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Estrogen Receptor Isoform-Specific Regulation of Endogenous Gene Expression in Human Osteoblastic Cell Lines Expressing Either ERα or ERβ

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Abstract Estrogen (17β-estradiol, E2) plays pivotal roles in the function and maintenance of the skeleton, including the bone-forming osteoblasts (OBs). The functions of E2 are largely mediated through two distinct estrogen receptor isoforms, ERα and ERβ, both of which are expressed in OBs. The level of each isoform dominates at early or late stages of OB differentiation. To date, only a limited comparison between the transcriptional targets of ERα and ERβ on endogenous gene expression has been reported. We have developed new stable cell lines, which contain doxycycline (Dox)-inducible ERα and ERβ, in the U2OS human osteosarcoma to determine the global transcriptional profile of ERα- and ERβ-regulation of endogenous gene expression. The U2OS-ERα and U2OS-ERβ cell lines were treated with Dox and either vehicle control or E2 for 24 h. Gene expression analysis was performed using a microarray containing ~6,800 full-length genes. We detected 63 genes that were regulated solely by ERα and 59 genes that were only regulated solely by ERβ. Of the ERα-regulated genes, 81% were upregulated and 19% were inhibited. Similarly 76% of the ERβ-regulated genes were upregulated whereas 24% were inhibited by E2. Surprisingly, only 17 genes were induced by both ERα and ERβ. Real-time PCR was employed to confirm the expression of a selected number of genes. The regulation of a number of known E2-responsive genes in human OBs, as well as many interesting novel genes, is shown. The distinct patterns of E2-dependent gene regulation in the U2OS cells by ERα and ERβ shown here suggest that during OB differentiation, when either isoform dominates, a unique pattern of gene responses will occur, partially due to the differentiation state and the ER isoform present. J. Cell. Biochem. 90: 315–326, 2003.

Key words: estrogen; ERα; ERβ; microarray; transcription; osteoblasts; RT-PCR

Estrogen is a key regulator in mediating important physiological processes in tissues, including uterus, breast, brain, and bone [Rickard et al., 2000]. The functions of estrogen are largely mediated through two distinct estrogen receptor (ER) isoforms, ERα and ERβ [Mangelsdorf et al., 1995; Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997]. Following estrogen binding, the ER functions to modulate gene expression, either by directly binding to specific response elements (i.e., estrogen response elements, EREs) or indirectly via protein–protein interactions in the regulatory regions of the estrogen-target genes. Activation of transcription by the ligand-bound receptor occurs through its association with specific transcriptional coactivators, such as the SRC/p160 family [Onate et al., 1995; Voegel et al., 1996; Li et al., 1997]. Both ERα and ERβ share significant amino acid homology within their ligand binding and DNA binding domains.

Abbreviations used: E2, 17β-estradiol; OB, osteoblast; ER, estrogen receptor; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; Dox, doxycycline; ERE, estrogen response element; SRC, steroid receptor coactivator; RT-PCR, reverse transcriptase polymerase chain reaction; LBD, ligand binding domain; BNB, biopsy nuclear binding; BSA, bovine serum albumin; PR, progesterone receptor; ALP, alkaline phosphatase; TGFβ, transforming growth factor β; vWF, von Willebrand factor; XIAP, X chromosome-linked inhibitor of apoptosis protein; ANOVA, analysis of variance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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However, significant differences between these ER isoforms exist in tissue specificity and in response to various ER agonists and antagonists [Paech et al., 1997].

Estrogen plays key roles in the development, metabolism, and overall homeostasis of the skeleton, which involves the regulatory actions of the bone forming osteoblasts (OB) and the bone resorbing osteoclasts [Waters et al., 2001]. Although there are many reports regarding the estrogen-regulation of specific genes in OBs [Rickard et al., 2000], little information concerning global transcriptional effects of estrogen in OBs is available. Furthermore, only a few reports exist on comparing the regulation of endogenous genes between ERα and ERβ in any estrogen-target cell. The aim of this study was to characterize the regulation of transcription of endogenous genes by the ER isoforms in a novel osteoblastic cell system expressing either ERα or ERβ using microarray technology as well as RT-PCR. This information becomes more pertinent with the report that the protein levels of both ERα and ERβ change during the differentiation of preosteoblastic cells to mature, bone-forming osteoblastic cells, resulting in a predominance of one of the isoforms [Arts et al., 1997; Onoe et al., 1997]. Our results demonstrate significant differences in the expression of specific endogenous genes between ERα and ERβ, including new estrogen-responsive genes in OBs. These data support that the particular isoform present as well as the state of OB differentiation will contribute to the unique pattern of estrogen-induced gene responses in OB cells.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human U2OS osteosarcoma cells were cultured in phenol red-free Dulbecco's Modified Eagle's medium (DMEM)/F12 media containing 10% (v/v) fetal bovine serum (FBS) supplemented with 1× antibiotic/antimycotic (Invitrogen, Carlsbad, CA). The U2OS stable transfectants containing either ERα or ERβ (described in the text) were cultured in the same media supplemented with 5 mg/L blasticidin S (Boehringer Mannheim, Indianapolis, IN) and 500 mg/L zeocin (Invitrogen). Charcoal-stripped fetal bovine serum (CS-FBS) was purchased from Hyclone Laboratories (Logan, UT). The pure ER antagonist ICI 182,780 was generously provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK), 17β-estradiol (E2) and doxycycline (Dox) were purchased from Sigma (St. Louis, MO), and [^{3}H]-E2 from DuPont-New England Nuclear (Boston, MA).

Development of Dox-Inducible ERα and ERβ Cell Lines

The T-REx™ System (Invitrogen) was used to generate ERα- and ERβ-inducible cell lines using the ER-negative U2OS osteosarcoma parental cell line. This system utilizes two vectors, the pcDNA6/TR© vector, a regulatory plasmid that expresses the tetracycline repressor (TetR), and pcDNA4/TO© which contains a CMV promoter driving the expression of a gene of interest under the control of Tet-operator sequences. In the absence of the Dox inducer, the TetR binds the Tet-operator sequences and silences transcription of the cloned gene. Addition of Dox to the culture media releases the TetR from the Tet-operator sequences allowing expression of the gene of interest.

