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Estrogen Receptor α/β Isoforms, but not βcx, Modulate Unique Patterns of Gene Expression and Cell Proliferation in Hs578T Cells

Frank J. Secreto,[1]* David G. Monroe,[1] Shamit Dutta,[1] James N. Ingle,[2] and Thomas C. Spelsberg[1]

[1]Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, Minnesota 55905
[2]Department of Oncology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, Minnesota 55905

Abstract The actions of 17β-estradiol (E2) and selective estrogen receptor modulators (SERMs) have been extensively investigated regarding their ability to act through estrogen receptor-α (ERα) to perturb estrogen receptor positive (ER+) breast cancer (BC) growth. However, many BCs also express ERβ, along with multiple estrogen receptor (ER) splice variants such as ERβcx, an ERβ splice variant incapable of binding ligand. To gain a more comprehensive understanding of ER action in BC cells, we stably expressed ERα, ERβ, or ERβcx under doxycycline (Dox) control in Hs578T cells. Microarrays performed on E2 or 4OH-tamoxifen (4HT) treated Hs578T ERα and ERβ cells revealed distinct ligand and receptor-dependent patterns of gene regulation, while the induction of ERβcx did not alter gene expression patterns. E2 stimulation of Hs578T ERβ cells resulted in a 27% decrease in cellular proliferation, however, no significant change in proliferation was observed following the exposure of Hs578T ERα or ERβ cells to 4HT. Expression of ERβcx in Hs578T cells did not effect cellular proliferation. Flow cytometry assays revealed a 50% decrease in E2-stimulated Hs578T ERβ cells entering S-phase, along with a 17% increase in G0/G1 cell-cycle arrest. We demonstrate here that ERα and ERβ regulate unique gene expression patterns in Hs578T cells, and such regulation likely is responsible for the observed isoform-specific changes in cell proliferation. Hs578T ER expressing cell-lines provide a unique BC model system, permitting the comparison of ERα, ERβ, and ERβcx actions in the same cell-line. J. Cell. Biochem. 101: 1125–1147, 2007.

Key words: estrogen receptor; breast cancer; microarrays; gene expression; proliferation

Estrogen receptor positive (ER+) breast cancer (BC) account for ~70% of all diagnosed primary BC in the United States [Dotzlaw et al., 1997; Fuqua et al., 2003], and roughly 17% of all diagnosed BC are ERα−/ERβ+ [Saji et al., 2005; Murphy and Watson, 2006]. The SERM, tamoxifen (TAM), remains the most frequently prescribed drug for treatment of ER+ BC. Although TAM has a high level of efficacy in ER+ BC tumors, most patients eventually develop a resistance to this selective estrogen receptor modulator (SERM). Additionally, nearly half of all ER+ BC fail to initially respond to TAM [Dorssers et al., 2001]. In vitro studies have demonstrated that the level of ERα protein is significantly reduced in MCF-7 cells following prolonged exposure to 4HT. The exact mechanism as to how TAM inhibits BC proliferation, and the cause(s) of this TAM resistance remains obscure [Shaw et al., 2006]. Acquired resistance is likely due to a shift in one or more of the components involved in TAM-directed ER signaling, and cannot fully be explained by changes in ER expression [Ring and Dowsett, 2004].

Estrogen (E2) and SERMs bind to both estrogen receptor α (ERα) and ERβ, resulting in receptor activation and subsequent association with specific gene promoters or enhancers. Together with nuclear co-regulator proteins, the DNA bound ER complex acts to induce or repress targeted gene transcription [Shang and

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*Correspondence to: Frank J. Secreto, Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905.
E-mail: secreto.frank@mayo.edu

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Brown, 2002; Taranta et al., 2002; Shao and Brown, 2004]. Approximately 59% of ER+ BC's express both ER isoforms, including a substantial amount of ER mRNA splice variants, for example, ERβcx [Koduri and Poola, 2001; Palmieri et al., 2002; Poola et al., 2002]. Unlike ERα and ERβ, however, ERβcx cannot bind ligand, due to a change in the orientation of helix 12, resulting from the alternative splicing of exon 8 [Ogawa et al., 1998; Leung et al., 2006]. Changes in the combination of the particular ratio of ER isoforms, the presence or absence of ER splice variants, and the variable expression levels of certain nuclear co-regulators in a particular BC, are thought to explain the inconsistent response that ER+ BC often display following exposure to SERMs or E2 [Jarvinen et al., 2000; Mann et al., 2001; Omoto et al., 2001; Murphy et al., 2002; Shang and Brown, 2002; Fuqua et al., 2003; Iwase et al., 2003; Fleming et al., 2004; Pearce and Jordan, 2004; Saji et al., 2005].

Recent interest has grown concerning the role of ERβ in modulating the actions of 4HT and E2 in ER+ BC gene regulation and tumor proliferation. [Iwao et al., 2000; Jarvinen et al., 2000; Lazennec et al., 2001; Omoto et al., 2001; Roger et al., 2001; Speirs, 2002; Hayashi et al., 2003; Carder, 2004; Esslimani-Sahla et al., 2004; Hopp et al., 2004; Myers et al., 2004; Paruthiyil et al., 2004; Koehler et al., 2005]. Previous studies have demonstrated that ERα expressing MCF-7 and T47D BC cells transfected with ERβ, resulted in an E2-dependent decrease in the rate of cellular proliferation [Paruthiyil et al., 2004; Strom et al., 2004; Chang et al., 2006]. Also, E2-dependent tumor formation was inhibited in mouse xenografts involving MCF-7 cells stably expressing ERβ [Paruthiyil et al., 2004]. Several case studies involving patients afflicted with ER+ BC suggested a positive correlation between the levels of ERβ expression and disease outcome and survival [Mann et al., 2001; Murphy et al., 2002; Iwase et al., 2003; Esslimani-Sahla et al., 2004; Fleming et al., 2004; Hopp et al., 2004; Myers et al., 2004; Nakopoulou et al., 2004]. Although these studies utilized a relatively small number of patients, the results coincide with most of the reports using BC cell and mouse model systems.

Despite a growing body of work devoted to examining ERβcx, the role of ERβcx in BC progression remains controversial. Although ERβcx cannot bind ligand, it was reported to form heterodimers in solution with ERα, which could explain the inhibition of ERα dependent gene expression by ERβcx [Ogawa et al., 1998]. E2-stimulated MCF-7 cells stably transfected with ERβcx showed a decreased rate of cellular proliferation when compared to E2-stimulated MCF-7 parental cells. Additionally, ERβcx expressing MCF-7 cells displayed a reduction in anchorage-independent colony formation [Omoto et al., 2003]. Further, the clinical significance regarding the role of ERβcx in BC remains unresolved. Different studies have suggested both a positive and negative correlation regarding the expression of ERβcx and an ER+ BC tumor response to 4HT [Saji et al., 2002; Esslimani-Sahla et al., 2005], as well as the effect of ERβcx expression on tumor progression [Palmieri et al., 2004; Esslimani-Sahla et al., 2005]. Increased ERβcx expression has been linked to an increase in the expression of progesterone receptor (PgR) [Clark, 1983; Saji et al., 2002]. However, the data from a second sample of biopsies reported in the same study indicated the opposite correlation between ERβcx and PgR. However, both of these sets of data were drawn from a relatively small sample population, n = 115 [Saji et al., 2002].

Although E2's role in promoting ER+ BC cell proliferation is well established, most studies used cell-lines expressing only ERα, or ERα-positive BC cell-lines in which ERβ was co-expressed. As noted, about 17% of all BC express ERβ in the absence of ERα [Saji et al., 2005; Murphy and Watson, 2006]. Therefore, the mechanisms responsible for the variances in the ER+ BC cell proliferation may involve ER-isoform specific differences in E2 and 4HT-dependent gene expression patterns, along with unique gene regulation resulting from the presence of alternative ER splice variants. In order to address this question, we utilized the ER-Hs578T cell-line derived from a human breast carcinoma [Hackett et al., 1977], and developed 3 novel Hs578T BC cell-lines, stably expressing ERα, ERβ, or ERβcx under doxycycline (Dox) regulation. Although several well established ERα expressing BC cell-lines (MCF-7, t47D) have been in use for decades, to date, no well characterized human BC cell-line that solely expresses ERβ or ERβcx have been reported. The extent of ER isoform-dependent gene expression differences in response to stimulation with E2, 4HT, or Dox alone (ERβcx) was achieved by microarray analyses. We

surmised that ER isoform-specific patterns of gene transcription would likewise lead to divergent regulation of various downstream cellular pathways, resulting in a specific ER-isoform phenotypic response. Development of the BC cells described in this manuscript enabled us to investigate the potential differences that E2, 4HT, or the expression of ERβcx may exert on endogenous gene expression, as well as the rate of BC cell proliferation in an ER-isoform/splice variant dependent manner.

METHODS

Cell Culture and Chemicals

Human Hs578T BC 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 Hs578T stable transfectants containing either ERα or ERβ were cultured in the same media supplemented with 5 mg/L blasticidin S (Boehringer Mannheim, Indianapolis, IN) and 500 mg/L zeocin (Invitrogen). Hs578T ERβcx expressing cells were cultured in the same media supplemented with 5 mg/L blasticidin S and 100 mg/L hygromycin B (Invitrogen). All cells undergoing ligand stimulation were cultured in DMEM/F12 media containing 10% (v/v) charcoal stripped (CS) FBS along with 1× antibiotic/antimycotic (Invitrogen). The pure ER antagonist, ICI 182,780 (ICI), was generously provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). E2, 4HT, and Dox were purchased from Sigma-Aldrich (St. Louis, MO).

Development and Characterization of Human Polyclonal ERβcx Antibody

Due to the lack of a commercially available ERβcx antibody, we contracted with Bio-Synthesis, Inc. (Lewisville, TX) in order to produce a polyclonal antibody raised against the unique ERβcx polypeptide, MKMETLLPEATMEQ [Ogawa et al., 1998]. ERβcx antibodies were produced for this study using two individual rabbits, and the amount of ERβcx protein was quantified using ELISA assays following the 6th week bleed. Two affinity purified samples of ERβcx antibody (BSYN 4858, IgG = 0.508 mg/ml, BSYN 4857, IgG = 0.408 mg/ml) were assayed by western analyses for affinity and specificity to transiently expressed ERβ and ERβcx protein. U2OS osteosarcoma cells were plated at ~70% density in 10% FBS supplemented media, and transfected with 5 μg of full-length FLAG-tagged ERβ or ERβcx expression constructs for 24 h using FuGene6 according to the manufacturers instructions (Roche, Pleasanton, CA). BSYN 4858 and 4857 recognized a single protein band ~55 kDa in U2OS cells transiently transfected with a full-length ERβcx expression vector (data not shown). Neither ERβcx antibody produced a band in protein isolated from U2OS cells transiently transfected with a full-length ERβ expression vector. All studies presented here utilized ERβcx BSYN 4858 rabbit polyclonal affinity purified antibodies.

