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Estrogen Response in the hFOB 1.19 Human Fetal Osteoblastic Cell Line Stably Transfected With the Human Estrogen Receptor Gene

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Abstract The gene coding for the human wild-type estrogen receptor (ER) was stably transfected into the human fetal osteoblastic cell line hFOB 1.19, a clonal cell line which is conditionally immortilized with a temperature sensitive mutant of SV40 large T antigen (ts*A58*). Five subclones were obtained which express various levels of ER mRNA and protein. The subclone with the highest level of functional (nuclear bound) ER, hFOB/ER9, contained 3,931 (±1,341) 17β-estradiol molecules bound/nucleus as determined by the nuclear binding (NB) assay. Using the dextran coated charcoal (DCC) method, the level of total cytosolic ER measured was 204 (±2) fmol/mg protein. This subclone was examined further for estradiol (E_2) responsiveness. The ER expressed in hFOB/ER9 cells was shown to be functional using a transiently transfected ERE-TK-luciferase construct. Expression of luciferase from this construct increased ~25-fold in hFOB/ER9 cells following 10^{-9}M E_2 treatment. This effect on ERE-TK-luciferase expression was both dose and steroid dependant. Further, treatment of hFOB/ER9 cells with 10^{-9}M E_2 resulted in a 2.5–4.0-fold increase in endogenous progesterone receptor (PR) levels detected by steroid binding assays, and a noticeable increase in both the A and B forms of PR by western blot assay. The establishment of this estrogen responsive human osteoblastic cell line should provide an excellent model system for the study of estrogen action on osteoblast function.

Key words: osteoblasts, estrogen receptor, stable transfection, SV40 large T antigen

INTRODUCTION

The beneficial effects of estrogen (E_2) in the treatment of bone loss in postmenopausal osteoporosis are well established [Riggs et al., 1972; Lindsay, 1981; Barzel, 1988]. However, the mechanisms involved in the direct action of E_2 on human bone cells remain unclear. Low levels of ER expression have been detected in cultured normal human osteoblastic (hOB) cells [Eriksen et al., 1988] as well as human osteosarcoma cell lines HOS TE85 [Komm et al., 1988] and SA OS [Etienne et al., 1990], and ROS 17/2.8 rat osteosarcoma cells [Komm et al., 1988]. Direct effects of E_2 on TGF-β gene expression in human osteosarcoma cells [Komm et al., 1988] and hOB cells [Oursler et al., 1991] have been observed. In addition, E_2 has been shown to affect type I procollagen gene expression in human osteosarcoma cells [Komm et al., 1988] and *c-fos* protooncogene expression in hOB cells [Harris et al., 1992]. Subtle effects of E_2 on alkaline phosphatase [Gray et al., 1987], type I procollagen [Ernst et al., 1989], insulin-like growth factor-I [Gray et al., 1989; Ernst et al., 1989], and 1,25-dihydroxyvitamin D_3 receptor levels [Liel et al., 1992] in rodent osteoblastic cell models have been reported. Recent studies involving peripheral blood monocytes indicate that elevated levels of interleukin-1 (IL-1) are secreted by cells from osteoporotic patients [Pacifici et al., 1989], suggesting that IL-1 may play a role in osteoporosis. Alternately, studies involving mouse model systems suggest that E_2 effects on interleukin-6 expression may play a pivitol role in bone physiology [Jilka et al., 1992; Passeri et al., 1993]. However, since bone remodeling only occurs to a very limited extent in older rodents, it is unclear whether E_2 effects on gene expression observed in rodent model systems can be extrapolated to humans. Other species specific differences may exist with regard to the cellular responses to E_2 treatment.

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In order to avoid the uncertainty associated with rodent osteoblastic cell systems with regard to their application to studying human osteoblast physiology, we developed the hFOB 1.19 human fetal osteoblastic cell line [Harris et al., 1995]. However, since hFOB 1.19 cells were found to express very low levels of ER, we developed subclones of hFOB 1.19 line which are stably transfected with an ER expression vector (hFOB/ER). This paper describes the hFOB/ER stable transfectants and their responses to E_2 treatment. These responses, which are expected in E_2 target cells, include increases in endogenous PR levels and upregulation of ERE-TK-luciferase expression following E_2 treatment of hFOB/ER cells. The hFOB/ER cells should be applicable for studies of estrogen effects on osteoblast gene expression and physiology.