The pcDNA6/TR© vector was linearized via *FspI* restriction digest and transfected into U2OS cells at a density of 50% using lipofectamine PLUS reagent (Invitrogen). Forty-eight hours later, the cells were split into standard media supplemented with 5 mg/L blasticidin S. Individual cell clones were isolated using a cloning cylinder and tested for induction of a pcDNA4/TO vector containing a cloned *luciferase* gene. One clone, which exhibited 348-fold Dox-inducibility of the *luciferase* gene, was chosen for future studies. Epitope (FLAG)-tagged ERα and ERβ receptors, cloned in the pcDNA4/TO© vector and described previously [Monroe et al., 2003], were linearized via *PvuI* restriction digest, transfected, and cloned as described above. The resultant cell lines expressing inducible ERα and ERβ were named U2OS-ERα and U2OS-ERβ, respectively.

Transfection Analysis of the U2OS-ERα and U2OS-ERβ Lines

The U2OS-ERα and U2OS-ERβ cell lines were tested for transcriptional function by transfecting 1 μg ERE-TK-LUC (an E2-responsive vector driving the production of firefly luciferase) and 1 μg pRL-TK (expressing Renilla luciferase purchased from Promega, Madison, WI) using methods described previously [Monroe et al., 2003]. The cells were treated with varying concentrations of Dox for 24 h, as

described in the text, and treated for an additional 24 h with either ethanol vehicle or 10 nM E2 in DMEM/F12 media containing 10% (v/v) CS-FBS. The cells were harvested and assayed using the Dual Luciferase Reporter System (Promega) and a Turner Design's 20/20 luminometer (Sunnyvale, CA). Promoter activity is described as the ratio of firefly luciferase to Renilla luciferase (expressed as relative light units; RLU).

Western Blot Analysis of the U2OS-ERα and U2OS-ERβ Lines

The U2OS-ERα and U2OS-ERβ cell lines were plated at a density of 50% in 10-cm culture dishes and treated with either 0, 50, or 100 ng/ml Dox for 24 h. Total protein extracts were prepared in RIPA buffer [1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) sodium dodecyl sulfate, and 1× protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN) in 1× phosphate buffered saline]. Equal amounts of protein (75 μg) were then analyzed by Western blot analysis. The proteins were detected using a α-FLAG-M2 antibody (Sigma) and visualized using enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Microarray Analysis

The U2OS-ERα and U2OS-ERβ cells were plated in 10-cm dishes at a cell density of 50% and treated with either ethanol control or 10 nM E2 for 24 h in duplicate. The total RNA was isolated using trizol reagent (Invitrogen) and 4 μg of RNA was used in microarray analysis on the HuGeneFL array (Affymetrix, Santa Clara, CA) containing probes for approximately 6,800 full-length human genes. Preparation of the labeled cDNA and microarray hybridization was performed by Microarray Core Facility at the Mayo Clinic (Rochester, MN). More information concerning the HuGeneFL array is available at the Affymetrix website (www.affymetrix.com).

Conventional and Real-Time RT-PCR Analysis

The U2OS-ERα and U2OS-ERβ cells were treated with either ethanol vehicle or 10 nM E2 for 24 or 48 h and total cellular RNA was harvested using trizol reagent (Invitrogen). The details of the real-time RT-PCR have been described previously [Monroe et al., 2003]. Reaction conditions for conventional RT-PCR analysis were identical to real-time RT-PCR analysis, except for the omission of Sybr Green (Molecular Probes, Eugene, OR) and the reduction of $MgCl_2$ concentration to 1.5 mM. The data is expressed relative to GAPDH for conventional RT-PCR and relative to β-actin for real-time RT-PCR. PCR primers (5′ and 3′, respectively) specific to each gene were: progesterone receptor, GACCAGATAACTCTCATTCAGTATTC and GTAAGTTGATAGAAACGCTGTGAGC; β-actin, TCACCCACACTGTGCCCATCTACGA and CAGCGGAACCGCTCATTGCCAATGG; pS2, GGCCCAGACAGAGACGTGTACAGTGG and GAGTAGTCAAAGTCAGAGCAGTCAATC; alkaline phosphatase (ALP), CCTCTCCAAGACGTACAACACC and CGGGAACGCTCAGTGGCTGCGC; osteocalcin, AGGGAGGTGTGTGAGCTCAATCCG and AGCAGAGCGACACCCTAGACCG; osteopontin, CCATACCAGTTAAACAGGCTGATTC and GAGATGGGTCAGGGTTTAGCCATG; von Willebr and factor, GATGGCTGTGATACTCACTTCTGC and CCCTCAGCCAGACACTTGTGTTC; mucin 1, GGCATCGCGCTGCTGGTGCTGG and GCCCGTAGTTCTTTCGGCGGCAC; cystatin S, AGTGGGTACAGCGTGCCCTTCAC and GAATTCACCAGGGACATTCTGTC; TIEG, GCCAACCATGCTCAACTTCG and TGCAGTTTTGTTCCAGGAATACAT; SA-1, ACACAATCCTCAGATGCAGATCTC and GATGGAGGCAGATCAATAACCATG; IAP-C, CCTGGATAGTCTACTAACTGCCGGA and GGTCGATCTGAAACATCTTCTGTGGG; angiotensinogen, CACTATGCCTCTGACCTGGACAAG and GTTAAGCTGTTGGGTAGACTCTGTG; and GAPDH [Rickard et al., 1998].

Proliferation Assay

The U2OS-ERα and U2OS-ERβ cell lines were seeded into 96-well plates at a density of 6,400 cells per well. Twenty-four hours later, the cells were treated with 100 ng/ml Dox (to induce either ERα or ERβ) for an additional 24 h in DMEM/F12 supplemented with 10% (v/v) CS-FBS. The cells were then treated (in triplicate) with E2 and/or ICI 182,780 at the concentrations indicated in the text and allowed to grow for 72 h. Dox and steroids were added fresh every 24 h to maintain the effective concentrations over the 72 h period. Twenty microlitres of the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) reagent was added to each well and allowed to incubate at 37°C for 30 min. The plate was read

at 490 nm on a SpectraMax 340 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) using the SoftMax Pro software (Molecular Devices Corp.).