Development and Initial Characterization of Dox-Inducible ERα, ERβ, and ERβcx Cell-lines

Hs578T Tet expressing cells were created in our laboratory using the T-REx™ System (Invitrogen) as previously described [Johnsen et al., 2002a]. Hs578T-Tet cells (ERα and ERβ negative) were stably transfected with full length FLAG-tagged ERα, ERβ, or ERβcx (original ERβcx clone generously provided by Shin-ichi Hayashi) expression constructs cloned into the pcDNA4/TO (ERα, ERβ) or pcDNA5/TO vector systems (Invitrogen) per the manufacturer's instructions. The addition of the FLAG-tags were performed as previously described [Monroe et al., 2003b]. The ER's were FLAG-tagged to permit accurate quantification of protein expression. All ER expression constructs were full-length sequenced to ensure the subsequently expressed protein was free of any PCR-based alterations. Additionally, the FLAG-tagged ERβcx expression construct was transiently transfected into COS-7 cells, along with ERα and a consensus ERE luciferase construct (tk-luc), in order to ensure that our ERβcx construct maintained its ability to inhibit E2-dependent ERα promoter activation. Cos-7 cells were plated at ~70% density and transiently transfected with 50 ng of full-length ERα expression construct, 250 ng of tk-luc, and 5, 50, or 500 ng of full-length ERβcx expression construct as described previously in Materials and Methods. The FLAG-tagged ERβcx expression construct displayed a dose-dependent ability to inhibit E2-stimulated ERα induction of tk-luc, thus indicating its functionality (data not shown). Individual clones were characterized by examining receptor expression via

western analysis and RT-PCR. Hs578T ERα, ERβ, or ERβcx cell lysates were isolated from control and 100 ng/ml Dox (Sigma-Aldrich) stimulated cells (24 h) by sonication (3 × 10 s pulse, 30 s incubation on ice between pulses, Misonix model W-370, Farmingdale, NY) in 8% (w/w) Sucrose, 0.24% (v/v) HEPES, 1× protease inhibitor cocktail (Roche), pH 7.2 homogenization buffer. Cell lysates were centrifuged at 10,000*g* for 2 min at 4°C, transferred to new Eppendorf tubes, and analyzed for total protein content (Bradford assay, Bio Rad, Hercules, CA).

Equivalent amounts of cell lysate (100 μg) were separated on SDS–PAGE 4–15% (w/w) gradient Criterion gels (Bio Rad), transferred to nitrocellulose, and analyzed against α-FLAG-M2 antibodies (Sigma) for ERα or ERβ protein expression. Expression of ERβcx protein was analyzed using the specific ERβcx antibody described above. Total RNA was isolated (Trizol) from Dox induced Hs578T ERα and ERβ cell-lines (cell-lines selected for Dox inducible expression of ER protein) stimulated w/wo 10^{-8} M E2 or 10^{-7} M 4HT for 24 h in CS 10% (v/v) FBS containing media. Total RNA (4 μg) was reverse-transcribed into cDNA as previously described [Monroe et al., 2003b]. The resulting cDNA was analyzed for E2-dependent induction and 4HT repression of pS2 gene transcription, as well as β-Actin (loading control), by PCR [1× PCR buffer (Promega, Madison, WI), 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 μ Taq (Promega), 1× Rediload (Invitrogen)]. Additionally, Hs578T ERα and ERβ cells were co-treated with E2 (10^{-8} M) and ICI, (10^{-7} M) and subsequently assayed by RT-PCR for pS2 mRNA expression. PCR primers specific to pS2 and β-Actin are listed in Table I.

Proliferation Assay

The Hs578T ERα, ERβ, and ERβcx 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 ERα, ERβcx, or ERβ) for an additional 24 h in DMEM/F12 supplemented with 10% (v/v) CS-FBS. Hs578T ERα and ERβ cells were treated (in triplicate) with vehicle (V), E2 (10^{-8} M), 4HT (10^{-7} M) and/or ICI (10^{-6} M) and allowed to grow for 72 h. Hs578T ERβcx cells were treated w/wo 100 ng/ml Dox for 72 h. Fresh media supplemented with Dox and steroids (Hs578T ERα or ERβ cells) was added every 24 h to maintain effective concentrations over the 72 h period. Twenty μl 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) and analyzed using SoftMax Pro software (Molecular Devices Corp.). All proliferation data is presented as a percentage of the Dox control.

Flow Cytometry Analyses

Hs578T ERα and ERβ cells were plated in 10-cm dishes at a cell density of 50% and stimulated with V or E2 (10^{-8} M) for 24 h. Cells were transferred to 50 ml centrifuge tubes, washed 3× in 1× PBS, resuspended in 3 μl of 1× PBS, and placed on ice. Three hundred microliters of ethanol (100%) was added drop-wise to each tube in order to permeabilize the cells, and all cells were subsequently incubated on ice for 1 h. Cells were rehydrated with the addition of 900 μl of 1× PBS, spun down, and washed 1× in 1× PBS. Wash steps were repeated twice, and cells were transferred to 12 × 75 mm round bottom tubes. The cells were resuspended in 300 μl of DNAse-free RNAse A solution (0.1 mg/ml in 0.1% (w/w) sodium citrate, Roche) and incubated for 15 min at 37°C. Finally, cells were stained with 300 μl of propidium iodide (0.1 mg/ml in 0.1 sodium citrate) in the dark for 15 min, and subsequently assayed for propidium stained DNA by the Flow Cytometry/Cell Sorting Lab at the Mayo Clinic, Rochester, MN (FACScan, Becton Dickinson, Franklin Lakes, NJ). Data was analyzed using ModFit software (Verity Software House, Topsham, ME).

Microarray Analyses

Hs578T ERα and ERβ cells were plated in 10-cm dishes at a cell density of 50% and treated with 100 ng/ml Dox for 24 h in DMEM/F12 CS media in duplicate (ERα) or triplicate (ERβ). Following the 24 h Dox treatment, Hs578T ERα and ERβ cells were stimulated with V, E2 (10^{-8} M), or 4HT (10^{-7} M) for an additional 24 h in fresh Dox containing media as described above. Hs578T ERβcx cells (triplicate samples) were stimulated w/wo 100 ng/ml Dox for 48 h, with fresh Dox containing CS-media being added following the initial 24 h stimulation. Total RNA was isolated using Trizol reagent (Invitrogen). Four micrograms of total RNA was

TABLE I. PCR Primer Sequences

Gene name	Gene symbol	Accession#	Primer sequences
Keratin 17	ker17	NM_000422	cagttcacctcctccagctc tcacctccagctcagtgttg
LGN protein	LGN	NM_013296	ttggaaggggaacgtctatg ctcttgcttctcccaccttg
Protein tyrosine phosphatase, non-receptor type 11	protP	NM_032904	atacgacgttggtggaggag atccgccaaaagtcattcac
Plectin 1, intermediate filament binding protein 500 kDa	Plectin	NM_000445	ggaaggtgtcagctcagagg gcatggcttggaagagagag
Integrin, alpha 6	ITGA6	NM_000210	aagtgtgcatggaggaaacc tcttttgtgggattccttgg
Complement component 3	C3	NM_000064	ggaaaaggaggatggaaagc acccaaagacaaccatgctc
Nuclear receptor interacting protein 1	NRIP1	NM_003489	cggaagaggctgtctgattc tttaggtgaggtggcaggac
Jagged 1	jagged1	NM_000214	aggggtattcaggacccaac taaccaaatcccgacaggag
Cyclin E2	cyclinE2	NM_004702	tactgactgctgctgccttg tgacaactgtccccctttc
Connective tissue growth factor	CTGF	NM_001901	gttccaagacctgtgggatg tggagattttgggagtacgg
Secreted frizzled-related protein 4	SFRP4	NM_003014	cttgccagtgtccacacatc cggctgttttcttcttgtcc
Retinol binding protein 4, plasma	RBP4	NM_006744	ccgagtcaaggagaacttcg tctggagaaaggaggctacg
Endothelin receptor type A	endoRA	NM_001957	tctccatctggatcctgtcc atcggttcttgtccatttcg
Inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	NM_002192	tttctgttggcaagttgctg cgggtctcttcttcaagtgc
v-maf musculoaponeurotic fibrosarcoma oncogene	vMAF	NM_005360	agctggtgaccatgtctgtg aggtggttctccatgactgc
Prostaglandin-endoperoxide synthase 2	PTGS2	NM_000963	tgagcatctacggtttgctg gaaaggtgtcaggcagaagg
Nuclear receptor coactivator 1	SRC1	NM_003743	ggcatcaatatgagatcaggc acgggctggtagaagcaggtg
Insulin-like growth factor binding protein 4	IGFBP4	NM_001552	gcccaagaggactgagactg gcttgagaggaaggacgatg
Interleukin 1, beta	IL1β	NM_000576	tggccctaaacagatgaagg tactcctcccctgtcaccac
Growth arrest-specific 1	GAS1	NM_002048	ccctcattcagctcaaccac gaagactttgccgcagtagg
Trefoil factor 1 (pS2)	pS2	NM_003225	ggcccagacagagacgtgtacagtgg gagtagtcaaagtcagagcagtcaatc
β-actin	actin	BC002409	tcacccacactgtgcccatcaacga cagcggaaccgctcattgccaatgg
Death-associated protein kinase 3	DAPK3	NM_001348	tgcacgacatcttcgagaac ataatgttttccggcttcag
Thioredoxin domain containing	TXNDC	NM_030755	aagaccttggattgccagtg cgccttttgaaggacaaag
Oncostatin M receptor	OSMR	NM_003999	ggaatgtgccacacactttg acattggtgccttcttccac
Pre-B-cell leukemia transcription Factor 2	PBX2	NM_002586	cagatgcagctgaagcagag cacttcttggcaagctcctc
PDGFA associated protein 2	PDAP2	BC023976	ccgcggctctatgttctatg ttcttatgcactcgcctcag

analyzed by microarray analyses using Human Focus Arrays (Affymetrix, Santa Clara, CA), containing probe sets for approximately 8,700 genes. Preparation of labeled cRNA and the ensuing microarray hybridization were performed by the Microarray Core Facility at the Mayo Clinic (Rochester, MN). Analyses of data generated by the microarray experiments were performed using GeneSpring (Agilent Technologies, Palo Alto, CA) and Expression Analysis Systematic Explorer (EASE) software [Hosack et al., 2003].

RT-PCR Confirmation of Microarray Analyses

Hs578T ERα and ERβ cells were cultured in DMEM/F12 CS media and stimulated with 100 ng/ml Dox for 24 h and subsequently treated with V, E2 (10^{-8} M), or 4HT (10^{-7} M) for an additional 24 h. Hs578T ERβcx cells were cultured in the same media supplemented with 100 ng/ml Dox for 48 h, with fresh Dox containing CS-media added following the initial 24 h stimulation. Total RNA was isolated using Trizol reagent (Invitrogen). RT-PCR was performed as

described above. PCR primers specific to each gene are listed in Table I.

RESULTS

Characterization of Stably Expressing ERα, ERβ, and ERβcx BC Cell-Lines

ER expressing clones were initially selected based upon Dox-inducible protein expression of the stably introduced ER. Figure 1A–C demonstrates Dox-inducible expression of ERα, ERβ, and ERβcx protein in stably transfected Hs578T cells, respectively. Note the lack of detectable ER expression in all of the control lanes, a result which was further supported by RT-PCR analyses using RNA isolated from Hs578T ERα, ERβ, and ERβcx cells stimulated w/wo Dox (data not shown). ERα and ERβ proteins were identified by Western analyses using α-FLAG-M2 antibodies. Due to the poor quality of ERβcx Western analyses using anti-FLAG-M2 antibodies, detection of ERβcx was accomplished with the use of a specific ERβcx antibody developed by our laboratory, as described in Materials and Methods. Three additional ERα and ERβ Hs578T cell-lines, along with two additional Hs578T ERβcx cell-lines, were analyzed for Dox-mediated receptor induction. All of these additional cell-lines demonstrated the ability to induce ER expression in a Dox-dependent manner (data not shown).