MATERIALS AND METHODS

Materials

The phenol-guanidine isothiocyanate (TRI reagent) solution for RNA isolation was purchased from Molecular Research Center (Cincinati, OH), radiolabeled nucleotides and steroids, such as [α-^{32}P]-dCTP, [^{3}H]-17β-estradiol, and [^{3}H]-R5020 (PR agonist), were purchased from Dupont-NEN (Boston, MA). The cloning vector pBluescript SK and Quickhyb buffer were purchased from Stratagene (LaJolla, CA), and Geneticin from Gibco-BRL (Gaithersburg, MD). Tissue culture media, 10× trypsin-EDTA reagent, and unlabeled 17β-estradiol were purchased from Sigma Chemical (St. Louis, MO). Hygromycin B and fetal bovine serum was purchased from Flow-ICN (Costa Mesa, CA).

Vector Construction

The cDNA sequence coding for the wild-type human estrogen receptor was excised from the HEGO vector [Tora et al., 1989] using Eco RI. This 1.9 Kb fragment was ligated into the Eco RI site of the pBluescript SK vector and excised once again using Eco RV and Bam HI. This new fragment was ligated into the Eco RV and Bam HI sites of the expression vector p636. The p636 vector is a derivative of pHYG [Sudgen et al., 1985] containing the CMV promoter [Thomsen et al., 1984] inserted into the Cla I/Hind III sites, the SV40 polyadenylation signal (Bam HI/Bcl I fragment) inserted into the Bam HI site, and the hygromycin B resistance gene driven by the thymidine kinase promoter. The ER expression vector resulting from the insertion of the ER cDNA into the p636 vector was designated pHEGO-HYG (see Fig. 1 for plasmid map).

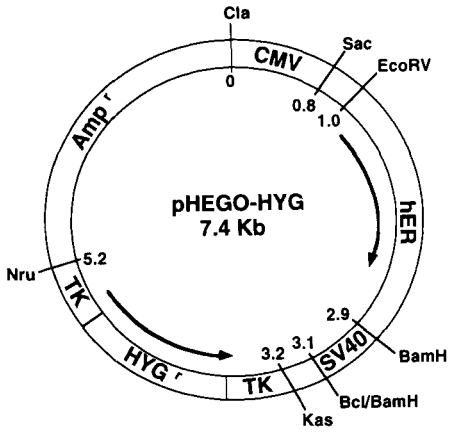

Fig. 1. Construction of the pHEGO-HYG vector. The human ER cDNA (hER) was inserted upstream of the CMV promoter and downstream of the SV40 polyadenylation signal. The direction of transcription for the ER and hygromycin resistance (HYG') genes are indicated with arrows. Selected restriction enzyme sites are denoted (Cla = Cla I, Sac = Sac I, BamH = BamH I, Bcl = Bcl I, Kas = Kas I, Nru = Nru I), as well as the map location (in kilobase pairs from the Cla I site).

Construction of the ERE-TK-luciferase vector was performed by digestion of pBLCAT2 [Luckow and Schutz, 1987] with Xba I and Xho I, yielding a 174 base pair fragment containing the TK promoter, which was ligated into the Nhe I/Xho I sites of the pGL2-basic luciferase expression construct (Promega, Madison, WI). This was followed by ligation of the ERE double-stranded oligonucleotide containing two copies of a consensus ERE into the Kpn I/Sac I sites of the TK-pGL2 vector. Each strand of the double-stranded ERE oligonucleotide contained oligonucleotides with the following sequences: 5'GGTCAGCGTGACCCGGGTCAGCGTGACCAGCT3', 5'GGTCACGCTGACCCGGGTCACGCTGACCGTAC3'.

Stable Transfection

Clonal hFOB 1.19 cells were transfected with the ER expression vector pHEGO-HYG (ATCC deposit No. 79994) by electroporation as described previously [Harris et al., 1995]. Briefly, 10 μg of pHEGO-HYG vector (linearized with Nru I) was added to the transfection mixture prior to electroporation. Transfected cells were plated in growth media (Dulbecco's modified Eagles medium [DMEM]/Ham F12 medium [F12] 1:1 v/v supplemented with 10% v/v charcoal stripped fetal bovine serum [csFBS]) and

incubated at 33.5°C for 48 h, then incubated in selective media containing 150 μg/ml hygromycin B for 7–10 days until resistant colonies were clearly visible. Resistant colonies (~200–400 cells) were trypsinized in glass cloning cylinders as described previously [Harris et al., 1995]. They were passaged and maintained in selective media containing 100 μg/ml hygromycin B until sufficient numbers of cells (~2×10^7) were obtained for cryopreservation. Routine growth conditions included incubation at 33.5°C and media changes every 2 days and changes to selective media containing 300 μg/ml Geneticin (G418-neomycin) instead of hygromycin B were performed every alternate media change to maintain neomycin resistance.