Biopsy Nuclear Binding Assay

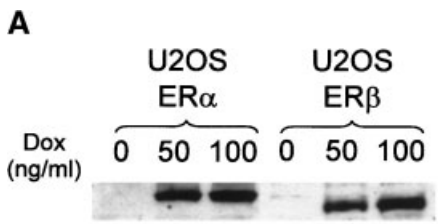
Specific E2 binding in the U2OS-ERα and U2OS-ERβ cells was determined using a biopsy nuclear binding (BNB) assay [Colvard et al., 1988; Eriksen et al., 1988; Harris et al., 1995]. Briefly, U2OS-ERα and U2OS-ERβ cells were grown to confluence and treated with 100 ng/ml Dox to induce either ERα or ERβ, respectively. The nuclei were then isolated by Dounce homogenization in nuclei buffer [10% glycerol (v/v), 0.1 Triton X-100 (v/v), 100 mM KCl, 50 mM Tris, 0.1% (w/v) BSA pH 7.4] and layered over nuclei buffer supplemented with 1.4 M sucrose. Following centrifugation, nuclei were incubated with [^{3}H]-E2 and non-specific binding was determined by incubation with the radiolabeled steroid plus a 100-fold molar excess of unlabeled steroid for 1 h at 4°C. The reaction was halted through addition of buffer [5 mM HEPES, 0.2 mM EDTA, 0.1% (w/v) BSA pH 7.4]. Quantitation of DNA and nuclear acceptor sites was determined using a microdiphenyl assay and scintillation counting, as previously described [Colvard et al., 1988].

RESULTS

Development and Characterization of ER-Expressing Osteosarcoma Cell Lines

To examine the individual actions of ERα and ERβ on E2-dependent transcription in OBs, a suitable cell line, stably expressing equivalent levels of either ERα or ERβ, was needed. The U2OS human osteosarcoma cell line was chosen mainly due to its OB-like properties, their rapid growth, ease of transfection, and the lack of detectable endogenous ER expression (data not shown). Therefore, we produced ER-expressing cell lines using the T-REx^{TM} System (Invitrogen), which allows control of the expression of the transgene using Dox as the inducer (see "Materials and Methods" for specifics). The resulting ERα and ERβ expressing lines were labeled as U2OS-ERα and U2OS-ERβ, respectively.

To assess the effectiveness of the Dox treatment on the cells, a titration of Dox was performed and protein levels of ERα and ERβ were analyzed using Western blot analysis. As shown in Figure 1A, optimal expression of both ERα and ERβ protein was observed at 100 ng/ml Dox, which was selected for the ER induction in subsequent studies. The appropriate comparison of the E2-dependent transcriptional profiles between the U2OS-ERα and U2OS-ERβ cell lines is dependent on relatively equal expression levels of the transgene between the two cell lines. As shown in Figure 1A, Western blot analysis suggests equivalent expression of ERα and ERβ in the two cell lines. The BNB assay, developed in our laboratory [Colvard et al., 1988; Eriksen et al., 1988; Harris et al., 1995], quantitates the number of functional receptors per nucleus based on the maximum number of receptors that are activated and bound to nuclear sites. Indeed, Figure 1B indicates using this BNB assay that the U2OS-ERα



B

Cell Lines	Mol / Nucleus
U2OS-ERα	8700 ± 1400
U2OS-ERβ	7300 ± 600
FOB/ERα9	3900 ± 1100
FOB/ERβ6	600
MCF7	3000

Fig. 1. Induction of ERα and ERβ protein and receptor quantitation. **A**: U2OS-ERα and U2OS-ERβ cells were treated with 0, 50, or 100 ng/ml of doxycycline (Dox) for 24 h prior to harvesting. Equal amounts of total cellular extract (75 μg) was separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Western blot analysis was performed using the α-FLAG-M2 antibody (Sigma) and visualized using enhanced chemiluminescence. The ERα and ERβ proteins were present at the expected size of approximately 66 and 54 kDa. **B**: Biopsy nuclear binding (BNB) assay was performed as described in "Materials and Methods." Receptor quantitation for the U2OS-ERα and U2OS-ERβ cell lines was performed in quadruplicate. All data are expressed as the mean ± standard error of molecules ER per nucleus. The experiment was repeated and similar results were observed. Data for the FOB/ERα9, FOB/ERβ6, and MCF7 cells are derived from the literature [Harris et al., 1995] and unpublished results.

and U2OS-ERβ cell lines express approximately 8,700 ± 1,400 and 7,300 ± 600 molecules (SEM) of ER per nucleus. Results of the BNB assay on three other cell lines expressing estrogen receptor (FOB/ERα9, FOB/ERβ6, and MCF7) are included for comparison.

To determine whether these cell lines support E2-inducible responses, an estrogen responsive promoter (ERE-TK-LUC) was transiently transfected into the U2OS-ERα and U2OS-ERβ cell lines. As seen in Figure 2A, an 8-fold induction of promoter activity was observed in both the U2OS-ERα and U2OS-ERβ cell lines following E2 treatment, confirming that these cells support similar levels of E2-dependent transcription from a transiently transfected reporter construct. To further demonstrate that these cells support appropriate E2-dependent gene regulation of endogenous genes in OBs, RT-PCR was performed on two known E2-responsive genes, progesterone receptor (*PR*) and *pS2* [Brown et al., 1984; Haslam and Levely, 1985; Rickard et al., 2002]. Indeed, similar inductions of PR and pS2 were observed in both the U2OS-ERα and U2OS-ERβ cell lines (Fig. 2B). These data support that ERα and ERβ regulate known OB E2-responsive endogenous genes and that these inductions are quantitatively similar between the U2OS-ERα and U2OS-ERβ cell lines.