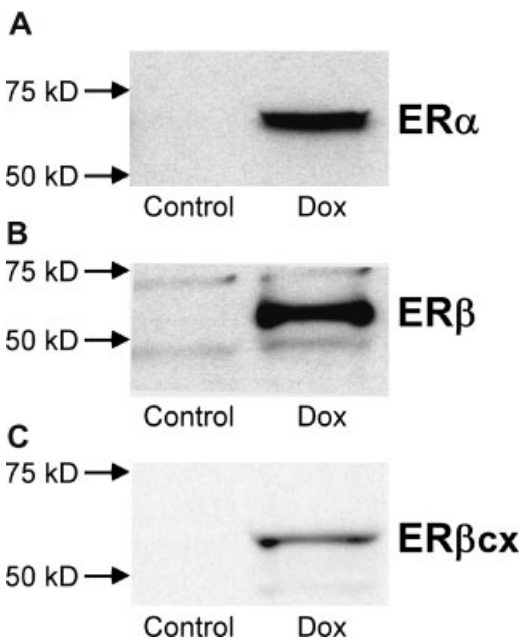


Fig. 1. Confirmation of ER protein expression in Hs578T ERα, ERβ, and ERβcx Cell-Lines. A previously constructed Hs578T-Tet cell-line was stably transfected with full length human ERα, ERβ, or ERβcx expression constructs and stimulated with 100 ng/ml Dox for 24 h. Cell lysates were harvested and subjected to western blot analyses using the α-FLAG-M2 Ab (ERα and ERβ) (Sigma) or a specific antibody raised against ERβcx (see Methods). All blots were developed using enhanced chemiluminescence kits (Amersham). ERα (**A**), ERβ (**B**), and ERβcx (**C**) proteins resolved at their expected sizes of 66, 54, and 51 kDa, respectively.

In order to establish whether our Hs578T clonal lines expressed functional ERα and ERβ protein, E2 and 4HT modulation of pS2 gene expression was assessed [Brown et al., 1984]. Figure 2A,B displays pS2 modulation in ERα and ERβ expressing Dox-induced Hs578T cell-lines, respectively. E2-stimulation of Hs578T ERα and ERβ cell-lines led to significantly increased pS2 mRNA expression (Fig. 2A,B). Exposure of Hs578T ERβ cells to 4HT resulted in nearly undetectable levels of pS2 mRNA, however, 4HT stimulation of Hs578T ERα cells did not significantly effect the level of pS2 mRNA levels compared to cells treated with Dox alone (Fig. 2A,B). Additional Hs578T ERα and ERβ clones were analyzed for ER function in order to address potential clonal variability. ER-mediated induction of pS2 mRNA expression observed in these cell-lines were similar to the results displayed by the fully characterized ERα and ERβ cell-lines (data not shown).

Effects of E2 and 4HT on Cellular Proliferation of Hs578T ERα and ERβ Cell-Lines

Studies focused on ERα-dependent regulation of BC cell proliferation involving the actions of E2 and SERMs have been ongoing for many years [Colozza et al., 2006]. Our ER expressing BC cell-lines described here are unique, as they permit the comparison of ER isoform-specific regulation of BC cell proliferation. Hs578T ERα and ERβ cell-lines were treated with V, E2, or 4HT for 72 h and assayed for changes in the rate of cellular proliferation using MTS tetrazolium assays. Hs578T ERα cells showed no changes in the rate of cellular proliferation with either E2 or 4HT stimulation (Fig. 3A). Stimulation of Hs578T ERα cells with higher concentrations of E2 (10^{-7} M) or 4HT (10^{-6} M) also resulted in no significant changes in proliferation (data not shown). It should be noted that the inability of 4HT to significantly effect the rate of cellular proliferation in Hs578T ERα cells was consistent with other ER-negative (ER-) BC cells stably transfected with ERα [Moggs et al., 2005]. Interestingly, Hs578T ERβ cells revealed

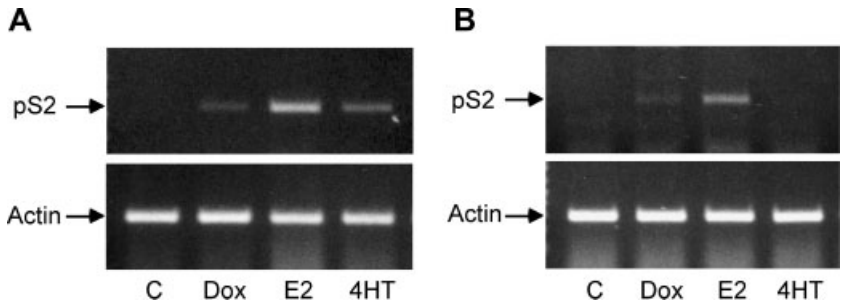
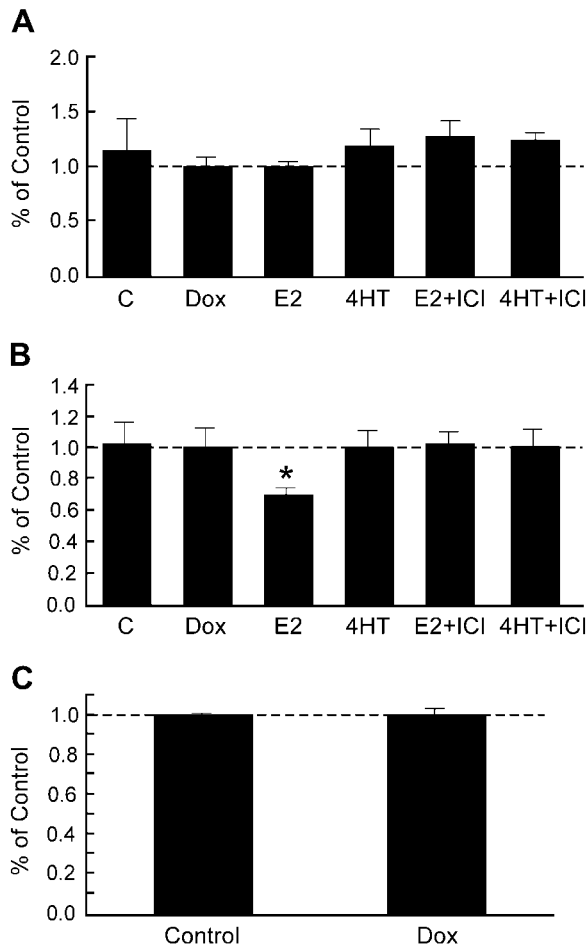


Fig. 2. Hs578T ERα and ERβ stable cell-lines express biologically active ER receptors. Total RNA was isolated (Trizol, Invitrogen) from Dox-induced Hs578T cell-lines expressing ERα or ERβ and subsequently stimulated with E2 (10^{-8} M) or 4HT(10^{-7} M) for 24 h. Four micrograms of total RNA was reverse transcribed into cDNA and subsequently analyzed by RT-PCR for pS2 mRNA expression in both Hs578T ERα (**A**) and ERβ (**B**) cell-lines. Actin controls were used to minimize sample to sample variation.

a 27% decrease in proliferation following stimulation with E2 (Fig. 3B). The E2-dependent decrease in proliferation was shown to be ERβ-dependent, as co-treatment with the pure ER antagonist ICI resulted in no change in the rate of proliferation as compared to V-stimulated cells (Fig. 3B). Additionally, treatment of Hs578T ERβ cells with 4HT resulted in no significant effect on the rate of proliferation (Fig. 3B). Higher concentrations of 4HT (10^{-6} M) also failed to induce a significant change in proliferation (data not shown). ER expression in the absence of ligand did not significantly effect the rate of cellular proliferation in either Hs578T ERα or ERβ cell-lines (Fig. 3A,B). Additional Hs578T ERα and ERβ clonal cell-lines displayed the same E2 and 4HT effects on proliferation as was seen in the fully characterized cell-lines examined in detail in this paper (data not shown). These results demonstrate that the E2-induced decrease in proliferation observed in the fully characterized Hs578T ERβ cells was not the result of clonal variation.



Effect of ERβcx on Cellular Proliferation of Hs578T Cells

Previous studies have demonstrated that ERβcx can inhibit E2-mediated increases in

Fig. 3. E2 Stimulation of Hs578T ERβ cells resulted in decreased cellular proliferation. Hs578T ERα and ERβ expressing cells were seeded in 96-well plates (6,400 cells/well), stimulated with CS media containing 100 ng/ml Dox for 24 h, and subsequently treated with E2 (10^{-8} M) or 4HT (10^{-7} M) alone or in combination with ICI (10^{-6} M) for 72 h. Hs578T cells expressing ERβcx were stimulated with 100 ng/ml Dox alone for the 72 h incubation. Following the 72 h incubation, 20 μl of MTS reagent (CellTiter 96, Promega) were added to each well and the plates subsequently incubated for 30 min at 37°C. Once sufficiently developed, the plates were analyzed on a SpectraMAX 340 plate reader at 490 nm (Molecular Devices). The data presented is a representative experiment with individual means comprised of six replications (ERα/ERβ) or three replications (ERβcx) per treatment. **Panels A** and **B**: Proliferation rates of Hs578T ERα cells (A) were not significantly effected by any treatment described, however, E2 stimulation of Hs578T ERβ cells (B) lead to a 27% decreased in proliferation which was completely abrogated following co-treatment with ICI. **Panel C**: The expression of ERβcx in Hs578T cells did not significantly effect cellular proliferation.

the rate of ER+ BC proliferation [Omoto et al., 2003]. To determine whether ERβcx can effect cellular proliferation independent of ERα or ERβ, Hs578T ERβcx cells were stimulated w/wo Dox for 72 h and subsequently analyzed for changes in cellular proliferation rates using MTS assays as described previously. Figure 3C demonstrates that the expression of ERβcx alone had no effect on the rate of proliferation of Hs578T cells.

Flow Cytometry Analyses

The decrease in cellular proliferation observed in E2-stimulated Hs578T ERβ cells could be verified and further defined by the assessment of an E2-dependent change in cell-cycle progression. Therefore, Hs578T ERα and ERβ cell-lines were stimulated with V or E2 for 72 h and analyzed by flow cytometry. The results of the flow analyses performed on E2-stimulated Hs578T ERα and ERβ cells (Fig. 4A) supported the findings of the MTS proliferation studies (Fig. 3). E2-stimulated Hs578T ERα cells failed to show any changes in cell cycle distribution, while similarly treated Hs578T ERβ cells displayed a ~50% decrease in cells entering S-phase combined with a 17% increase in G0/1 cell cycle arrest (Fig. 4B).