Transient Transfection

The ERE-TK-luciferase construct was transfected into hFOB/ER9 cells by the DEAE-dextran method of Lopata et al. [1984], except that the cells were incubated with the DNA/DEAE-dextran mixture for only 1 h and the DMSO shock was for 1 min. Briefly, the cells were grown to ~30% confluence in DMEM/F12 + 10%(v/v) csFBS, then pretreated for 48 h with 10^{-8}M ICI 182,780 to reduce the effect of residual E_2 in the csFBS. The cells were rinsed twice with HEPES buffered saline, once with serum free DMEM/F12 media, and incubated with the DNA/DEAE-dextran mixture (1 ml/100 mm plate) containing 2 μg of ERE-TK-luciferase vector and 0.2 μg of pHD101 CAT_3 (CMV-CAT) vector [Davis and Huang, 1988]. Following transfection, the cells were returned to DMEM/F12 + 10%(v/v) csFBS containing 10^{-8}M ICI 182,780 for 24 h. The cells were then rinsed twice with serum free DMEM/F12 and incubated in DMEM/F12 + 1%(v/v) csFBS with or without steroids for 48 h. After each treatment, the cells were harvested in TEN buffer (40 mM Tris 7.4, 1 mM EDTA, 150 mM NaCl) and the cell pellet was lysed in 1× reporter lysis buffer (Promega, Madison, WI). The concentration of protein in each cell lysate was determined by the Bradford method, and 20 μg of protein was analyzed for luciferase or chloramphenicol acetyltransferase (CAT) activity. Luciferase activity was measured following the addition of luciferase substrate buffer (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP) to each cell extract as specified by the manufacturer (Promega technical bulletin No. 161) and quantitating relative light units on a Turner model TD-20e luminometer. CAT activity was measured as specified by the manufacturer (Promega technical bulletin No. 84) following incubation of each cell extract for 2 h at 37°C in reaction buffer containing 0.13M Tris-HCl, pH 8.0, 0.25 μCi ^{14}C-chloramphenicol, and 25 μg n-butyryl coenzyme A substrate. The reaction buffer was extracted three times with Xylene and acetylated chloramphenicol was quantitated by scintillation counting. Luciferase activity in each cell extract was normalized to CAT activity as a control for transfection efficiency.

Steroid Binding Assays

Specific E_2 and Pg binding in hFOB/ER cells was measured using a micronuclear binding (NB) assay [Colvard et al., 1988; Eriksen et al., 1988] and the dextran-coated charcoal (DCC) assay [Thibodeau et al., 1981], which were performed as described previously. Briefly, hFOB/ER cells were grown to confluence in DMEM/F12 + 10% (v/v) csFBS. Then the cells were rinsed three times with serum free media (DMEM/F12 + 0.25% (w/v) BSA), treated with 10^{-9}M 17β-estradiol or ethanol vehicle in serum free media for 48 h, and treated again in fresh serum free media for another 48 h. Following the 96 h treatment period, the cells were rinsed three times with phosphate buffered saline (PBS), removed from the tissue culture flasks by trypsin/EDTA treatment, rinsed with 10 volumes of DMEM/F12 + 10% (v/v) csFBS medium at 4°C, and centrifuged at 900*g* for 10 min at 4°C. The cell pellet was rinsed with 10 mls of PBS and centrifuged at 900*g* for 10 min at 4°C again. The resultant cell pellets containing 4–8 × 10^6 cells for the nuclear binding assay or 40–60 × 10^6 cells for the DCC assay were incubated with radiolabeled steroids as described previously [Colvard et al., 1988; Eriksen et al., 1988; Thibodeau et al., 1981].

Northern Blot Analyses

Total RNA was isolated from hFOB/ER cells by phenol/guanidine isothiocyanate method of Chomczynski [1993], except that an additional extraction with 1 volume of chloroform was added after the phenol extraction. Purified RNA samples were denatured in glyoxal/dimethyl sulfoxide buffer and separated by glyoxal-agarose gel electrophoresis [McMaster and Charmichael, 1977]. The RNA was then blotted to nylon

filters by capillary diffusion in 20× SSC (3M NaCL, 0.3M sodium citrate, ph 7.0), and bound to nylon by vacuum baking at 80°C for 2 h. Hybridization was performed in a hybridization incubator at 65°C for 2 h in 10 ml Quickhyb buffer containing 100 μg/ml denatured calf thymus DNA. Each hybridization contained approximately 10^7 cpm (5–10 ng) of ^{32}P-labeled cDNA. Labeling of cDNAs was performed with a random primer labeling kit (Dupont-NEN, Boston, MA) as per the manufacturer's instructions using [^{32}P]-dCTP (3,000 Ci/mmol), and the labeled cDNAs were then purified by gel filtration chromatography. The northern blots were exposed to Kodak X-OMAT AR5 film with intensifying screens at −70°C then developed in a Kodak X-OMAT M20 film processor. Quantitation of band intensities were performed with a Shimadzu (Kyoto, Japan) CS 9000 flying spot scanning laser densitometer.