To determine whether the proliferation of the U2OS-ERα and U2OS-ERβ cell lines is regulated by E2 as previously reported for OB cells, a cell proliferation assay was performed. As seen in Figure 3, treatment of E2 (10^{-8} or 10^{-7} M) resulted in a ~40% decrease in proliferation in the U2OS-ERα cell line, which is completely reversed by cotreatment with the pure ER-antagonist ICI 182,780. Interestingly, E2 had no significant effect on proliferation in the U2OS-ERβ cell line at any concentration. ICI 182,780 treatment alone resulted in an increase in proliferation in both the U2OS-ERα and U2OS-ERβ cell lines, when compared to vehicle treatment. This indicates that mere expression of either of the ER isoform has an E2-independent growth inhibitory effect that is

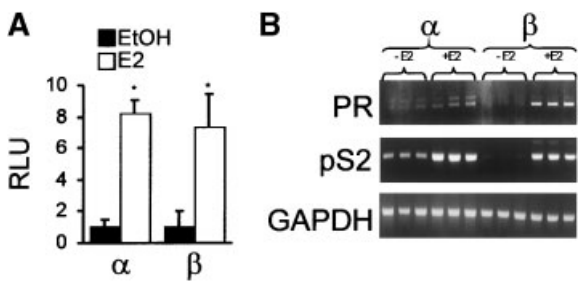


Fig. 2. The U2OS-ERα and U2OS-ERβ cell lines contain functional receptors. **A**: The U2OS-ERα (α) and U2OS-ERβ (β) cell lines at 50% confluence were cotransfected with 1 μg of ERE-TK-LUC and 1 μg of a Renilla luciferase, followed by 10 nM E2 and 100 ng/ml Dox for 24 h. The cells were harvested and assayed for luciferase activity. Luciferase values (relative light units, RLU) represent the mean ± the standard deviation and the asterisks represent significance at the $P < 0.001$ level (ANOVA) compared to the ethanol control for each cell line. **B**: The U2OS-ERα and U2OS-ERβ cells were treated with 10 nM E2 for 24 h and RNA was isolated. RT-PCR analysis was performed using primers specific for progesterone receptor (PR), pS2, and GAPDH. Each treatment group was performed in triplicate.

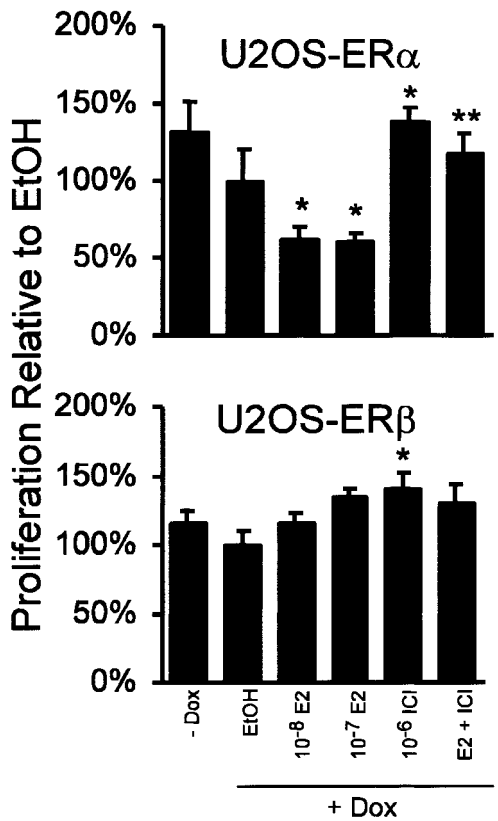


Fig. 3. E2 inhibits proliferation of the U2OS-ERα but not the U2OS-ERβ cell line. The U2OS-ERα and U2OS-ERβ cell lines were seeded in 96-well dishes and treated in triplicate with 100 ng/ml Dox and either E2 (10^{-8} or 10^{-7}), ICI 182,780 (10^{-6}), or a combination of E2 (10^{-8}) and ICI 182,780 (10^{-6}) for 72 h. The cell proliferation assay was performed as described in "Materials and Methods." The data is represented as the proliferation relative to the ethanol control (100%) and expressed as the mean ± the standard deviation. The single asterisk represents significance at the $P < 0.01$ level (ANOVA) compared to the ethanol control for each cell line. The double asterisk represents significance at the $P < 0.01$ level (ANOVA) compared to the E2-treated U2OS-ERα cell line. The experiment was repeated twice and a representative experiment is shown.

reversed by ICI 182,780 and thus is receptor-dependent. Overall, these results support that the growth inhibitory effects of E2 on the U2OS osteosarcoma are mediated through the ERα isoform and not the ERβ isoform.

Microarray Analysis of Estrogen-Regulated Genes

Identification of the array of genes regulated by E2 through ERα and ERβ in OBs would aid our understanding of the differential effects of E2 elicited through these two estrogen receptors. Although previous reports exist indicating ER isoform preference for select genes [Waters et al., 2001], a thorough characterization of the global transcriptional modulation by E2 between ERα and ERβ in an osteoblastic cell line has not been reported. Thus, we performed microarray analysis on the U2OS-ERα and U2OS-ERβ cell lines where the cells were treated with Dox for 24 h to induce the receptor, and then treated with E2 for an additional 24 h. The HuGeneFL (Affymetrix) microarray that contains approximately 6,800 full-length genes was used for this analysis. Modulation of gene expression in both U2OS-ERα and U2OS-ERβ cell lines was determined by comparison to a vehicle (ethanol) control for each cell line. Only those genes where one of the duplicates was regulated greater than 2-fold (induced or inhibited relative to the control) were included in the analysis to reduce the possibility of artifactual E2-regulation.

Using this stringent threshold value, Table I shows that a total of 80 genes were identified as ERα-regulated genes and 76 identified as ERβ-regulated genes. To better understand the regulation of endogenous gene expression by ERα and ERβ in an osteoblastic cell line, the E2 regulated genes were organized first by generalized function and then by the degree of E2-regulation (Tables II–IV). Of the ERα-regulated genes, 63 (79%) were specific to ERα (not regulated in the ERβ cell line), in which 51 (81%) were induced and 12 (19%) were inhibited by E2 treatment (Table II). Of the ERβ-regulated genes, 59 (78%) were specific to ERβ, in which 45 (76%) were induced whereas 15 (24%) were inhibited by E2 (Table III). Interestingly, Table IV shows that 17 genes are E2-regulated in both the U2OS-ERα and U2OS-ERβ cell lines (21 and 22%, respectively).