Microarray Analyses of E2 and 4HT Stimulated Hs578T ERα and ERβ Cell-lines

To investigate the ligand and ER isoform-dependent differences in gene expression, RNA was isolated from Dox-induced Hs578T ERα and ERβ cells stimulated with E2 and 4HT in CS media for 24 h, and subsequently analyzed

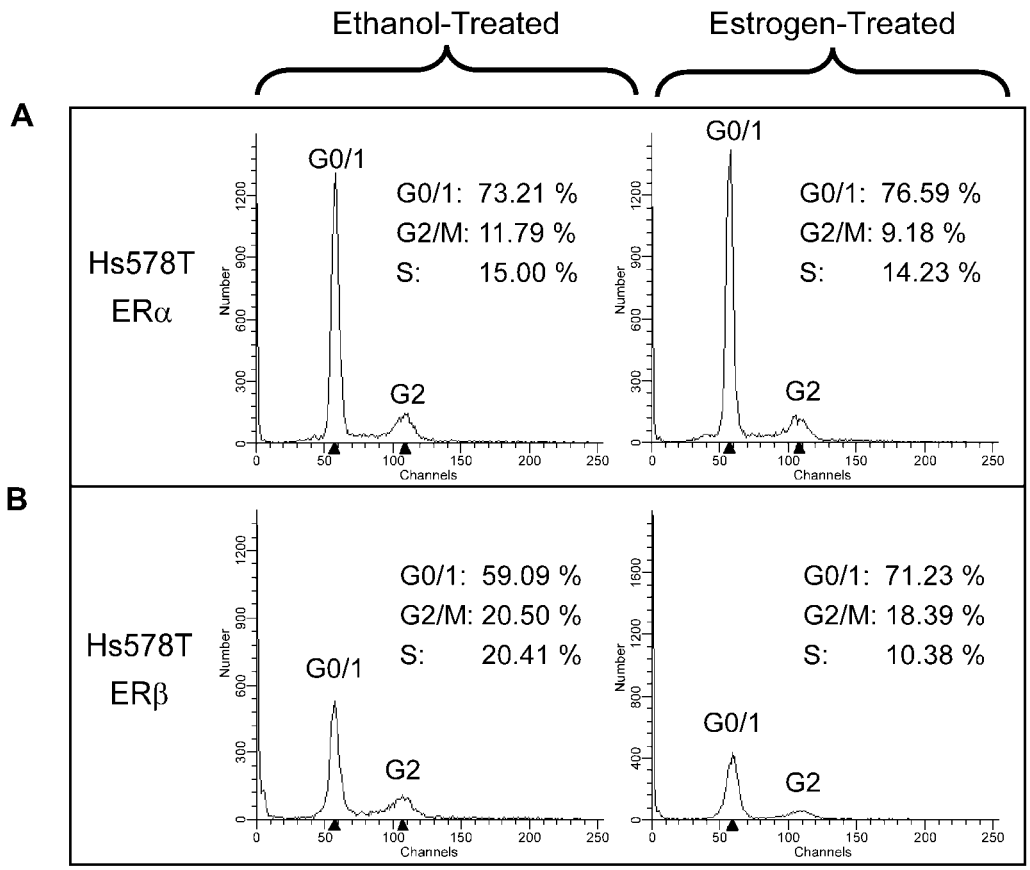


Fig. 4. E2 stimulation of Hs578T ERβ inhibits cell cycle progression. Hs578T ERα cells were stimulated w/wo E2 (10^{-8} M) for 72 h in CS media, permeablized in 50% (v/v) ethanol, and stained with 0.1 mg/ml propidium iodide. Cells were analyzed on a FACScan (Becton Dickinson) cell sorter, and all scans were conducted following a minimum 20,000 event threshold. Data was analyzed using ModFit software (Verity Software House), and the figure presented represents a single set of data that was repeated to ensure the findings consistency. **Panel A**: Hs578T ERα cells treated with E2 did not show any significant changes in cell cycle arrest (G0/1, G2/M) or DNA replication (S). **Panel B**: E2 Stimulation of Hs578T ERβ cells lead to a 50% decrease in DNA replication (S), and a 17% increase in G0/1 cell cycle arrest.

using Human Focus Gene Array (Affymetrix) microarrays. Specific E2 and 4HT mediated effects on gene expression were determined by comparison of total RNA isolated from E2 or 4HT treated cells with those treated with V. Only those genes whose expression were increased or decreased 2-fold or greater ($\geq$2-fold) were reported. Analyses of data compiled from gene arrays were accomplished with the aid of GeneSpring software (Silicon Genetics). Tables II and IV list all genes regulated by E2 ($\geq$2-fold) in Hs578T ERα and ERβ cells, respectively. Similarly, all genes regulated by 4HT ($\geq$2-fold) in Hs578T ERα and ERβ cells are listed in Tables III and V, respectively. Genes regulated in a similar fashion in both Hs578T ERα and ERβ cells are compiled in Table VI. Comparisons of gene expression between E2 and 4HT stimulated Hs578T ERα and ERβ cells revealed a distinct pattern of ligand specificity (Fig. 5A,B). Interestingly, E2 or 4HT stimulated gene regulation was shown to be highly ER isoform specific (Fig. 6A,B). In summary, the comparisons of ligand and isoform-dependent gene expression indicate that ERα and ERβ primarily regulate unique sets of genes. These observations are consistent with previous data involving ER expressing U2OS cell-lines [Kian Tee et al., 2004; Stossi et al., 2004; Monroe et al., 2005].

A number of E2 and 4HT regulated genes, as determined from the analyses of microarray data, were further verified by RT-PCR. A total of 28 genes were selected and subsequently assayed for ligand-dependent modulation of gene expression. Twenty-four (86%) of the genes displayed the same responses in expression as indicated by the microarray data (Fig. 7). A subset of the genes illustrated in Figure 7 were assayed in 3 additional ERα and ERβ Hs578T cell-lines. The same patterns of gene expression were observed in these cells compared to the fully characterized cell-lines (data not shown). Actin controls were used in order to minimize sample to sample variation.

To identify genes that may be responsible for the observed E2-dependent decrease in Hs578T ERβ proliferation (Fig. 3), clustering analyses were performed on microarray data obtained from E2-stimulated Hs578T ERβ cells. Table VIIA,B summarizes gene clustering analyses of our microarray data obtained with the aid of EASE software [Hosack et al., 2003]. Table VIIA lists genes recognized as proliferation (cell cycle) agonists from our gene array analyses which were down-regulated at least 2-fold by E2 in Hs578T ERβ cells, while Table VIIB shows E2-dependent increases in genes involved in cell cycle arrest. Only 2 of the proliferation categorized genes, IL-6 and SPHK-1, were regulated by Hs578T ERα cells in a similar manner compared to Hs578T cells expressing ERβ (Table VIIA).

Microarray Analyses of an Hs578T ERβcx Cell-line Stimulated w/wo Dox

Hs578T ERβcx cells were similarly examined for changes in gene expression. The cells were stimulated w/wo Dox in CS media for 48 h, with fresh Dox supplemented CS media being replaced following the initial 24 h stimulation. RNA was isolated from triplicate 100 mm plates as described previously. Analysis of ERβcx microarray data identified only five genes regulated at the $\geq$2-fold level (data not shown). Subsequent reanalysis of the ERβcx microarray data indicated that 67 genes were regulated at a $\geq$1.5-fold level of expression (data not shown). However, RT-PCR analyses of the ERβcx microarray data, including three genes regulated $\sim$2-fold greater than control treated cells, failed to support the microarray results (Fig. 8). In order to ensure that the 48 h time point was reflective of potential ERβcx mediated gene regulation, all five (Fig. 8) of the confirmations were repeated using RNA isolated from Hs578T ERβcx cells stimulated with Dox for 30 min, 2, 8, and 24 h. No significant gene expression changes were observed in any of the five genes tested at any of the additional time points (data not shown).

Although ERβcx has previously been shown to inhibit ERα binding to a consensus ERE [Ogawa et al., 1998], we were interested in whether or not ERβcx alone can form a complex with an ERE containing DNA oligomer. Gel shift analysis using in vitro translated full-length ERβcx protein failed to form a detectable complex with an ERE oligomer. Full length ERα and ERβ constructs were used as positive controls, and both proteins displayed visibly detectable shifted complexes (data not shown).

DISCUSSION

The particular roles exhibited by specific ER isoforms, especially their individual effects on gene expression and proliferation in BC cells,

TABLE II. E2 Stimulated Hs578T ERα Cells

Gene description	Fold change	Accession #
LGN protein	8.725	NM_013296
Integrin, alpha 6	6.845	NM_000210
Protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	5.076	NM_032904
Sec23 homolog A (S. cerevisiae)	4.848	NM_006364
Tropomodulin 3 (ubiquitous)	4.044	NM_014547
Protein phosphatase 2 (formerly 2A), regulatory subunit B″, alpha	3.875	NM_181897
v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	3.71	NM_005433
Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2	3.57	NM_025235
Prostaglandin-endoperoxide synthase 2	3.402	NM_000963
Pericentriolar material 1	3.345	NM_006197
Protein kinase, interferon-inducible double stranded RNA dependent	3.256	NM_002759
BRCA1 associated RING domain 1	3.253	NM_000465
Coated vesicle membrane protein	3.062	NM_006815
Breast cancer 1, early onset	3.025	NM_007294
Interleukin 6 signal transducer (gp130, oncostatin M receptor)	3.005	NM_175767
Rho-associated, coiled-coil containing protein kinase 1	2.994	NM_005406
Microtubule-associated protein 1B	2.966	NM_032010
Mitochondrial translational initiation factor 2	2.925	NM_002453
Baculoviral IAP repeat-containing 3	2.875	NM_182962
Homer homolog 1 (Drosophila)	2.856	NM_004272
Splicing factor, arginine/serine-rich 2, interacting protein	2.852	NM_004719
Hepatitis B virus x associated protein	2.832	NM_016578
PC4 and SFRS1 interacting protein 2	2.824	NM_033222
Leucine zipper transcription factor-like 1	2.795	NM_020347
Tripartite motif-containing 33	2.786	NM_033020
ERO1-like (S. cerevisiae)	2.776	NM_014584
Topoisomerase (DNA) I	2.73	NM_003286
Muscleblind-like (Drosophila)	2.697	NM_021038
Hypothetical protein FLJ22028	2.687	NM_024854
Alkylglycerone phosphate synthase	2.644	NM_003659
Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein 1	2.639	NM_005744
CGI-07 protein	2.619	NM_015938
fem-1 homolog b (C. elegans)	2.602	NM_015322
S-phase kinase-associated protein 2 (p45)	2.597	NM_032637
Mannosidase, alpha, class 2A, member 1	2.586	NM_002372
Chromosome 5 open reading frame 5	2.579	NM_016603
TIA1 cytotoxic granule-associated RNA binding protein	2.556	NM_022173
Chitobiase, di-N-acetyl-	2.55	NM_004388
Membrane-associated nucleic acid binding protein	2.539	NM_018835
Brefeldin A-inhibited guanine nucleotide-exchange protein 1	2.538	NM_006421
Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	2.525	NM_006482
RAD51-interacting protein	2.522	NM_006479
Amylo-1, 6-glucosidase, 4-alpha-glucanotransferase	2.516	NM_000646
Mitogen-activated protein kinase 6	2.513	NM_002748
Restin (reed-steinberg cell-expressed intermediate filament-associated protein)	2.512	NM_198240
Dihydrolipoamide dehydrogenase	2.504	NM_000108
Signal transducer and activator of transcription 1, 91 kDa	2.485	NM_139266
LIM domain containing preferred translocation partner in lipoma	2.476	NM_005578
Chromodomain helicase DNA binding protein 1	2.471	NM_001270
Glutamyl-prolyl-tRNA synthetase	2.457	NM_004446
Bromodomain containing 7	2.456	NM_013263
Heat shock protein (hsp110 family)	2.438	NM_014278
Retinoblastoma-like 1 (p107)	2.433	NM_183404
Radixin	2.429	NM_002906
Acyl-Coenzyme A binding domain containing 3	2.423	NM_022735
Heparan sulfate 2-O-sulfotransferase 1	2.407	NM_012262
Optineurin	2.401	NM_021980
Meningioma expressed antigen 5 (hyaluronidase)	2.397	NM_012215
Ribosomal protein S6 kinase, 90 kDa, polypeptide 3	2.388	NM_004586
Phospholipase C-like 1	2.388	NM_006226
H factor 1 (complement)	2.388	NM_000186
CDC42 binding protein kinase alpha (DMPK-like)	2.383	NM_014826
Cytidine monophosphate N-acetylneuraminic acid synthetase	2.375	NM_018686
DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	2.374	NM_004939
SWI/SNF related regulator of chromatin, subfamily a, member 3	2.361	NM_139048
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	2.358	NM_000176
Ras homolog gene family, member Q	2.354	NM_012249
Eukaryotic translation initiation factor 1A	2.337	NM_001412
Tetratricopeptide repeat domain 3	2.336	NM_003316
Synaptojanin 1	2.335	NM_003895
Kinesin family member 23	2.334	NM_138555
Serine/threonine kinase 3 (STE20 homolog, yeast)	2.333	NM_006281
CDC5 cell division cycle 5-like (S. pombe)	2.312	NM_001253
Matrin 3	2.312	NM_018834

