Support of a bi-faceted role of estrogen receptor β (ER β) in ER α -positive breast cancer cells

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Abstract

The expression of estrogen receptor α (ER α) in breast cancer identifies patients most likely to respond to endocrine treatment. The second ER, ER β , is also expressed in breast tumors, but its function and therapeutic potential need further study. Although *in vitro* studies have established that ER β opposes transcriptional and proliferative functions of ER α , several clinical studies report its correlation with proliferative markers and poorer prognosis. The data demonstrate that ER β opposes ER α are primarily based on transient expression of ER β . Here, we explored the functions of constitutively expressed ER β in ER α -positive breast cancer lines MCF7 and T47D. We found that ER β , under these conditions heterodimerized with ER α in the presence and absence of 17 β -estradiol, and induced genome-wide transcriptional changes. Widespread anti-ER α signaling was, however, not observed and ER β was not antiproliferative. Tamoxifen antagonized proliferation and ER-mediated gene regulation both in the presence and absence of ER β . In conclusion, ER β 's role in cells adapted to its expression appears to differ from its role in cells with transient expression. Our study is important because it provides a deeper understanding of ER β 's role in breast tumors that coexpress both receptors and supports an emerging bi-faceted role of ER β .

Key Words

- ▶ estrogen receptor α
- ▶ estrogen receptor β
- ▶ breast cancer
- ▶ SERM
- ▶ gene expression

Endocrine-Related Cancer
(2014) 21, 143–160

Introduction

The role of estrogen receptor α (ER α (*ESR1*)) as a biological marker and target in breast cancer therapy is clear. ER α antagonists, such as tamoxifen, or estrogen ablation using aromatase inhibitors are efficient therapeutic approaches in the treatment of ER α -positive breast cancer. A second ER, ER β (*ESR2*), was discovered in 1996 (Kuiper *et al.* 1996, Mosselman *et al.* 1996) and is the predominant ER in normal breast (Kuiper *et al.* 1996). Clinical studies show that though ER β expression decreases during tumor progression (Roger *et al.* 2001, Palmieri *et al.* 2002, Speirs *et al.* 2002, Koehler *et al.* 2005, Zhao *et al.* 2008), a large

proportion, between 39 and 77%, of all breast cancer tumors coexpress both ERs (Fuqua *et al.* 2003, Shaaban *et al.* 2003, O'Neill *et al.* 2004, Speirs *et al.* 2004, Saunders 2006, Honma *et al.* 2008, Novelli *et al.* 2008, Shaaban *et al.* 2008, Li *et al.* 2010a, Marotti *et al.* 2010, Murphy & Leygue 2012, Powell *et al.* 2012, Braun *et al.* 2013). ER β is therefore a potential marker and target, in these tumors, that could enhance the use of endocrine therapy. Albeit both ERs are activated by estrogen, their ligand-binding domains allow for receptor-selective ligands. Their DNA-binding domains are highly conserved and they share between 46 and 73%

of chromatin-binding sites (Matthews & Gustafsson 2003), although distinct regions of binding have also been defined for each receptor (Charn *et al.* 2010, Zhao *et al.* 2010, Grober *et al.* 2011). The receptors' N-terminal domains are structurally different and lead to different abilities in their interaction with coregulators (Warnmark *et al.* 2001), resulting in a higher transactivation capacity for ER α (Mosselman *et al.* 1996).

The function of ER β in breast cancer is not clearly understood (Leygue & Murphy 2013) and ER β is currently not used in the diagnosis or treatment of breast cancer patients. One obstacle in the field is that available breast cancer cell lines do not express sufficient levels of endogenous ER β for consistent mechanistic and functional studies (Holbeck *et al.* 2010), and mRNA levels in the tissues are also persistently low in comparison with ER α (Sayers *et al.* 2012). To explore its role, ER β thus has to be introduced exogenously. The majority of studies has used transient or inducible induction of ER β and indicates that ER β in ER α -positive breast cancer cells counteracts the proliferative and transcriptional functions of ER α (Lazennec *et al.* 2001, Paruthiyil *et al.* 2004, Strom *et al.* 2004, Chang *et al.* 2006, Williams *et al.* 2008, Horimoto *et al.* 2011). Although some clinical studies link ER β expression to better outcomes (Omoto *et al.* 2001, Nakopoulou *et al.* 2004, Sugiura *et al.* 2007), others have correlated ER β expression to the proliferation marker Ki67 in the primary breast tumors (Jensen *et al.* 2001, O'Neill *et al.* 2004, Honma *et al.* 2013) and associated its expression with a higher risk of relapse in node-positive breast cancer patients (Novelli *et al.* 2008). In ER α -negative tumors, ER β expression has been correlated to a higher aneuploidy, indicating a more aggressive phenotype (Fuqua *et al.* 2003). The body of data correlating ER β with both anti-proliferative and proliferative parameters has led to the suggestions of a bi-faceted role for ER β (Leygue & Murphy 2013). One clinical study indicated that tamoxifen treatment of ER β -positive tumors may be beneficial: ER β was associated with better survival after long-term tamoxifen treatment in post – but not pre-menopausal women (Honma *et al.* 2008). A deeper understanding of the role and mechanism of ER β may help us to improve the treatment for breast cancer patients.