Validation of the accuracy of this microarray data was achieved through the confirmation of regulation for select genes using real-time RT-PCR. These genes were selected based on not only their high degree of regulation by estrogen but also their cellular functions in OB cells. ALP is a well-known E2-responsive osteoblast gene marker [Holinka et al., 1986]. Microarray analysis predicted E2-regulation of ALP expression in the U2OS-ERα cell line (Table II), which was confirmed by RT-PCR, with a 10-fold induction of gene expression observed following 48 h E2 treatment (Fig. 4A). Interestingly, a 3-fold increase in ALP expression by E2 treatment was detected by RT-PCR in the U2OS-ERβ cell line (Fig. 4A). The lower dynamic range of microarray analysis versus the extremely sensitive RT-PCR analysis is one possible explanation for the failure of the microarray to detect ALP-regulation in the U2OS-ERβ cell line. Two other classical bone markers, osteocalcin (Fig. 4B) and osteopontin (Fig. 4C), demonstrated modest increases, some of which were statistically significant in gene expression following 48 h E2 treatment in both cell lines (5-fold and 2-fold, respectively). Osteocalcin was not present in the microarray and osteopontin-regulation by E2 (2-fold) is below the dynamic range of the microarray assay.

The microarray also predicted the regulation of genes not known to be expressed in OBs. The RT-PCR analysis supported the microarray analysis demonstrating that von Willebrand factor (Fig. 4D) and the epithelial form of mucin 1 (Fig. 4E) are regulated 63- and 33-fold, respectively, by E2 in the U2OS-ERα cell line but not in the U2OS-ERβ cell line. Conversely, E2-dependent gene expression for the nuclear

TABLE I. Compilation of Gene Chip Data From the U2OS-ERα and U2OS-ERβ Cell Lines

	U2OS-ERα	U2OS-ERβ
Total no. of E2 regulated genes	80	76
Uniquely regulated genes	63 (79%; 63 of 80)	59 (78%; 59 of 76)
% Upregulated	81% (51 of 63)	76% (45 of 59)
% Downregulated	19% (12 of 63)	24% (14 of 59)
Commonly regulated genes	17 (21%; 17 of 80)	17 (22%; 17 of 76)

TABLE II. Genes Uniquely Regulated in the U2OS-ERα Cell Line

Gene	Accession no.	E2 reg (±range)
Bone genes		
Cartilage matrix protein	M55683	2.8 ± 0.2
Intestinal ALP	J03930	2.0 ± 0.4
Collagen type XVIIIα1	L22548	2.0 ± 0.2
Human ALP-1	J04948	1.7 ± 0.9
Parathyroid hormone-like protein	M24351	−2.1 ± 0.9
Parathyroid hormone-related protein	M17183	−1.8 ± 0.2
Cancer related/autoantigens		
Imogen 38	Z68747	2.9 ± 0.1
Glioma pathogenesis-related protein	U16307	2.9 ± 0.6
Deleted in colorectal cancer	X76132	2.3 ± 0.1
Pericentriol material-1	L27841	1.9 ± 0.4
Nuclear proteins		
Nuclear factor-interleukin 6β	M83667	4.0 ± 0.3
Serine-arginine domain protein, SRrp129	Y11251	3.2 ± 0.1
Retinoblastoma binding protein 1 isoform 1	S57153	2.9 ± 0.4
GATA-6	U66095	2.9 ± 0.8
Thyroid receptor interactor, TRIP8	L40411	2.4 ± 0.2
TRAF-interacting protein, I-TRAF	U59863	2.3 ± 0.4
Myocyte-specific enhancer factor 2	X68505	2.2 ± 0.6
Transcriptional activator hSNF2a	D26155	2.1 ± 0.0
Forkhead domain protein	U36922	1.9 ± 0.4
Homolog of yeast mutL, hPMS1	U13695	1.9 ± 0.9
XRCC4	U40622	1.9 ± 1.0
SnoA protein, ski-related	X15217	1.6 ± 0.6
Cockayne syndrome complementation group A protein, CSA	U28413	1.5 ± 1.0
Id1	S78825	−2.1 ± 0.1
Cardiovascular/hematologic		
Von Willebrand factor	M10321	4.9 ± 0.2
α-Myosin heavy chain	Z20656	2.6 ± 0.1
Factor XIII subunit A	M14539	2.5 ± 0.1
Proteoglycan core protein	X17042	2.4 ± 0.4
Vascular permeability factor	M27281	2.1 ± 0.9
Angiotensin type 2 receptor	U20860	−3.8 ± 1.0
Heparin binding protein	M60047	−3.1 ± 0.4
Metabolism/signaling		
Glut. Cyclotransferase	X71125	3.1 ± 0.1
Protein phophatase inhibotor 2	U68111	3.0 ± 0.3
Protein serine/threonine phosphatase 1β	X80910	2.3 ± 0.6
11-β-hydroxysteroid dehydrogenase type 2	U26726	2.0 ± 0.6
Galactokinase, GALK1	L76927	1.9 ± 0.6
Serine dehydratase	J05037	1.9 ± 0.2
Inositol polyphosphate 5-phosphatase	Z31695	1.9 ± 0.2
Inositol polyphosphate 4-phosphate type II-α	U96922	−2.5 ± 1.6
Protein tyrosine kinase, NET PTK	L40636	−1.9 ± 0.5
Cytokines/receptors		
Growth hormone-releasing hormone receptor	L01406	3.3 ± 0.1
Follistatin	M19481	2.3 ± 0.1
Activin β-A subunit	X57579	−2.4 ± 0.2
Cell cycle/apoptosis		
Nuc2 homolog	S78234	3.0 ± 0.1
Growth arrest specific protein, Gas	L13720	2.8 ± 0.1
XIAP associated factor-1	X99699	2.5 ± 0.2
Checkpoint suppresor 1	U68723	2.3 ± 0.3
Structural/transporters		
Epithelial mucin (splice variant 9)	J05582	4.1 ± 0.8
Oviductal glycoprotein	U09550	3.2 ± 0.4
Ig active epsilon1 5	L00022	3.1 ± 0.2
Na/Cl-dependent serotonin transporter	L05568	2.9 ± 0.0
Potassium channel Kv2.1	L02840	2.9 ± 0.8
Sperm membrane protein, BS-63	U64675	2.8 ± 0.1
Flavoprotein β subunit	X71129	2.6 ± 0.1
Lung amiloride sensitive Na channel	X76180	2.6 ± 0.1
Anti-hepatitis A IgG variable region	M87789	2.1 ± 0.2
Pancreatic mucin	J05582	1.9 ± 0.4
Neuronal kinesin	U06698	−2.1 ± 0.5
Plasminogen activator inhibitor type 1	X04729	−2.0 ± 0.4
Enhancer of filamentation, HEF1	L43821	−1.9 ± 1.0
Unidentified/unknown		
KIAA0137	D50927	3.6 ± 0.0
KIAA0259	D87448	2.2 ± 0.8
KIAA0073	D38552	1.8 ± 0.4