TABLE II. (*Continued*)

Gene description	Fold change	Accession #
Activator of S phase kinase	2.307	NM_006716
odz, odd Oz/ten-m homolog 1(Drosophila)	2.3	NM_014253
Retinoblastoma-like 2 (p130)	2.296	NM_005611
Twisted gastrulation homolog 1 (Drosophila)	2.294	NM_020648
Fatty-acid-Coenzyme A ligase, long-chain 3	2.292	NM_004457
Retinoblastoma binding protein 2	2.288	NM_005056
Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	2.288	NM_002210
Topoisomerase (DNA) II alpha 170 kDa	2.28	NM_001067
Decorin	2.277	NM_133507
Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	2.273	NM_006209
Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	2.267	NM_014331
Thioredoxin domain containing	2.265	NM_030755
Solute carrier family 35 member A3	2.265	NM_012243
Tumor rejection antigen (gp96) 1	2.264	NM_003299
Solute carrier family 39 (zinc transporter), member 6	2.264	NM_012319
Leukocyte-derived arginine aminopeptidase	2.223	NM_022350
Dihydrolipoamide branched chain transacylase	2.215	NM_001918
PC4 and SFRS1 interacting protein 2	2.209	NM_033222
NIMA (never in mitosis gene a)-related kinase 4	2.208	NM_003157
Signal transducer and activator of transcription 1, 91 kDa	2.208	NM_139266
Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF)	2.204	NM_177423
Methylmalonyl Coenzyme A mutase	2.203	NM_000255
Pellino homolog 1 (Drosophila)	2.199	NM_020651
Similar to rab11-binding protein	2.197	NM_019045
Colon carcinoma related protein	2.195	NM_016206
Solute carrier family 16 (monocarboxylic acid transporters), member 1	2.193	NM_003051
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1	2.186	NM_033252
Protein kinase C-like 2	2.185	NM_006256
Nudix (nucleoside diphosphate linked moiety X)-type motif 4	2.183	NM_199040
Discs, large homolog 1 (Drosophila)	2.18	NM_004087
Fatty-acid-Coenzyme A ligase, long-chain 4	2.179	NM_022977
RAD17 homolog (S. pombe)	2.178	NM_133344
Vacuolar protein sorting 54 (yeast)	2.174	NM_016516
Ubiquitin protein ligase E3A	2.172	NM_130839
Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45 kDa	2.168	NM_000430
Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170 kDa	2.166	NM_003750
PRKC, apoptosis, WT1, regulator	2.165	NM_002583
Muscleblind-like 2 (Drosophila)	2.162	NM_144778
Zinc finger protein 325	2.16	NM_016265
RAB23, member RAS oncogene family	2.157	NM_183227
Heat shock 105/110 kDa protein 1	2.145	NM_006644
Signal sequence receptor, gamma (translocon-associated protein gamma)	2.138	NM_007107
TTK protein kinase	2.138	NM_003318
Myeloid/lymphoid or mixed-lineage leukemia, translocated to 2	2.137	NM_005935
Microfibrillar-associated protein 3	2.136	NM_005927
Epidermal growth factor receptor pathway substrate 8	2.135	NM_004447
Kelch-like 2, Mayven (Drosophila)	2.134	NM_007246
SWI/SNF related regulator of chromatin subfamily c, member 1	2.128	NM_003074
Protein kinase, cAMP-dependent, catalytic, beta	2.125	NM_182948
UPF3 regulator of nonsense transcripts homolog B (yeast)	2.12	NM_080632
Solute carrier family 5 (inositol transporters), member 3	2.113	NM_006933
Disrupter of silencing 10	2.113	NM_020368
Stromal antigen 2	2.105	NM_006603
SWI/SNF related regulator of chromatin subfamily a, member 5	2.105	NM_003601
Ubiquitin specific protease 1	2.102	NM_003368
TAF13 RNA polymerase II	2.091	NM_005645
Splicing factor, arginine/serine-rich 11	2.086	NM_004768
SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	2.086	NM_005496
RNA-binding region (RNP1, RRM) containing 2	2.079	NM_184244
ATP-binding cassette, sub-family D (ALD), member 3	2.079	NM_002858
DEK oncogene (DNA binding)	2.075	NM_003472
Synovial sarcoma translocation, chromosome 18	2.072	NM_005637
Vacuolar protein sorting 4B (yeast)	2.07	NM_004869
Pre-B-cell colony-enhancing factor	2.069	NM_182790
Casein kinase 1, gamma 3	2.068	NM_004384
5′-nucleotidase, ecto (CD73)	2.067	NM_002526
Transposon-derived Buster1 transposase-like protein	2.067	NM_021211
Kinesin-associated protein 3	2.065	NM_014970
Crystallin, zeta (quinone reductase)	2.063	NM_001889
Fibroblast growth factor 2 (basic)	2.061	NM_002006
Sperm specific antigen 2	2.06	NM_006751
Wiskott-Aldrich syndrome protein interacting protein	2.059	NM_003387
Zinc finger protein 262	2.058	NM_005095
Stromal antigen 1	2.055	NM_005862

(*Continued*)

TABLE II. (*Continued*)

Gene description	Fold change	Accession #
Baculoviral IAP repeat-containing 2	2.052	NM_001166
Copine III	2.051	NM_003909
RIO kinase 3 (yeast)	2.05	NM_145906
Insulin-degrading enzyme	2.048	NM_004969
Sjogren syndrome antigen A2	2.047	NM_004600
Lumican	2.045	NM_002345
Chloride channel, calcium activated, family member 2	2.045	NM_006536
Sorting nexin 2	2.037	NM_003100
A kinase (PRKA) anchor protein 11	2.036	NM_144490
Rearranged L-myc fusion sequence	2.035	NM_012421
General transcription factor IIIC, polypeptide 3, 102 kDa	2.034	NM_012086
Retinoblastoma 1 (including osteosarcoma)	2.03	NM_000321
Spinocerebellar ataxia 1	2.027	NM_000332
Putative dimethyladenosine transferase	2.026	NM_014473
Chromosome 1 open reading frame 9	2.017	NM_016227
Kinesin family member 11	2.014	NM_004523
Thyroid hormone receptor interactor 12	2.013	NM_004238
Mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	2.009	NM_002408
Aryl hydrocarbon receptor	2.007	NM_001621
Heat shock 90 kDa protein 1, alpha	2.004	NM_005348
Striatin, calmodulin binding protein 3	2.003	NM_014574
PDZ domain containing guanine nucleotide exchange factor (GEF) 2	2.002	NM_016340
Alport syndrome chromosomal region, gene 1	2.001	NM_015365
Diptheria toxin resistance protein required for diphthamide biosynthesis-like 1	0.499	NM_001383
Deleted in lung and esophageal cancer 1	0.494	NM_005106
Histone 2, H2aa	0.489	NM_003516
Chromosome 6 open reading frame 11	0.489	NM_005452
Nicotinamide N-methyltransferase	0.488	NM_006169
Sphingosine kinase 1	0.488	NM_021972
Thyroid hormone receptor-associated protein, 95-kDa subunit	0.487	NM_005481
CDP-diacylglycerol—inositol 3-phosphatidyltransferase	0.487	NM_145752
RNA binding protein with multiple splicing	0.483	NM_006867
Pseudoautosomal GTP-binding protein-like	0.482	NM_012227
Stomatin (EPB72)-like 1	0.482	NM_004809
Excision repair cross-complementing, complementation group 1	0.48	NM_001983
Dipeptidylpeptidase 3	0.479	NM_130443
Wingless-type MMTV integration site family, member 5B	0.473	NM_032642
Retinoid X receptor, beta	0.473	NM_021976
Transforming growth factor beta 1 induced transcript 1	0.473	NM_015927
Rab9 effector p40	0.472	NM_005833
G protein-coupled receptor kinase 6	0.472	NM_002082
BCL2-like 1	0.465	NM_138578
Peroxisomal long-chain acyl-coA thioesterase	0.463	NM_006821
Keratin 7	0.457	NM_005556
Peroxisomal acyl-CoA thioesterase	0.455	NM_183386
Isovaleryl Coenzyme A dehydrogenase	0.453	NM_002225
Plasminogen activator, urokinase	0.442	NM_002658
Leukemia inhibitory factor (cholinergic differentiation factor)	0.442	NM_002309
Calponin 1, basic, smooth muscle	0.442	NM_001299
Interleukin 6 (interferon, beta 2)	0.439	NM_000600
Plectin 1, intermediate filament binding protein 500 kDa	0.426	NM_000445
SCO cytochrome oxidase deficient homolog 2 (yeast)	0.423	NM_005138
Keratin 17	0.39	NM_000422
Fatty acid desaturase 3	0.371	NM_021727
Uridine phosphorylase 1	0.227	NM_181597

remains controversial. Recent studies have emphasized the importance of ERβ and to some extent, ERβcx, in BC carcinogenesis, as well as TAM resistance and tumor aggressiveness [Esslimani-Sahla et al., 2004; Palmieri et al., 2004]. However, the results from clinical studies aimed at deciphering the correlation between ERβ, ERβcx, and BC tumor progression are inconsistent. A significant understanding has been gained regarding the mechanism(s) by which ERα regulates gene expression in BC. However, only recently have efforts been directed at deciphering the potential roles that ERβ and ERβcx may play in modulating ligand-dependent/independent gene regulation and subsequent modulation of BC growth and progression [Esslimani-Sahla et al., 2004; Palmieri et al., 2004].

In order to elucidate the actions of ERα, ERβ, and ERβcx on BC growth, our laboratory developed novel BC (Hs578T) cell-lines expressing ERα, ERβ, or ERβcx. Hs578T cells were used in this study because of their lack of endogenous ER expression [Wang et al., 1996],

TABLE III. 4HT Stimulated Hs578T ERα Cells

Gene description	Fold change	Accession #
Bone marrow stromal cell antigen 2	11.63	NM_004335
Retinol binding protein 4, plasma	8.031	NM_006744
G protein-coupled receptor 56	7.71	NM_005682
Fibrinogen, gamma polypeptide	6.419	NM_000509
Secreted frizzled-related protein 4	4.95	NM_003014
Cellular retinoic acid binding protein 2	3.721	NM_001878
Putative lymphocyte G0/G1 switch gene	3.403	NM_015714
Endothelin receptor type A	3.094	NM_001957
Interferon-induced protein with tetratricopeptide repeats 1	2.718	NM_001548
LIM domain protein	2.678	NM_003687
Neuroblastoma, suppression of tumorigenicity 1	2.561	NM_005380
GTP cyclohydrolase 1 (dopa-responsive dystonia)	2.557	NM_000161
Growth differentiation factor 11	2.531	NM_005811
Carboxypeptidase M	2.421	NM_001874
Protein phosphatase 1B (formerly 2C)	2.394	NM_002706
Interferon-induced protein with tetratricopeptide repeats 4	2.374	NM_001549
Proteasome (prosome, macropain) subunit, beta type, 8	2.367	NM_004159
2′, 5′-oligoadenylate synthetase 1, 40/46 kDa	2.351	NM_002534
G protein-coupled receptor 48	2.274	NM_018490
Mucosal vascular addressin cell adhesion molecule 1	2.268	NM_007164
Protein phosphatase 2 (formerly 2A), regulatory subunit B″, alpha	2.26	NM_002718
NY-REN-58 antigen	2.205	NM_016122
Prenylcysteine oxidase 1	2.2	NM_016297
Sialyltransferase 4B (beta-galactoside alpha-2,3-sialyltransferase)	2.16	NM_006927
Hyperpolarization activated cyclic nucleotide-gated potassium channel 2	2.13	NM_001194
Glutamine-fructose-6-phosphate transaminase 2	2.115	NM_005110
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	2.092	NM_012141
Phosphodiesterase 3B, cGMP-inhibited	2.091	NM_000753
Pleiomorphic adenoma gene 1	2.049	NM_002655
Lutheran blood group (Auberger b antigen included)	2.042	NM_005581
Chromosome 1 open reading frame 29	2.036	NM_006820
Stearoyl-CoA desaturase (delta-9-desaturase)	2.035	NM_005063
Solute carrier family 22 (organic cation transporter), member 14	2.032	NM_004803
CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A)	2.025	NM_004715
Membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	2.025	NM_005374
Insulin-like growth factor binding protein 5	0.5	NM_000599
2,4-dienoyl CoA reductase 1, mitochondrial	0.498	NM_001359
5-azacytidine induced gene 2	0.494	NM_022461
Neurofilament, light polypeptide 68 kDa	0.492	NM_006158
Ferredoxin 1	0.48	NM_004109
Natriuretic peptide receptor B/guanylate cyclase B	0.465	NM_000907
Chromosome condensation 1-like	0.455	NM_001268
Keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)	0.454	NM_000526
Prostaglandin-endoperoxide synthase 2	0.445	NM_000963
Bradykinin receptor B1	0.423	NM_000710
Aquaporin 3	0.413	NM_004925
Keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	0.408	NM_005557
Keratin 13	0.405	NM_002274
Ubiquitin specific protease 20	0.399	NM_006676
Reticulon 1	0.396	NM_021136
Tripartite motif-containing 8	0.382	NM_030912
Inhibin, beta A (activin A, activin AB alpha polypeptide)	0.352	NM_002192
Glioblastoma amplified sequence	0.341	NM_001483
Lymphocyte cytosolic protein 2	0.333	NM_005565
v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	0.244	NM_005360
Guanine nucleotide binding protein (G protein)	0.222	NM_002071