Western Blot Analyses

Cell extracts from vehicle control and E_2 treated hFOB/ER9 cells were prepared and analyzed by western blot analysis as described previously [Johnson et al., 1994], except that the primary antibody A/B-52 [Estes et al. 1987] was used for PR detection. Briefly, the cells were grown to confluence and treated with or without 10^{-9}M E_2 in serum free media for 96 h as described previously for the NB and DCC assays. The cells were than harvested in TEN buffer and 1/20th of the cells were lysed in 1× reporter buffer (Promega, Madison, WI) and the concentration of protein was measured using the Bradford assay. Cell extracts containing equal amounts of protein in SDS sample buffer were fractionated by gel electrophoresis, transferred to polyvinylidene difluoride membrane, and analyzed by Western blot assay.

RESULTS

Expression of ER mRNA and E_2 Binding Activity in hFOB/ER Subclones

Following stable transfection of the ER expression vector pHEGO-HYG (see Fig. 1 for plasmid map) into hFOB 1.19 cells, the hFOB/ER subclones were screened for expression of ER mRNA by northern analyses. Five subclones of hFOB/ER cells were found to express significant amounts of the expected 1.9 Kb ER mRNA (see Fig. 2). A broad range of ER steady state mRNA levels were expressed among the various hFOB/ER

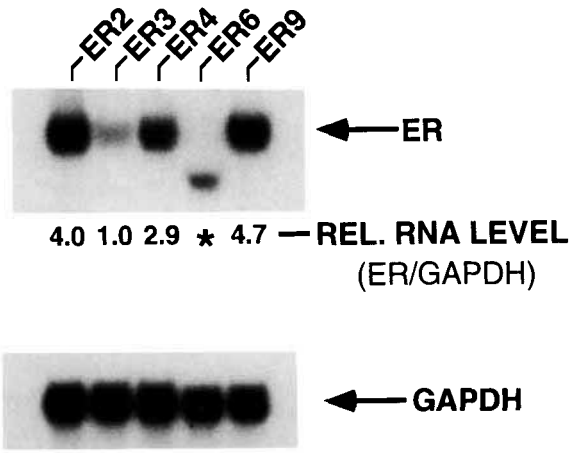

Fig. 2. Northern analysis of the hFOB/ER subclones. Total RNA (10 μg) isolated from the indicated hFOB/ER subclones was fractionated by glyoxal-agarose electrophoresis, blotted to a nylon filter, and hybridized with the ER and glyceraldehyde 3-dehydrogenase (GAPDH) cDNA probes. The ER mRNA steady state levels were determined by densitometry of the autoradiogram, normalized to GAPDH levels, and expressed as a ratio (ER/GAPDH) of relative mRNA levels (REL. RNA LEVEL). The asterisk indicates a truncated size ER mRNA.

subclones. The highest level of ER mRNA expression was in hFOB/ER9, which was ~5-fold more than the level of ER mRNA expression in hFOB/ER3, which had the lowest level. The hFOB/ER6 subclone expressed an aberrant size mRNA, so this subclone was not examined further.

To determine if the hFOB/ER cells exhibit functional receptors, we performed NB assays with each subclone. These data (see Table I) show that the number of E_2 nuclear binding sites in each subclone was proportional to the amount of ER mRNA expressed in each subclone, with the highest number of E_2 binding sites (3,931 ± 1,341) in hFOB/ER9, and the lowest number (825 ± 18) in hFOB/ER3. These levels of ER are consistent with the range of ER levels measured in hOB cells using the same NB assay [Eriksen et al., 1988]. It should be noted that clones ER2, ER4, and ER9 have relatively high levels of ER compared to the mean level (1,615 ± 411) reported for hOB cells [Eriksen et al., 1988].