The E2-regulated genes in the U2OS-ERα cell line are functionally categorized and the accession number and degree of E2-regulation (±range) is given.

TABLE III. Genes Uniquely Regulated in the U2OS-ERβ Cell Line

Gene	Accession no.	E2 reg (±range)
Cancer related/autoantigens		
Melanoma differentiation associated gene-7	U16261	3.0 ± 0.1
p126	U15131	−2.2 ± 0.2
Nuclear proteins		
SA-1	Z75330	3.4 ± 2.1
E14/A-T proteins	X91196	2.4 ± 0.4
Deleted in colorectal cancer	S81294	2.4 ± 0.4
Complement component C3 α and β subunits	K02765	2.3 ± 0.0
GC box binding protein	D31716	2.0 ± 0.1
Regulator of non-sense transcript stability, RENT1	U65533	2.0 ± 0.5
E2F-5	U31556	−2.1 ± 0.1
Cardiovascular/hematologic		
Angiotensinogen	K02215	3.1 ± 0.1
Pancreatic elastase IIA	M16652	2.5 ± 1.2
Thrombospondin 2	L12350	2.3 ± 0.8
Hermansky–Pudlak syndrome protein	U65676	2.3 ± 0.0
Alpha-fetoprotein	V01514	2.1 ± 0.2
Thrombin receptor	M62424	−2.0 ± 0.0
Metabolism/signaling		
BDR-1, member of the recoverin family	D16227	3.5 ± 0.2
Ras-like	Z95624	3.5 ± 1.2
Estrogen synthetase	X13589	2.9 ± 0.6
3,5′ cyclic AMP phosphodiesterase	Z46632	2.9 ± 1.8
Carboxypeptidase M	J04970	2.8 ± 0.4
CDP-diacylglycerol synthase	U60808	2.8 ± 0.4
Guanylate cyclase	L13436	2.7 ± 0.1
Natural killer G2-A	X54867	2.7 ± 0.1
Tumor necrosis factor-inducible protein, TSG-14	M31166	2.6 ± 0.2
Sialyltransferase SThM	U14550	2.5 ± 0.3
Calbindin 27	M19878	2.5 ± 0.1
Lysyl oxidase	L16895	2.4 ± 0.4
Glycerol kinase	X68285	2.3 ± 0.5
Metalloproteinase	L23808	2.2 ± 0.1
Adipocyte lipid-binding protein	J02874	2.2 ± 0.2
GTP cyclohydrolase I	U19523	2.1 ± 0.3
Flavin-containing monooxygenase, FMO1	M64082	2.1 ± 0.1
CD33	M23197	1.8 ± 0.4
11-β-hydroxysteroid dehydrogenase	M76665	−2.2 ± 0.2
Rab28	X94703	−2.1 ± 0.1
COX6B	AC002115	−2.1 ± 0.1
Cytokines/receptors		
Lysophosphitidic acid receptor homolog	U80811	4.2 ± 0.0
B2-bradykinin receptor	X86163	2.7 ± 0.4
Adipophilin	X97324	2.3 ± 0.1
TGFβ2	Y00083	−3.0 ± 1.5
TGFβ2	M19154	−2.4 ± 0.8
Monocyte chemoattractant protein-4 precursor	U46767	−1.7 ± 0.9
Cell cycle/apoptosis		
Inhibitor of apoptosis protein homolog C	U37546	5.7 ± 0.4
MACH-β-1	X98176	2.3 ± 0.1
Progression associated protein	Y07909	−2.9 ± 0.1
Structural/transporters		
Intestinal mucin 3	M55405	6.1 ± 0.1
Heavy neurofilament subunit, NF-H	X15306	3.9 ± 0.1
pS2	X52003	3.4 ± 0.1
Dystrobrevin-ε	U46746	3.0 ± 0.5
Statherin	M32639	2.4 ± 0.4
Bullous phemphigoid antigen, BPAG1	M69225	2.1 ± 0.5
ABC3 transporter	U78735	2.0 ± 0.2
RING protein	Y07828	1.7 ± 0.8
Fibulin-2	X82494	−2.3 ± 0.4
Elafin	L10343	−1.9 ± 0.1
Unidentified/unknown		
C-rich secretory protein 2	X95239	3.0 ± 0.7
239FB mRNA	U57911	2.1 ± 0.1
MN1 protein	Z70281	−2.8 ± 0.4
Protein A alternatively spliced form 2	U47928	−2.3 ± 0.3

The E2-regulated genes in the U2OS-ERβ cell line are functionally categorized and the accession number and degree of E2-regulation (±range) is given.