ease of transfection, and their high rate of cell proliferation and metastatic potential [Thompson et al., 1992]. Recently, microarray analyses have revealed that Hs578T cells exhibit a mesenchymal profile of gene expression [Charafe-Jauffret et al., 2006], however, Hs578T ER expressing cells display many similarities compared to BC cells expressing endogenous ER. These include the E2-induction of pS2, along with the regulation of ITGA6 and protein phosphatase 2A, the latter of which had previously been reported to occur in MCF-7, but not in ER- Hs578T cell-lines [Bliss et al., 1995; Gopalakrishna et al., 1999].

The ERs were flag-tagged for quantitative purposes, and their expression was shown to be inducible following treatment with Dox. ERα and ERβ function was determined by their ability to regulate pS2 gene expression in an E2 and 4HT-dependent manner. E2 stimulation of Hs578T ERα and ERβ cell-lines resulted in the increased expression of pS2, while 4HT was

TABLE IV. E2 Stimulated Hs578T ERβ Cells

Gene description	Fold change	Accession #
Complement component 3	12.83	NM_000064
Nuclear receptor interacting protein 1	11.39	NM_003489
Chemokine (C-X-C motif) ligand 14	8.585	NM_004887
Interleukin 7 receptor	6.345	NM_002185
Bone morphogenetic protein 2	5.269	NM_001200
Putative lymphocyte G0/G1 switch gene	3.811	NM_015714
Sema domain, immunoglobulin domain (Ig)	3.558	NM_004636
Cystatin SN	3.443	NM_001898
Rho GTPase activating protein 6	3.432	NM_001174
Prostaglandin-endoperoxide synthase 2	3.357	NM_000963
WNT1 inducible signaling pathway protein 1	3.354	NM_003882
LIM domain only 2 (rhombotin-like 1)	3.308	NM_005574
A disintegrin and metalloproteinase domain 15 (metargidin)	3.248	NM_003815
Angiopoietin-like 4	3.181	NM_016109
Placenta-specific 1	3.084	NM_021796
WNT1 inducible signaling pathway protein 2	3.042	NM_003881
Angiotensinogen proteinase inhibitor, clade A	3.041	NM_000029
Rho GDP dissociation inhibitor (GDI) alpha	3.026	NM_004309
Carbonic anhydrase XII	2.913	NM_001218
Alkaline phosphatase, placental (Regan isozyme)	2.788	NM_001632
CCAAT/enhancer binding protein (C/EBP), delta	2.666	NM_005195
v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2.658	NM_005235
Bone morphogenetic protein 5	2.644	NM_021073
Glutaredoxin (thioltransferase)	2.626	NM_002064
Spindlin	2.56	NM_006717
Insulin-like growth factor binding protein 5	2.462	NM_000599
Heat shock 27 kDa protein family, member 7 (cardiovascular)	2.416	NM_014424
Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	2.405	NM_004267
Distal-less homeobox 4	2.405	NM_001934
Sialyltransferase 4B (beta-galactoside alpha-2,3-sialyltransferase)	2.357	NM_006927
Phosphodiesterase 4B, cAMP-specific	2.305	NM_002600
pim-1 oncogene	2.3	NM_002648
EH-domain containing 2	2.277	NM_014601
BCL2-associated athanogene 5	2.238	NM_004873
Tyrosine hydroxylase	2.223	NM_000360
Zinc finger protein 236	2.222	NM_007345
Interleukin 6 signal transducer (gp130, oncostatin M receptor)	2.205	NM_002184
Heat shock transcription factor 1	2.169	NM_005526
Protein phosphatase 2, regulatory subunit B (PR 52), beta isoform	2.153	NM_004576
Natural killer-tumor recognition sequence	2.146	NM_005385
CD34 antigen	2.144	NM_001773
Phosphodiesterase 3B, cGMP-inhibited	2.127	NM_000753
Secreted frizzled-related protein 1	2.117	NM_003012
Fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	2.099	NM_004480
Carbonic anhydrase I	2.063	NM_001738
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	2.051	NM_004566
Lymphoid enhancer-binding factor 1	2.042	NM_016269
Tumor protein D52-like 1	2.038	NM_003287
Carbonic anhydrase IX	2.034	NM_001216
Aryl hydrocarbon receptor nuclear translocator-like	2.032	NM_001178
Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	2.031	NM_006209
Dehydrogenase/reductase (SDR family) member 3	2.018	NM_004753
Flap structure-specific endonuclease 1	0.496	NM_004111
Proliferating cell nuclear antigen	0.491	NM_00259
SWI/SNF related, actin dependent regulator of chromatin	0.488	NM_003078
Phorbol-12-myristate-13-acetate-induced protein 1	0.488	NM_021127
A disintegrin and metalloproteinase domain 19 (meltrin beta)	0.483	NM_023038
FOS-like antigen 1	0.48	NM_005438
Sphingosine kinase 1	0.478	NM_021972
SMC2 structural maintenance of chromosomes 2-like 1 (yeast)	0.478	NM_006444
Pregnancy specific beta-1-glycoprotein 1	0.476	NM_006905
Caspase 9, apoptosis-related cysteine protease	0.468	NM_001229
Synovial sarcoma translocation gene on chromosome 18-like 2	0.464	NM_016305
Renal tumor antigen	0.463	NM_014226
Dmx-like 1	0.455	NM_005509
Actin filament associated protein	0.453	NM_021638
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	0.452	NM_003786
Suppressor of cytokine signaling 2	0.448	NM_003877
Plasminogen activator, urokinase	0.448	NM_002658
Plexin domain containing 1	0.446	NM_020405
Laminin, beta 1	0.432	NM_002291
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	0.426	NM_000609
Interleukin 6 (interferon, beta 2)	0.422	NM_000600
Cyclin E2	0.421	NM_004702

TABLE IV. (*Continued*)

Gene description	Fold change	Accession #
Dapper homolog 1, antagonist of beta-catenin (xenopus)	0.419	NM_016651
DNA replication complex GINS protein PSF2	0.417	NM_016095
Synaptosomal-associated protein, 25 kDa	0.417	NM_003081
Osteoprotegerin	0.415	NM_002546
Prostaglandin E receptor 4 (subtype EP4)	0.387	NM_000958
Musculin (activated B-cell factor-1)	0.385	NM_005098
Connective tissue growth factor	0.384	NM_001901
Disintegrin-like and metalloprotease with thrombospondin type 1 motif, 5	0.376	NM_007038
Insulin-like growth factor binding protein 3	0.373	NM_000598
M-phase phosphoprotein 1	0.371	NM_016195
A disintegrin-like and metalloprotease (reprolysin type)	0.364	NM_006988
Diacylglycerol kinase, delta 130 kDa	0.359	NM_003648
Caldesmon 1	0.356	NM_004342
Dickkopf homolog 1 (Xenopus laevis)	0.346	NM_012242
Collagen, type VIII, alpha 1	0.315	NM_001850
Mesoderm specific transcript homolog (mouse)	0.303	NM_002402
Regulator of G-protein signalling 5	0.269	NM_003617
Ectodermal-neural cortex (with BTB-like domain)	0.267	NM_003633
Oxytocin receptor	0.262	NM_000916
Regulator of G-protein signalling 4	0.233	NM_005613
Jagged 1 (Alagille syndrome)	0.136	NM_000214

unable to reduce pS2 expression below Dox-stimulated levels in Hs578T ERα cells. Unliganded ERα has been reported to associate with the estrogen response element present in the human pS2 promoter, thus potentially explaining the observed ligand independent pS2 mRNA induction in Dox-stimulated Hs578T ER expressing cells, especially in the ERα expressing cell-lines [Metivier et al., 2003].

Treatment of ERα or ERβ cell-lines with 4HT had little effect on the rate of cellular proliferation. Although an upward trend was apparent in 4HT stimulated Hs578T ERα cells, co-treatment with ICI failed to diminish the slight, but non-significant increase in cellular proliferation. Therefore, this trend cannot be adequately explained as being solely an ERα-mediated phenotype. Interestingly, Hs578T ERβ cells stimulated with E2 displayed a significant decrease (~27%) in cellular proliferation, while similarly treated Hs578T ERα cells showed no significant effect. Activation of ERβ has been reported to inhibit the proliferation of MCF-7 cells by repressing c-myc, cyclin D1, and cyclin A gene expression, while increasing the transcription of $p21^{Cip1}$ and $p27^{Kip1}$, leading to G2 cell cycle arrest [Paruthiyil et al., 2004]. Our data indicates the E2-dependent inhibition of Hs578T ERβ proliferation resulted in a ~50% decrease in cells entering S-phase, with a concurrent 17% increase in G0/1 cell cycle arrest. E2-regulated genes which may be responsible for the observed decrease in Hs578T ERβ include the notch ligand jagged-1 and cyclin E2, both of which have been implicated in tumor progression and decreased patient survival rates [Reedijk et al., 2005; Yan et al., 2006]. In any case, the observed E2-dependent decrease in Hs578T ERβ cell proliferation is supported by several recent publications [Paruthiyil et al., 2004; Strom et al., 2004; Helguero et al., 2005], although all of these reports examined the role of ERβ in cell-lines expressing both ER isoforms, or when ERβ was introduced to BC cells expressing endogenous ERα.

Several clinical studies have attempted to decipher the role of endocrine therapy on the proliferation of ERβ+/ERα− BC, and have suggested a positive correlation between certain genes involved in proliferation (Ki67, cyclin A) and the expression of ERβ [Jensen et al., 2001; O'Neill et al., 2004; Murphy and Watson, 2006]. To our knowledge, however, this is the first report of an E2-dependent decrease in cellular proliferation in a BC cell model stably expressing only ERβ. Conversely, the lack of any significant E2-mediated increase in Hs578T ERα cellular proliferation is consistent with other studies were ERα has been re-expressed in ER- BC cell-lines [Moggs et al., 2005].