Since the hFOB/ER9 cells contained the highest number of E_2 binding sites, this subclone was examined further for E_2 responsiveness. DCC assays performed using hFOB/ER9 cells indicated that the ER K_D = 0.15 nM at equilibrium, and that the level of ER was 204 (±2) fmol/mg cytosol protein in this subclone (see

TABLE 1. Nuclear Binding Assay Results

Subclone	Untransfected cells	ER2	ER3	ER4	ER9
Relative RNA level[a]	UD[d]	4.0	1.0	2.9	4.7
Relative E_2 binding[b]	< 0.2	3.7	1.0	3.7	4.8
Activated ERs per nucleus[c]	0-121[e]	3,076 ± 280	825 ± 18	3,082 ± 967	3,931 ± 1,341

[a]Arbitrary densitometry units from northern blots using ER/GAPDH ratio.
[b]Arbitrary units corresponding to relative amount of [^{3}H]-17β-estradiol molecules/nucleus with the lowest value (in ER3) set at 1.0.
[c]Number of bound 17β-estradiol molecules/nucleus; 1:1 stoichiometry for E_2:ER. Values are mean ± standard deviation (n = 4 determinations), except ER9 values are mean ± SEM (n = 4 NB assays).
[d]Undetectable with total RNA.
[e]Range of values from 4 NB assays. This range is below accurate detection limits.

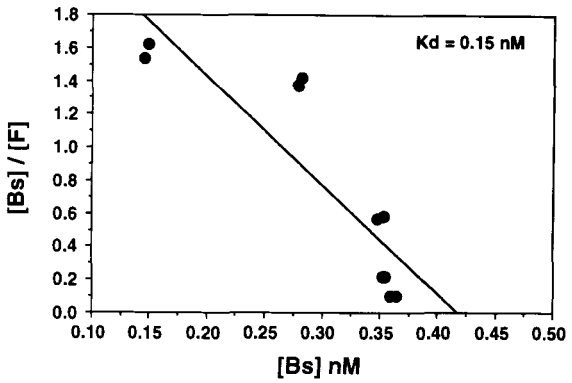

Fig. 3. Scatchard analysis of specific E_2 binding. Using the DCC assay, specific binding of ^{125}I-estradiol in hFOB/ER9 cell extracts was measured. Specific binding (Bs)/free (F) vs. specific binding are shown, and the calculated dissociation constant (K_d) is indicated. The amount of ER in the cell extract was calculated to be 204 (±2) fmol/mg cytosol from the concentration of ^{125}I-estradiol at saturation (0.417 nM) and the amount of cytosolic protein (2.04 mg) in the reaction.

Fig. 3). The measured K_D for the ER in this subclone is consistent with values measured in other osteoblastic cells, whereas the level of ER measured using this assay are quite high compared to hOB cells [Eriksen et al., 1988]. However, the DCC assay measures total E_2 binding in the cytosol, whereas the NB assay measures functional nuclear binding of E_2. Therefore, the NB assay probably provides a better estimate of the number of functional ER per cell.

Effect of E_2 on ERE-TK-Luciferase Expression

Since the hFOB/ER subclones were found to express ER which functionally bound E_2, it was also necessary to determine whether the expressed ER was capable of transcriptional activation. Expression from a transiently transfected ERE-TK-luciferase reporter construct was measured in hFOB/ER9 cells. This reporter construct contains two copies of a consensus estrogen response element (ERE), linked to the thymidine kinase (TK) promoter in a luciferase expression vector. Expression from the transfected ERE-TK-luciferase construct (see Fig. 4A) increased ~25-fold following treatment with 10^{-9}M E_2 relative to control (vehicle treated) cells. In contrast, the E_2 upregulation of luciferase expression was almost completely abrogated by cotreatment with 10^{-7}M ICI 182,780, a potent estrogen antagonist. In the parental hFOB 1.19 cell line, 10^{-9}M E_2 treatment had no effect luciferase expression.

To determine if the increase in expression from the ERE-TK-luciferase construct was dose and steroid dependent, transiently transfected hFOB/ER9 cells were treated with various doses of E_2 or other steroids. Luciferase expression (see Fig. 4B) did not significantly increase when the cells were treated with 10^{-11}M E_2, but dramatically increased (20–50-fold) in a dose dependant manner when treated with 10^{-10}–10^{-8}M E_2. In contrast, treatment of hFOB/ER9 cells with 10^{-7}M 4-hydroxytamoxifen (a partial E_2 agonist/antagonist) resulted in only a modest (~3-fold) increase in luciferase expression, and treatment with 10^{-7}M testosterone had no effect on expression. Interestingly, cells which did not receive the ICI 182,780 pretreatment had very high basal levels of luciferase expression, which was only modestly increased with 10^{-9}M E_2 treatment. This is not surprising, however, given that the wild type receptor has a higher affinity for estrogen than the mutant (HEO) form [Tora et al., 1989], and that charcoal stripped serum is suspected to contain residual amounts of E_2 [Tora et al., 1989]. In any case, the high basal levels of luciferase expression in cells without pretreatment were reduced dramatically (>90%) when the cells were treated with 10^{-7}M of the antiestrogen ICI 182,780.