TABLE IV. Genes Commonly Regulated in the U2OS-ERα and U2OS-ERβ Cell Line

Gene	Accession no.	U2OS-ERα	U2OS-ERβ
Cancer related/autoantigens			
Testis-specific protein	U58096	1.4 ± 0.8	1.8 ± 0.4
Nuclear proteins			
Pur-α	M96684	3.1 ± 0.0	2.6 ± 0.6
RIP140	X84373	1.7 ± 0.8	3.0 ± 0.1
Metabolism/signaling			
Protein kinase C β1	X06318	4.8 ± 2.0	1.7 ± 2.6
Pyruvate dehydrogenase kinase isoform 4	U54617	1.6 ± 0.6	2.9 ± 0.1
NF1 protein isoform, neurofibromin isoform	D12625	1.5 ± 1.1	2.5 ± 0.6
Cytokines/receptors			
Type 1 inositol 1,4,5-triphosphate receptor	D26070	2.2 ± 0.1	2.7 ± 0.4
Interleukin-8 receptor β	L19593	2.5 ± 0.6	2.6 ± 0.1
Cell cycle/apoptosis			
Inhibitor of apoptosis 1	U45878	1.4 ± 1.8	4.2 ± 0.9
Structural/transporters			
Cystatin S	X54667	3.2 ± 0.1	2.5 ± 0.1
Cystatin D	NM001900	3.0 ± 0.7	4.6 ± 0.1
Dopamine transporter	M95167	3.6 ± 0.2	2.3 ± 0.4
Dynein heavy chain	Z83806	1.5 ± 1.7	3.2 ± 0.2
Lectin-like type II integral membrane protein	L14542	2.7 ± 0.6	3.6 ± 0.4
GT mitochondrial solute carrier protein homolog	M31659	2.3 ± 0.8	1.3 ± 1.4
Unidentified/unknown			
Ig active ε-1,5 UP, VDJ region subgroup VH-I	L00022	3.1 ± 0.2	2.4 ± 0.1
Super cysteine-rich protein	U63332	1.3 ± 1.7	3.3 ± 1.3

The E2-regulated genes in the U2OS-ERα and U2OS-ERβ cell lines are functionally categorized and the accession number and degree of E2-regulation (± range) is given.

protein SA-1 (Fig. 4G), inhibitor of apoptosis protein homolog C (IAP-C; Fig. 4H), and angiotensinogen (Fig. 4I) appear largely specific to the U2OS-ERβ cell line, supporting the microarray analysis. Thus, an ER isoform-specific action of E2 is demonstrated for these genes. Cystatin S appeared strongly E2-regulated in both U2OS-ERα and U2OS-ERβ on the microarray (Table IV). Indeed, RT-PCR confirmed the microarray results demonstrating a 137- and 24-fold induction of cystatin S in the U2OS-ERα and U2OS-ERβ cell lines, respectively (Fig. 4F). A number of other genes from the microarray analysis were also confirmed using RT-PCR (data not shown), demonstrating that microarray analysis provides valid and accurate measures of E2-regulation in these cell lines, at least with a limited sensitivity greater than 2-fold.

DISCUSSION

Estrogen is an important regulator in the anabolic functions of OBs. Although both ERα and ERβ have been detected in OBs, few reports exist that differentiate between the contribution of the ER isoforms individually to the regulation of endogenous genes. Therefore, we developed inducible cell lines expressing ERα and ERβ in the U2OS human osteosarcoma cell line in order to measure ERα- and ERβ-dependent gene regulation in OBs on a large scale. In this study using a combination of microarray and RT-PCR, we have demonstrated that ERα and ERβ regulate largely unique patterns of gene expression in these newly developed U2OS-ERα and U2OS-ERβ osteoblastic cell lines. To limit the possibility of false positives due to the reported limited sensitivity of the microarray method [Shiffman et al., 2000; de Jong et al., 2002], only those genes where one of the duplicates was regulated at least greater than 2-fold on the microarray was included in the analysis.

Of the approximately 6,800 genes on the microarray, only 80 and 76 were regulated by E2 treatment above this stringent threshold value in the U2OS-ERα and U2OS-ERβ cell lines, respectively. More specifically, the data demonstrate that of the ER-regulated genes, roughly 80% of the total genes regulated in either the U2OS-ERα or the U2OS-ERβ cell lines are not regulated in the other cell line and thus are uniquely regulated. It is perhaps not surprising that large differences in gene expression between the ER isoforms exist, since E2 had large differential effects on cell proliferation in the ERα and ERβ expressing cell lines.

It is well accepted that microarray analysis exhibits a lower dynamic range of detection of