ERβcx is expressed in many ER+ BC's, and thus has been examined as a possible marker for BC progression/metastasis. Results of such studies remain inconclusive, as ERβcx/ERα+ BC biopsies displayed differing regulation of ERα-mediated genes, including the E2-mediated regulation of PgR [Saji et al., 2002]. Also, the cumulative results of several clinical

TABLE V. 4HT Stimulated Hs578T ERβ Cells

Gene description	Fold change	Accession #
Interleukin 8	3.56	NM_000584
Phosphodiesterase 3B, cGMP-inhibited	3.279	NM_000753
Survival of motor neuron 1, telomeric	3.111	NM_000344
Interleukin 1, beta	3.027	NM_000576
Growth arrest-specific 1	2.931	NM_002048
Friend leukemia virus integration 1	2.805	NM_002017
CD209 antigen-like	2.762	NM_014257
Transcription termination factor, RNA polymerase II	2.588	NM_003594
A kinase (PRKA) anchor protein (gravin) 12	2.505	NM_005100
EGF-containing fibulin-like extracellular matrix protein 1	2.431	NM_004105
Integrin, alpha 6	2.38	NM_000210
Heat shock transcription factor 1	2.353	NM_005526
Suppression of tumorigenicity	2.302	NM_013437
TEA domain family member 1 (SV40 transcriptional enhancer factor)	2.258	NM_021961
Potassium channel, subfamily K, member 4	2.167	NM_016611
Sphingosine kinase 1	2.165	NM_021972
Rho GDP dissociation inhibitor (GDI) alpha	2.128	NM_004309
Neuropilin 2	2.121	NM_003872
CD28 antigen (Tp44)	2.113	NM_006139
BTB and CNC homology 1, basic leucine zipper transcription factor 2	2.101	NM_021813
Solute carrier family 26 (sulfate transporter), member 2	2.1	NM_000112
Cryptochrome 1 (photolyase-like)	2.086	NM_004075
Casein beta	2.027	NM_001891
Myoglobin	2.026	NM_005368
Baculoviral IAP repeat-containing 4	2.022	NM_001167
Serum/glucocorticoid regulated kinase	2.006	NM_005627
FK506 binding protein 4, 59 kDa	0.5	NM_002014
Trinucleotide repeat containing 5	0.496	NM_006586
Wolfram syndrome 1 (wolframin)	0.494	NM_006005
Retinitis pigmentosa 2 (X-linked recessive)	0.491	NM_006915
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	0.488	NM_004398
Secreted frizzled-related protein 1	0.483	NM_003012
Cargo selection protein (mannose 6 phosphate receptor binding protein)	0.482	NM_005817
Hexokinase 1	0.481	NM_000188
Beta-site APP-cleaving enzyme	0.48	NM_012104
Polymerase (DNA directed) sigma	0.48	NM_006999
Translocase of inner mitochondrial membrane 17 homolog B (yeast)	0.479	NM_005834
Stromal cell protein	0.478	NM_018845
Mst3 and SOK1-related kinase	0.478	NM_016542
Sialyltransferase 4C (beta-galactoside alpha-2,3-sialyltransferase)	0.477	NM_006278
2,4-dienoyl CoA reductase 1, mitochondrial	0.477	NM_001359
Tumor protein p53 binding protein, 1	0.477	NM_005657
LIM domain binding 2	0.476	NM_001290
Tumor protein p73-like	0.474	NM_003722
Slit homolog 3 (Drosophila)	0.469	NM_003062
Cyclin G2	0.469	NM_004354
Purinergic receptor P2Y, G-protein coupled, 5	0.466	NM_005767
Zinc finger protein 36, C3H type, homolog (mouse)	0.462	NM_003407
T-cell, immune regulator 1, ATPase, lysosomal V0 protein a isoform 3	0.462	NM_006019
Dimethylarginine dimethylaminohydrolase 2	0.46	NM_013974
General transcription factor IIH, polypeptide 2, 44 kDa	0.458	NM_001515
Phosphatidylinositol transfer protein, cytoplasmic 1	0.457	NM_012417
Immunoglobulin superfamily, member 4	0.457	NM_014333
Tax interaction protein 1	0.455	NM_014604
Aldo-keto reductase family 1, member C3	0.455	NM_003739
Inhibin, beta A (activin A, activin AB alpha polypeptide)	0.454	NM_002192
Similar to rat tricarboxylate carrier-like protein	0.45	NM_030971
Polymerase (DNA-directed), delta 4	0.449	NM_021173
Enolase 2, (gamma, neuronal)	0.447	NM_001975
FOS-like antigen 2	0.447	NM_005253
Inhibin, beta B (activin AB beta polypeptide)	0.446	NM_002193
Retinoic acid receptor, alpha	0.442	NM_000964
Inositol polyphosphate-4-phosphatase, type II, 105 kDa	0.441	NM_003866
Homer homolog 1 (Drosophila)	0.441	NM_004272
Solute carrier family 9 isoform 3 regulatory factor 2	0.438	NM_004785
Choline kinase	0.438	NM_001277
Nuclear receptor coactivator 1	0.438	NM_003743
Intercellular adhesion molecule 3	0.437	NM_002162
Transcription factor AP-2 gamma	0.429	NM_003222
N-myc downstream regulated gene 1	0.426	NM_006096
Nuclear factor of kappa light polypeptide gene enhancer in B-cells	0.426	NM_020529
t-complex-associated-testis-expressed 1-like	0.424	NM_006520
Carbonic anhydrase IX	0.423	NM_001216
TNF receptor-associated factor 4	0.422	NM_004295

TABLE V. (*Continued*)

Gene description	Fold change	Accession #
Solute carrier family 17 member 7	0.419	NM_020309
Kinetochore associated 2	0.419	NM_006101
Chromosome X open reading frame 12	0.416	NM_003492
Stanniocalcin 2	0.415	NM_003714
Carbonic anhydrase XII	0.413	NM_001218
Stearoyl-CoA desaturase (delta-9-desaturase)	0.413	NM_005063
Growth arrest and DNA-damage-inducible, beta	0.41	NM_015675
Plexin B3	0.408	NM_005393
Histone 1, H2bd	0.407	NM_021063
Paired-like homeodomain transcription factor 1	0.403	NM_002653
Phosphatidylcholine transfer protein	0.4	NM_021213
MARCKS-like protein	0.396	NM_023009
Zic family member 1 (odd-paired homolog, Drosophila)	0.394	NM_003412
GM2 ganglioside activator protein	0.393	NM_000405
MAD1 mitotic arrest deficient-like 1 (yeast)	0.387	NM_003550
Four and a half LIM domains 2	0.383	NM_001450
Spinocerebellar ataxia 7	0.383	NM_000333
Latent transforming growth factor beta binding protein 1	0.378	NM_000627
Interleukin 7 receptor	0.376	NM_002185
PTK9L protein tyrosine kinase 9-like (A6-related protein)	0.375	NM_007284
Sperm associated antigen 4	0.368	NM_003116
Neurofilament, light polypeptide 68 kDa	0.367	NM_006158
Glypican 1	0.367	NM_002081
Regulator of G-protein signalling 5	0.363	NM_003617
Solute carrier family 10 member 3	0.35	NM_019848
F-box and leucine-rich repeat protein 4	0.343	NM_012160
Insulin-like growth factor binding protein 5	0.331	NM_000599
Interleukin 16 (lymphocyte chemoattractant factor)	0.33	NM_004513
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.314	NM_005398
Ubiquitin specific protease 39	0.302	NM_006590
Anaphase-promoting complex subunit 10	0.299	NM_014885
Solute carrier family 9 isoform 3 regulatory factor 1	0.294	NM_004252
E4F transcription factor 1	0.293	NM_004424
unc-51-like kinase 1 (C. elegans)	0.292	NM_003565
Gap junction protein, alpha 1, 43 kDa (connexin 43)	0.274	NM_000165
Transglutaminase 2	0.256	NM_004613
Histone 1, H2bh	0.251	NM_003524
Sialic acid binding Ig-like lectin 7	0.237	NM_014385
TGFB1-induced anti-apoptotic factor 1	0.209	NM_004740
PDZ domain containing 1	0.177	NM_002614
Insulin-like growth factor binding protein 4	0.171	NM_001552
Natriuretic peptide receptor B/guanylate cyclase B	0.13	NM_000907
Prostaglandin-endoperoxide synthase 2	0.0526	NM_000963

studies revealed a discordant linkage between the presence of ERβcx and patient survival rates [Palmieri et al., 2004; Esslimani-Sahla et al., 2005]. Previously published data aimed at deciphering the role of ERβcx in BC proliferation, involving the introduction of ERβcx into ERα expressing MCF-7 cells, resulted in an inhibition of ERα-induced proliferation [Omoto et al., 2003]. Interestingly, our studies show that the stable expression of ERβcx in Hs578T cells did not alter the rate of cellular proliferation, lending support to previous data indicating that ERβcx cannot function as a typical nuclear hormone transcription factor. The mechanism by which ERβcx inhibits ERα activity, including its ability to regulate proliferation, likely involves the sequestering of nuclear co-regulator proteins necessary for ERα-mediated gene expression, or directly by forming heterodimers with ERα, thereby inhibiting the ability of ERα to bind DNA [Ogawa et al., 1998].

Gene array analyses of Hs578T ERα and ERβ cell-lines, treated with E2 or 4HT, revealed that the pattern of E2-regulated gene expression was largely unique to either ER isoform. Several studies have examined the action of the ERβ on endogenous gene expression in 4HT and E2-stimulated BC cells [Hayashi et al., 2003; Omoto et al., 2003]. However, these studies utilized ERα expressing MCF-7 cells stably transfected with ERβ, and therefore, a comparison of ER isoform-specific gene expression was not possible. Our laboratory and others have recently reported that E2-stimulation of human fetal osteoblast (hFOB) and U2OS ERα and ERβ expressing cells resulted in largely unique patterns of gene expression [Waters et al., 2001; Rickard et al., 2002; Monroe et al., 2003a; Kian Tee et al., 2004; Stossi et al., 2004;

TABLE VI. Commonly Regulated E2 and 4HT Dependent Genes in Hs578T ERα and ERβ Cells

Gene description	Fold change E2	Fold change 4HT	Accession #
A: Hs578T ERα E2 and 4HT regulated genes			
Protein phosphatase 2, reg. Subunit B″, alpha	3.875	2.26	NM_181897
Prostaglandin-endoperoxide synthase 2	3.402	0.445	NM_000963
B: Hs578T ERβ E2 and 4HT regulated genes			
Interleukin 7 receptor	6.345	0.376	NM_002185
Prostaglandin-endoperoxide synthase 2	3.357	0.0526	NM_000963
Rho GDP dissociation inhibitor (GDI) alpha	3.026	2.128	NM_004309
Carbonic anhydrase XII	2.913	0.413	NM_001218
Insulin-like growth factor binding protein 5	2.462	0.331	NM_000599
Heat shock transcription factor 1	2.169	2.353	NM_005526
Phosphodiesterase 3B, cGMP-inhibited	2.127	3.279	NM_000753
Secreted frizzled-related protein 1	2.117	0.483	NM_003012
Carbonic anhydrase IX	2.034	0.423	NM_001216
Sphingosine kinase 1	0.478	2.165	NM_021972
Gene description	**Fold change ERα**	**Fold change ERβ**	**Accession #**
C: Hs578T ERα and ERβ E2 regulated genes			
Prostaglandin-endoperoxide synthase 2	3.402	3.357	NM_000963
Interleukin 6 signal transducer	3.005	2.205	NM_175767
Autotaxin	2.273	2.031	NM_006209
Aryl hydrocarbon receptor	2.007	2.032	NM_001621
Sphingosine kinase 1	0.488	0.478	NM_021972
Plasminogen activator, urokinase	0.442	0.448	NM_002658
Interleukin 6 (interferon, beta 2)	0.439	0.422	NM_000600
D: Hs578T ERβ and ERβ 4HT regulated genes			
Stearoyl-CoA desaturase (delta-9-desaturase)	2.035	0.413	NM_005063
Insulin-like growth factor binding protein 5	0.5	0.331	NM_000599
2,4-dienoyl CoA reductase 1, mitochondrial	0.498	0.477	NM_001359
Neurofilament, light polypeptide 68 kDa	0.492	0.367	NM_006158
Natriuretic peptide receptor B/guanylate cyclase	0.465	0.13	NM_000907
Prostaglandin-endoperoxide synthase 2	0.445	0.0526	NM_000963
Inhibin, beta A	0.352	0.454	NM_002192

Monroe et al., 2005]. Data obtained from microarray analyses of E2-stimulated Hs578T ERα and ERβ BC cells supports the ER isoform specificity observed in similarly treated U2OS cell-lines. Additionally, the 4HT treated cells revealed a largely unique subset of regulated genes in an isoform-dependent manner, which may explain the divergent responses elicited by ER+ BC tumors following TAM therapy [Jirstrom et al., 2005; Gururaj et al., 2006].