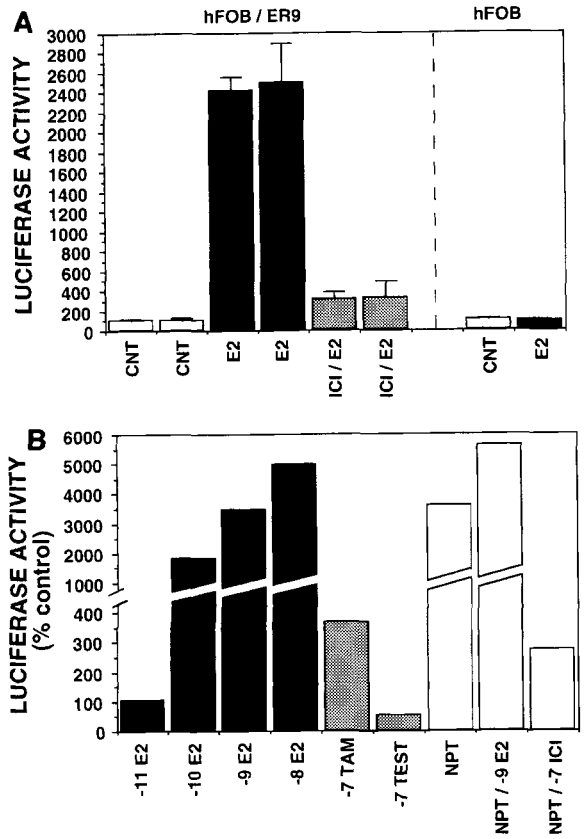

Fig. 4. Analysis of E_2 effects on ERE-TK-luciferase expression. The relative level of luciferase activity measured in transfected hFOB/ER9 cell extracts is shown. All experimental groups were pretreated for 48 h with 10^{-8}M ICI 182,780 to attenuate the effects of residual E_2 in csFBS, except for the NPT (not pretreated) groups. **A:** The luciferase activities are shown for hFOB/ER9 cells treated with vehicle control (CNT), 10^{-9}M E_2 (E2), or cotreatment with 10^{-7}M ICI 182,780 and 10^{-9} E_2 (ICI/E2), as well as hFOB 1.19 (hFOB) cells treated with vehicle control or 10^{-9}M E_2. The mean value (arbitrary units) from two seperate transfection experiments with hFOB/ER9 cells are shown with the error bars denoting standard deviation (n = 3). **B:** The luciferase activity relative to the vehicle control (100%) cell extracts are shown. The doses (in log molar) of E_2, 4-hydroxytamoxifen (TAM), testosterone (TEST), or ICI 182,780 (ICI) are indicated. The values from one representative transfection experiment out of four are shown. Using SAS linear regression statistical analysis of the data from all four experiments, luciferase activity increased linearly with doses of E_2 from 10^{-11}–10^{-8}M ($P < .001$), and from 10^{-10}–10^{-8} ($P < .03$).

Effect of E_2 on PR levels in hFOB/ER9

We have previously reported that E_2 treatment of normal human osteoblast-like (hOB) cells results in increased levels of endogenous PR expression [Eriksen et al., 1988]. To determine if endogenous PR expression is upregulated in E_2-treated hFOB/ER cells, NB, DCC, and Western blot assays were performed (as described in the Materials and Methods section) using the hFOB/ER9 subclone. These data (see Fig. 5) indicate that the number of progesterone (Pg) binding sites localized to the nucleus increased ~4-fold following treatment with 10^{-9}M E_2, as measured by the NB assay (see Fig. 5A). Further, this upregulation of PR expression following 10^{-9}M E_2 treatment was completely abolished by cotreatment with 10^{-7}M of the antiestrogen ICI 182,780. Using the DCC assay, the number of total Pg receptors increased ~2.5-fold following 10^{-9}M E_2 treatment (see Fig. 5B). In addition, Western blot analyses (see Fig. 5C) indicate that both the A and B forms of PR are undetectable in control (vehicle treated) hFOB/ER9 cells, whereas both forms of PR are easily detectable in cells treated with 10^{-9}M E_2.