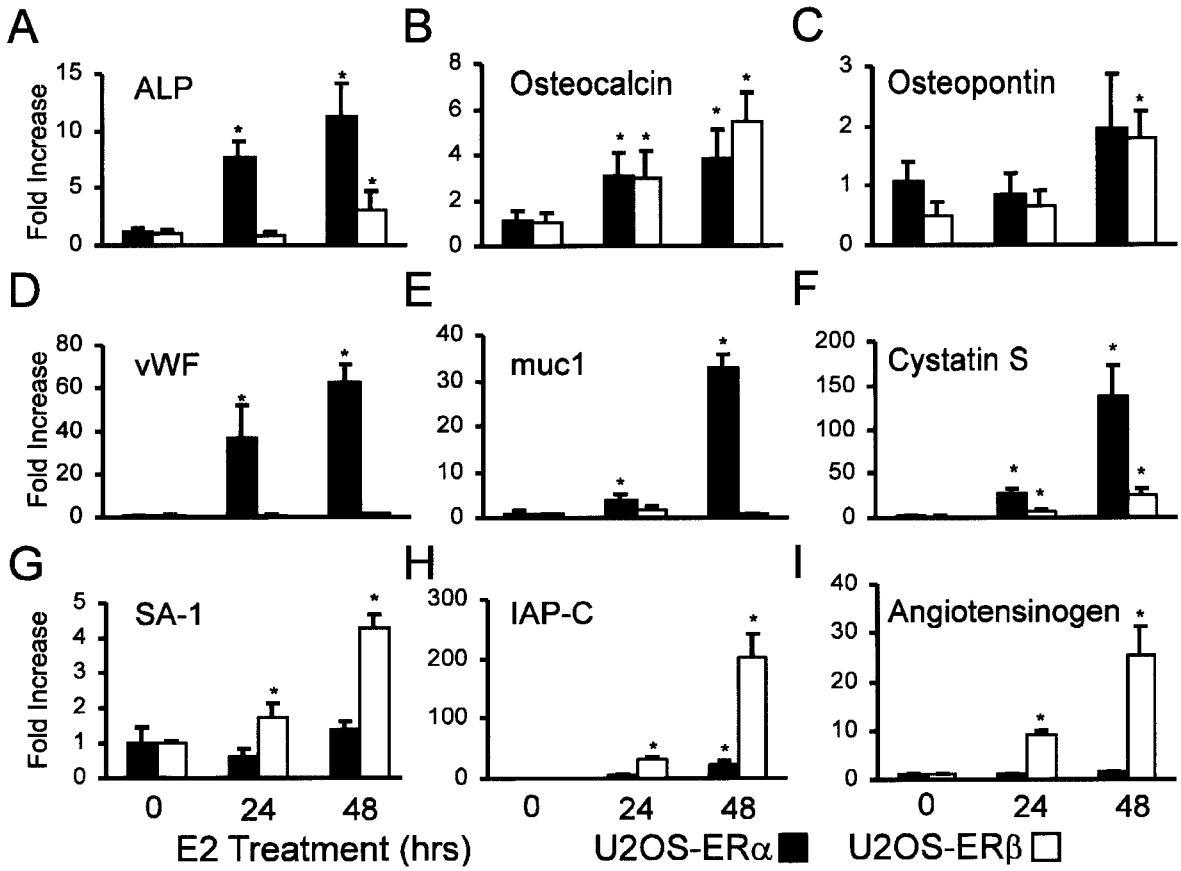


Fig. 4. Confirmation of E2-regulation of select genes from the microarray analysis. The U2OS-ERα and U2OS-ERβ cell lines were treated with 100 ng/ml Dox and either vehicle control or 10 nM E2 for 0, 24, and 48 h. Cells were harvested and real-time RT-PCR was performed using primers specific for (**A**) alkaline phosphatase (ALP), (**B**) osteocalcin, (**C**) osteopontin, (**D**) von Willebrand factor (vWF), (**E**) the epithelial form of mucin 1 (muc1), (**F**) cystatin S, (**G**) SA-1, (**H**) inhibitor of apoptosis protein homolog C (IAP-1), and (**I**) angiotensinogen. The *β-actin* gene was used as a control as described in "Materials and Methods." Data are expressed as the mean ± standard error. Each treatment group was performed in triplicate and the asterisks denote significance at the $P < 0.01$ level (ANOVA) compared to vehicle treated cells for each cell line. Each experiment was repeated at least twice and a representative experiment is shown.

gene expression when compared to the more sensitive RT-PCR analysis [Shiffman et al., 2000; de Jong et al., 2002]. Thus, to verify the microarray results with a more sensitive analysis of gene expression, select E2-regulated genes were analyzed using real-time RT-PCR analysis. For example, the regulation of ALP, a known E2-responsive gene [Holinka et al., 1986], was observed as E2-regulated on the microarray and was successfully confirmed using real-time RT-PCR. Other bone-related genes, such as cartilage matrix protein and one type of collagen, were also identified as E2-regulated on the microarray. Similarly, cytokines having known effects in bone and produced by OB cells, such as parathyroid hormone-related protein and TGFβ [Mundy, 1997], were also shown to be E2-regulated on the microarray, confirming previous reports from our laboratory and by others [Oursler et al., 1996; Robinson and Spelsberg, 1997; Khosla et al., 1999]. The regulation of a number of other genes on the microarray described in this report correlate well with the confirmatory RT-PCR analysis (data not shown). These data demonstrate that our microarray analysis successfully identified E2-responsive expression profiles of a number of genes whose regulation by E2 is known in OBs and other genes whose regulation by E2 is unknown. However, due to its lowered sensitivity, microarray analysis should only be considered a qualitative indicator

of gene regulation and useful in the identification of potential E2-responsive genes for further study.

Interestingly, a number of cardiovascular/hematologic-related genes displayed a marked regulation by E2 in both the U2OS-ERα and U2OS-ERβ cell lines. For example, the gene expression profile for von Willebrand factor (vWF) exhibited a strong 4.9-fold induction by E2 in the U2OS-ERα cell line on the microarray analysis, which was confirmed by real-time RT-PCR. It is curious why vWF is expressed in this osteosarcoma and is regulated by E2. vWF is a large secreted protein involved in blood clotting through interaction with factor VIII [Budde and Schneppenheim, 2001]. Since vWF is known to associate with several types of collagen during clotting [Budde and Schneppenheim, 2001], a possible function of vWF in bone is in the bone extracellular matrix, which contains abundant collagen. A number of other cardiovascular/hematologic-related genes such as factor XIII subunit A, vascular permeability factor, angiotensinogen type 2 receptor, and heparin binding protein were also regulated by E2 in the U2OS-ERα cell line. Gene expression profiles for angiotensinogen and thrombin receptor were regulated by E2 in the U2OS-ERβ cell line. Regulation of these cardiovascular/hematologic-related genes in this osteosarcoma may reflect the necessity for increased bone vascularization during active bone remodeling or may be involved in the revascularization during bone fracture healing. However, further study is needed to properly identify the role of these genes in osteoblastic cell growth and function.

In conclusion, these novel ER isoform expressing cell lines display E2-regulation that is comparable with normal OB cells. The microarray analysis demonstrates that the majority (80%) of E2-regulated genes in either the U2OS-ERα or U2OS-ERβ cell lines are uniquely regulated in that particular cell line, indicating that the transcriptional effects of E2 via either ERα or ERβ are largely distinct in OBs. Furthermore, RT-PCR supported the microarray data at least within the limits of sensitivity of the microarray. This study identified new target genes for ERα and ERβ in human OBs as well as novel information on the regulation of known E2-target genes by each ER isoform. Finally, these data support a major role of the particular ER isoform, in addition to the state of OB differentiation, in generating a unique E2 response in OB cells. These U2OS-ERα and U2OS-ERβ cell lines therefore represent a valuable new model for the identification and study of ERα- and ERβ-dependent phenomena in OBs and the action of selective estrogen receptor modulators on gene expression.

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