Although ERβcx cannot bind ligand, it can modulate both ERα and ERβ-dependent gene expression when co-expressed in certain cell types. Recently, E2-stimulated HEK293 cells transiently co-transfected with ERβ and ERβcx displayed a significant increase in pS2 mRNA expression as compared to cells transfected with ERβ alone [Leung et al., 2006]. Microarray analyses of E2-stimulated MCF-7 cells stably expressing ERβcx showed a marked difference

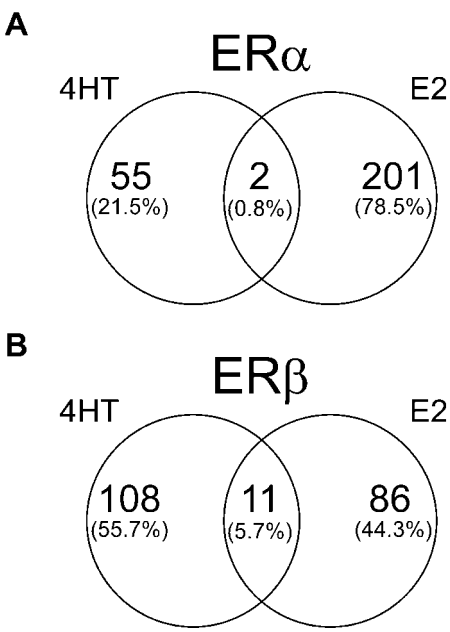


Fig. 5. E2 and 4HT stimulation of Hs578T ERα and ERβ cells displayed largely unique ligand-dependent patterns of gene regulation. Hs578T ERα and ERβ cells were stimulated with 100 ng/ml Dox for 24 h in CS media, and subsequently treated with E2 (10^{-8} M) or 4HT (10^{-7} M) for an additional 24 h. Total RNA was isolated (Trizol, Invitrogen) and 4 μg were subjected to microarray analyses (Human Focus Array, Affymetrix). Venn diagrams comparing E2 and 4HT treated Hs578T ERα and ERβ cell lines were generated using GeneSpring 7 software (Silicon Genetics). **Panels A** and **B**: Genes regulated specifically by E2 and 4HT are represented by the numbers appearing outside of the intersecting circles. Those genes regulated by either E2 or 4HT are represented by the numbers appearing inside the overlapping circles.

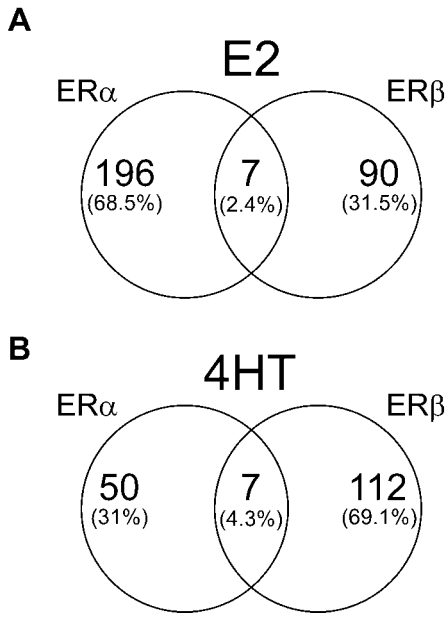


Fig. 6. E2 and 4HT stimulation of Hs578T ERα and ERβ cells displayed largely unique ER isoform-dependent patterns of gene regulation. Hs578T ERα or ERβ cells were treated and analyzed as described in Figure 5. **Panels A** and **B**: The number of ERα and ERβ specific genes are represented by the numbers appearing outside of the intersecting circles. The number of genes which were regulated by either ER isoform appear inside the overlapping circles.

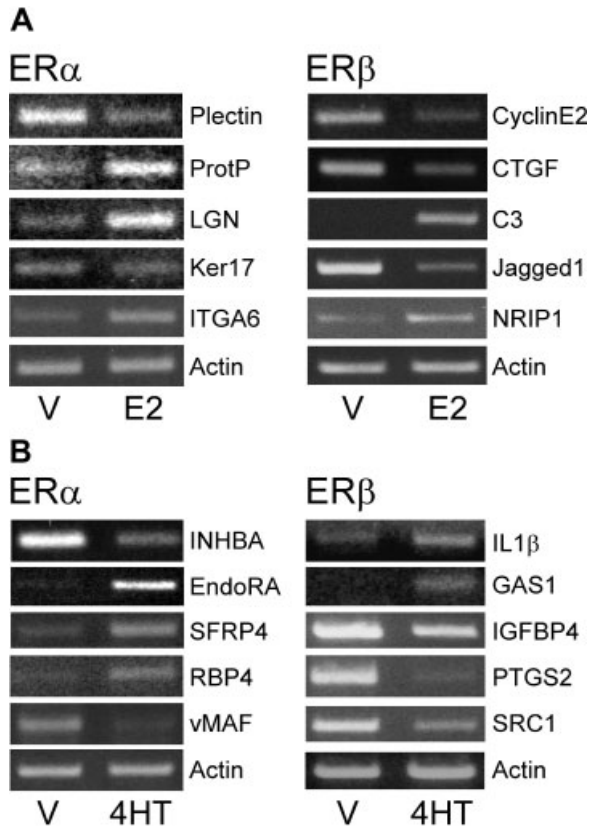


Fig. 7. RT-PCR confirmation of Hs578T ERα and ERβ microarray data. Hs578T ERα and ERβ cells were cultured in CS media supplemented with Dox (100 ng/ml) for 24 h, followed by stimulation with V, E2 (10^{-8} M), or 4HT (10^{-7} M) for an additional 24 h. Total RNA was isolated (Trizol, Invitrogen), and 4 μg reverse transcribed into cDNA. **Panel A**: Five representative genes regulated by E2 in Hs578T ERα and ERβ cells, as determined by microarray analyses, were amplified by PCR and visualized by ethidium bromide staining in 1.5% (w/w) Agarose gels. Actin controls were used to ensure equivalent loading. **Panel B**: Five representative genes regulated by 4HT in Hs578T ERα and ERβ cells, as determined by microarray analyses, were assayed as described in Figure 6A.

in the profile of gene expression as compared to E2-stimulated MCF-7 parental cells [Omoto et al., 2003]. However, the MCF-7/ERβcx studies used small custom made cDNA arrays, and the authors data regarding the lack of any significant difference in E2-stimulated MCF/ERβ gene expression profiles, compared to E2-stimulated MCF parental cells, is not supported

TABLE VII. Hs578T ERβ E2-Regulated Genes Involved in Proliferation

Gene	Fold change in ERβ	Fold change in ERα
A: Proliferation (cell cycle) agonists		
FOSL-1	↓2.1	NR
IL-6	↓2.4	↓2.3
Jagged-1	↓7.4	NR
PCNA	↓2.0	NR
Cyclin-E2	↓2.4	NR
MPHOSPH-1	↓2.7	NR
PFS-2	↓2.4	NR
SOCS-2	↓2.2	NR
SPHK-1	↓2.1	↓2.0
OPG	↓2.4	NR
CTCG	↓2.6	NR
SMC2L-1	↓2.1	NR
B: Proliferation (cell cycle) antagonists		
SFRP-1	↑2.1	NR
IGFBP-5	↑2.5	NR

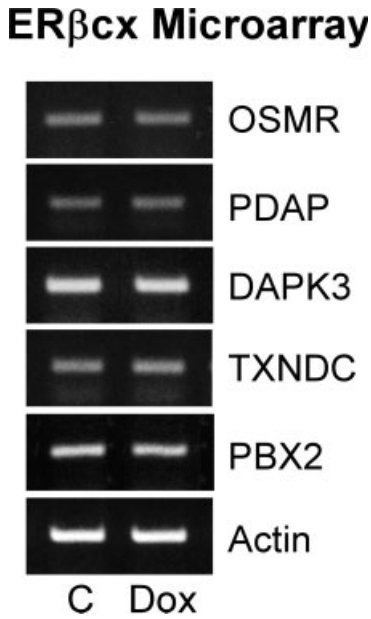


Fig. 8. RT-PCR failed to confirm Hs578T ERβcx mircroarray data analyzed at the 1.5-fold threshold. Hs578T ERβcx cells were cultured in CS media supplemented w/wo Dox (100 ng/ml) for 48 h, with fresh CS ± Dox media being replaced following the initial 24 h incubation. RNA was isolated as described in Figure 7. Three Dox-dependent genes regulated ≥2-fold and 2 genes regulated approxmiately <2-fold were amplified by PCR and visualized as described in Figure 7. No Dox-dependent regulation was observed in the five genes selected for RT-PCR confirmation.

by published data obtained using ER expressing U2OS cells [Monroe et al., 2005].

To date, it has not been reported whether the expression of ERβcx in an ER- BC cell-line could alter gene expression profiles. The ability to control the expression of ERβcx in the Hs578T Dox inducible ERβcx cell-line enabled us to analyze what effects ERβcx expression may have on Hs578T gene expression. In the studies presented here, ERβcx alone did not significantly alter the gene expression profile of Hs578T cells. The explanation for the lack of observed ERβcx-mediated gene regulation is unknown, but is likely due to the same reasons which explained the lack of any ERβcx effect on the rate of Hs578T cellular proliferation, especially the inability of ERβcx to bind DNA akin to the actions of a typical nuclear hormone receptor.

ERα remains the primary target of endocrine therapy in women diagnosed with hormone sensitive BC. In postmenopausal women, therapies include TAM, which competes with E2 for ER binding, along with E2 reducing aromatase inhibitors and the ER downregulator fluvestrant [Ingle, 2004; Ingle et al., 2006]. The fact that endocrine agents have varying levels of activity and incomplete cross-resistance, demonstrated by the ability to obtain responses when given sequentially, illustrates the complexity of the biology in ERα-positive tumors. Our studies indicate that ERβcx probably does not by itself play a role in regulating the response of BC to endocrine therapy. However, the fact that ERβcx is expressed in many BC tumors, and that ERβcx has been reported to interact with both ERα and ERβ, suggests that ERβcx may be involved in mediating E2/SERM responses. This could be accomplished by either a direct interaction with ER's, or through a change in ERβ parental gene expression leading to higher levels of the alternatively spliced ERβcx product. In turn, this would result in a reduction of both ERβ-mediated gene expression and subsequent downstream ERβ-dependent cellular phenotypes. The findings of this paper demonstrate not only markedly different patterns of gene regulation by ERα and ERβ isoforms, but also an ER isoform specific action on BC cell proliferation.

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