentiation-dependent manner. Endocrinology 134:614–620.

Rickard D, Russell G, Gowen M (1992): Oestradiol inhibits the release of tumor necrosis factor but not interleukin-6 from adult human osteoblasts in vitro. Osteoporosis Int 2:94–102.

Rifas L, Kenney JS, Marcelli M, Pacifici R, Cheng S-L, Dawson LL, Avioli LV (1995): Production of interleukin-6 in human osteoblasts and human bone marrow stromal cells: Evidence that induction by interleukin-1β and tumor necrosis factor-α is not regulated by ovarian steroids. Endocrinology 136:4056–4067.

Riggs BL, Melton LJ III (1992): The prevention and treatment of osteoporosis. N Engl J Med 327:620–627.

Scatchard G (1949): The attraction of proteins for small molecules and atoms. Ann NY Acad Sci 51:660–672.

Shalhoub V, Conlon C, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB (1992): Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. J Cell Biochem 50:425–440.

Somjen D, Weisman Y, Harell A, Berger E, Kaye AM (1989): Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone. Proc Natl Acad Sci USA 86:3361–3365.

Stein B, Yang MX (1995): Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-κB and C/EBPβ. Mol Cell Biol 15:4971–4979.

Stein GS, Lian JB (1993): Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblastic phenotype. Endocr Rev 14:424–442.

Suzuki S, Koga M, Takaoka K, Ono K, Sato B (1993): Effects of retinoic acid on steroid and vitamin D_3 receptors in cultured mouse osteosarcoma cells. Bone 14:7–12.

Turner RT, Riggs BL, Spelsberg TC (1994): Skeletal effects of estrogen. Endocr Rev 15:275–300.

Verhaar HJJ, Damen CA, Duursma SA, Schieven BAA (1994): A comparison of the action of progestins and estrogens on the growth and differentiation of normal adult human osteoblast-like cells in vitro. Bone 15:307–311.

Wakeling AE, Bowler J (1992): ICI 182,780, a new antioestrogen with clinical potential. J Steroid Biochem Mol Biol 45:173–177.

Watts CKW, King RJB (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.

Weiss MJ, Henthorn PS, Lafferty MA, Slaughter C, Raducha M, Harris H (1986): Isolation and characterization of cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. Proc Natl Acad Sci USA 83:7182–7186.