DISCUSSION

There are a number of human osteoblastic cell systems which express ER, such as HOS TE85, SAOS, and hOB cells. However, the osteosarcoma lines proliferate rapidly in culture, but do not display contact inhibition or exhibit the full range phenotypic characteristics associated with normal osteoblastic cells. Alternately, hOB cells are phenotypically normal, yet proliferate very slowly, display contact inhibition, and can only be maintained in culture for a limited period. Therefore, the ability to study the effects of E_2 treatment on osteoblastic cells is somewhat limited by the lack of rapidly proliferating, yet phenotypically normal osteoblastic cell model. We previously reported the development and characterization of the human fetal osteoblastic cell line hFOB 1.19 [Harris et al., 1995]; a rapidly proliferating clonal cell line which is conditionally immortalized with a temperature-sensitive mutant of the SV40 large T antigen. The hFOB 1.19 cell line also exhibits the full range of osteoblastic phenotypic characteristics and has a significantly extended lifespan in culture.

Since hFOB 1.19 cells express negligible levels of ER (see Table I), the ER expression vector pHEGO-HYG expression vector was stably transfected into this cell line to facilitate the study of E_2 effects on ostoblast physiology. In the present study we report the development of several E_2-responsive human fetal osteoblastic (hFOB/ER) subclones. Further, the effects of E_2 treatment on PR and ERE-TK-luciferase expression in the subclone hFOB/ER9 are described.

Analyses of five hFOB/ER subclones by Northern blot and nuclear binding (functional recep-

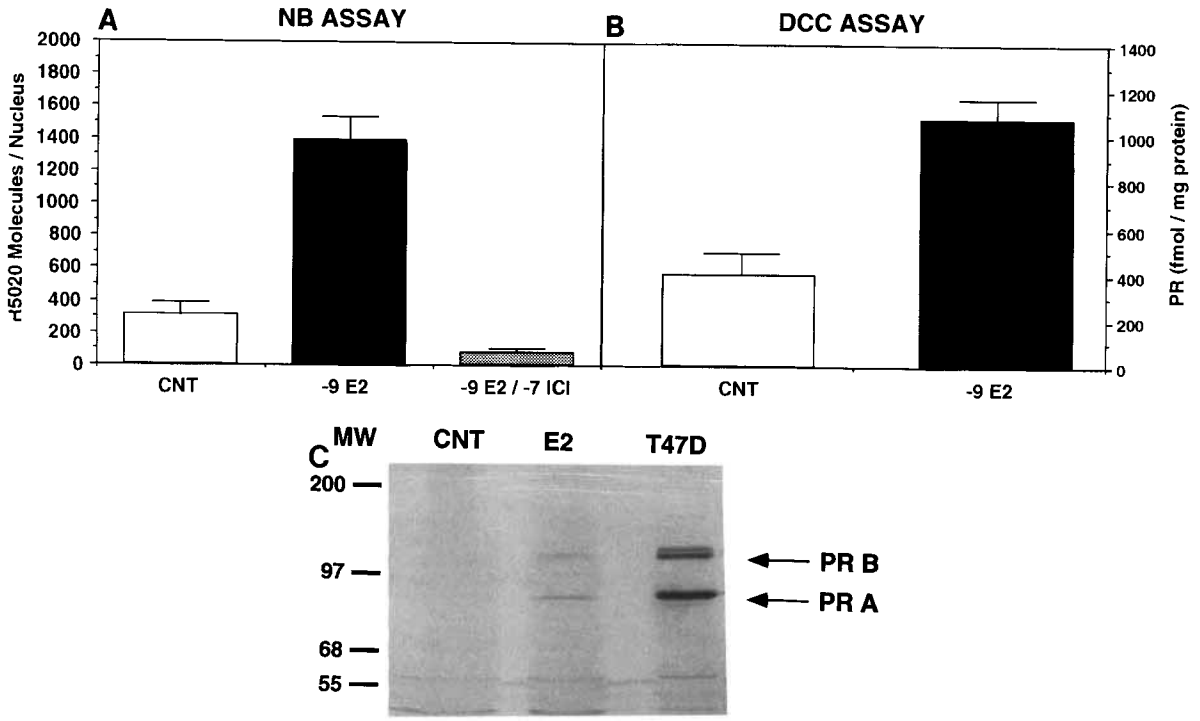

Fig. 5. Analysis of E_2 effects on PR levels in hFOB/ER9 cells. The amount of specific Pg binding was measured in control (CNT) or E_2-treated (E_2) hFOB/ER9 cells by the NB assay (**A**) or the DCC assay (**B**) as described in Materials and Methods. Control and E_2 treatments were ethanol vehicle and 10^{-9}M E_2, respectively, for 96 h. Each bar denotes the mean value from three separate assays ±SEM. Western blot analysis (**C**) was performed with 350 μg of cell extract from vehicle control (CNT) and 10^{-9}M E_2-treated (E2) cells as described in the methods section. Extracts from the positive control T47D breast tumor cell line (T47D) and molecular weight standards (MW) were included as well. The positions of the A and B forms of PR are indicated with arrows. A representative blot from three is shown.