We set out to investigate the effect of constitutive expression of ER β in breast cancer cells. Our aim in this study was to complement the studies of transient or short-term effects of ER β and gain additional insight into its mechanism and potential clinical applications.

Subjects and methods

Cell culture, ER β expression, and treatments

Duplicate T47D-ER β and T47D-control and triplicate MCF7-ER β and MCF7-control mixed-cell populations were generated by lentiviral transductions with CMV-driven, FLAG-tagged full-length ER β cDNA (530 aa) or empty vector, respectively, and selected as described previously (Hartman *et al.* 2009). Two or three separate transduction replicates in the shape of mixed-cell populations, for each control and ER β cell line, were used for all experiments. The T47D and MCF7 cells were passaged and serum starved before ligand treatments, as described previously (Williams *et al.* 2008, Katchy *et al.* 2012). 17 β -estradiol (E₂; Sigma) at 10 nM concentration was used as agonist for both receptors, PPT and DPN (Tocris Bioscience, Bristol, UK), at 1–10 nM as selective ligands for ER α and ER β , respectively, KB101471 (gift from KaroBio, Huddinge, Sweden) at 0.5 nM and LY3201 (gift from Eli Lilly) at 1 nM were used as selective ligands for ER β . Tamoxifen (Sigma) at 1 μ M and ICI (Tocris Bioscience) at 10 nM were used as antagonists. All ligand dilutions were made in ethanol or DMSO (vehicle).

RNA extraction, cDNA synthesis, and qPCR

RNA was isolated using TRIzol (Invitrogen) or QIAzol (Qiagen), purified with RNeasy Mini Kit and treated with DNase I (both Qiagen), according to manufacturer's instructions. Synthesis of cDNA, from 0.5 or 1 μ g total RNA, and qPCRs and analysis were performed as described previously (Williams *et al.* 2008, Katchy *et al.* 2012). The samples were run in triplicate, with at least two mixed-cell populations per each cell type (ER β and control respectively) and negative controls. The primer sequences are provided upon request. The gene expression was normalized to 18S rRNA, GAPDH, or ARHGDI expression.

Western blotting and immunoprecipitation

Western blotting was carried out according to procedures described earlier (Edvardsson *et al.* 2011). The following antibodies and dilutions were used: ER α 1:1000 (HC-20, Santa Cruz Biotechnology), ER β 1:1000 (NBP1-04936, Novus Biologicals, Littleton, CO, USA), β -actin 1:20 000 (AC-15, Sigma), anti-mouse IgG 1:6000 (NA931V, GE Healthcare, Piscataway, NJ, USA), anti-rabbit IgG 1:6000 (NA934V, GE Healthcare), and anti-chicken IgY 1:6000

(31401, Thermo Scientific, Rockford, IL, USA). For immunoprecipitation (IP) experiments, cells were harvested from 15-cm plates. The cells were incubated in RIPA buffer 5 min, washed twice with cold PBS, collected in microcentrifuge tubes, passed through a syringe, and incubated at least 4 h on a nutator at 4 °C. Supernatants were collected as described earlier, and protein concentration was determined. Equal amounts of protein were used for each IP, which was performed with Anti-FLAG M2 affinity gel (Sigma–Aldrich), as described by the manufacturer. Essentially, part of the input was saved before incubating the lysates overnight on a nutator at 4 °C. The samples were boiled in Laemmli sample buffer for 5 min before performing western blot as described earlier.

Ligand-binding and luciferase assays

Ligand-binding assays were carried out with tritium-labeled E₂, essentially as described earlier (Edvardsson et al. 2013). Luciferase estrogen-response element (ERE) transactivation assays were carried out with the cells seeded in the 12-well plates at an approximate confluency of 80%. Each well was transfected with 500 ng ERE–TATA–Luc plasmid and 50 ng RSV–gal plasmid using Lipofectamine LTX and Opti-MEM (Invitrogen). The cells were treated with vehicle and E₂ as described previously, before washing with PBS and lysing. Lysis buffer and luciferase reagents were obtained from BioVision (Luciferase Reporter Assay Kit, Milpitas, CA, USA). Luciferase and X-gal activity was read on a Victor X4 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). Luciferase activity was normalized to X-gal activity.