tor) assays indicate that each of the lines contain different levels of ER expression. As expected, the amount of specific E_2 binding measured by the NB assays in each subclone was proportional to the steady state level of ER mRNA measured by northern analyses. The NB assay provides an estimate of the number of ERs which specifically and functionally bind E_2, whereas Northern analyses reflect the steady state level of ER mRNA which can be translated into ER. The data obtained from these experiments indicate that the ER mRNA transcribed from the stably transfected pHEGO-HYG vector is translated into ER protein which functionally binds E_2. Further, these hFOB/ER subclones express levels of ER which are consistent with the range of ER levels measured in hOB cells [Eriksen et al., 1988], whereas the parental hFOB 1.19 cell line expresses negligible levels of ER. While it is unclear why the parental hFOB cell line would express lower levels of ER than hOB cells, we have previously reported that the level of functional ER expressed in various strains of hOB cells varied widely from 0 to ~4,000 17β-estradiol molecules bound per nucleus [Colvard et al., 1989]. Thus, the clonal hFOB parental cell line may have been derived from a strain of osteoblasts which had particularly low ER expression. Alternately, the low levels of ER expression could be due the fact that the hFOB cells were derived from human fetal bone rather than adult bone.

Further analysis of E_2-responsiveness in the hFOB/ER9 clone, which expresses the highest level of ER, indicates that the expressed ER is capable of transcriptional activation. Specifically, E_2 treatment of hFOB/ER9 cells resulted in a dramatic increase in expression from a transfected ERE-TK-luciferase reporter construct, whereas in the parental hFOB 1.19 cell line luciferase expression did not increase. Further, the upregulation of ERE-TK-luciferase expression in hFOB/ER9 was both dose dependent and steroid specific.

Analysis of E_2 effects on endogenous gene expression in hFOB/ER9 cells indicates that PR

expression is upregulated following E_2 treatment. The level of PR in E_2-treated hFOB/ER9 cells increases ~4.0-fold as measured by the NB assay, and ~2.5-fold as measured by the DCC assay. The data from both assays show that PR levels increase following E_2 treatment, but, as expected, the extent of this increase in somewhat different in each assay since the two assays measure different aspects of the receptor. The DCC assay measures total Pg binding in the cytosol, whereas the NB assay measures functional (nuclear) Pg binding. Thus, E_2 treatment of hFOB/ER9 cells may affect functional Pg binding more than it affects total binding. Interestingly, in the ER-deficient osteosarcoma cell line HTB 96 (U2 OS), transfected with a ER expression vector, endogenous PR expression is not upregulated by E_2 treatment [Watts et al., 1989]. In contrast, stable transfection of an ER expression vector into rat fibroblast cell line (RAT1 + ER) results in the activation of the previously silent (repressed) PR gene [Kaneko et al., 1993]. Therefore, whereas the induction of PR gene expression is observed in hOB cells, as well as hFOB/ER and Rat1 + ER cells, expression of ER in HTB 96 osteosarcoma cells is not sufficient to induce PR expression or the expression of other endogenous genes. These data suggest that, in addition to ER, other gene products are required for transcriptional activation of endogenous genes by ER. Therefore, it appears that hFOB/ER cells express the necessary genes and the proper environment required to respond to E_2 through the regulation of E_2-responsive endogenous genes. In fact, recent unpublished studies indicate that the level of insulin-like growth factor binding protein-4 in hFOB/ER9 cell conditioned medium increases following E_2 treatment (R. Okazaki and B.L. Riggs, personal communication). Clearly, since hFOB/ER9 cells have relatively high ER levels, confirmatory data using other E_2-responsive cell lines or in vivo data would facilitate the interpretation of future findings regarding E_2 effects on osteoblast gene expression.

In summary, this paper describes the establishment of E_2-responsive human fetal osteoblastic (hFOB/ER) cells. The development of such a novel human cell line which is clonal, rapidly proliferating, and expresses the full range of osteoblastic phenotypic characteristics should provide an excellent model system in which to study the effects of E_2 treatment on osteoblast physiology, cytokine and growth factor production, and osteoblast differentiation.

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