Microarray analysis and bioinformatics

Microarray experiments and analyses were carried out basically as described previously (Williams et al. 2008, Edvardsson et al. 2011). Genes of interest and subjects, for confirmation using qPCR, were chosen among the genes with *B* values over 0 and an *M* value (=log₂ (Cy5/Cy3)) higher than |0.4|. Regulations that were not detected on at least three out of four arrays were discarded. Overrepresentation/enrichment analyses were carried out in Pathway Studio (Ariadne Genomics, Rockville, MD, USA), using the software's Gene Ontology gene sets and the ResNet motif-based database, and the transcription factor (TF) target gene set was provided by Broad Institute's Molecular Signatures Database based on TRANSFAC motifs (Subramanian et al. 2005). *P* values indicated in these data are calculated with Fisher's exact test. Microarray data is

available on NCBI's GEO data repository, under the accession numbers GSE45047 and GSE45557.

Cell counting and MTS assay

The cells were seeded at indicated density in 25-cm² flasks and grown over the indicated number of days before counting of viable cells using Trypan-blue staining, as described previously (Dey et al. 2012). For the MTS assay, cells were seeded in a 96-well plate at a density of 2500–5000 cells/well and thereafter treated as described earlier with indicated ligands. The MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) was carried out according to the manufacturer's instructions and as described previously (Dey et al. 2012). Each treatment was performed in three to five technical replicates in the same plate. The plates were read on a SpectroMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Wound-healing assay

Cell migration was measured using wound-healing (*in vitro* scratch) assays. The assay was carried out with cells seeded in a 12-well plate. Upon confluency, a scratch was made with a pipette tip and pictures of the scratch were taken with microscope camera at 0 and 24 h. The cells were treated with vehicle or E₂ in 0.5% DCC–FBS-supplied medium. ImageJ (Rasband 1997) was used to analyze the scratch area, which was used to calculate migration.

PARP cleavage

The cleavage of PARP was examined using anti-PARP antibody (#9542, Cell Signaling, Danvers, MA, USA), dilution 1:1000, detecting full PARP (116 kDa) and cleaved fragment at 89 kDa. Relative cleavage was calculated, normalized to β -actin. Western blotting was performed as described previously. Ligand treatment was done according to the standard procedure with the addition of cisplatin at 10 μ g/ml at 0 h.

Statistical analysis

Statistical significance of data was assayed using two-sample Student's *t*-test with two-tailed distribution, assuming homoscedasticity. The error bars show one s.d. and unless otherwise stated, asterisks are used as follows: **P*<0.05, ***P*<0.01, and ****P*<0.001.

Results

We assessed the functional and transcriptional effects of stable ER β expression in two models for the luminal subtype of breast tumors: the human epithelial ER α -positive breast cancer cell lines: T47D and MCF7 (Ross & Perou 2001, Lacroix & Leclercq 2004, Neve *et al.* 2006, Kao *et al.* 2009). The cell lines are derived from ductal carcinoma and adenocarcinoma, respectively, and both are dependent on ER α and estrogen for growth. Previous studies have characterized the ligand-activated gene regulation by ER α and corresponding promotion of cell proliferation and cell survival, along with anti-proliferative effect of ER antagonists (Frasor *et al.* 2003, 2004). The effects of transient or inducible ER β expression in these cell lines have also been characterized (Paruthiyil *et al.* 2004, Strom *et al.* 2004, Chang *et al.* 2006, Williams *et al.* 2008).

Lentivirus-transduced cells expressed functional ER β that forms heterodimer with ER α

We used lentivirus transduction followed by selection with blasticidin to generate T47D and MCF7 cells stably expressing full-length, FLAG-tagged ER β in replicated mixed-cell populations, in duplicates for T47D and in triplicates for MCF7 cells. ER β transcript and protein levels were significantly increased in the ER β -transduced cells

compared with the barely detectable levels in parental or control-transduced cells (Fig. 1A and B). Competitive ligand-binding assay with tritium-labeled E₂ showed that the T47D-ER β cells contained more than double as many ligand-binding receptors as the T47D control cells (Fig. 1C), indicating an ER β :ER α ratio of $\sim$ 2:1. MCF7-ER β cells also exhibited increased ligand binding after expression of ER β , and siRNA of ER α visualized the contribution by ER β (Fig. 1D). In MCF7 cells, an ERE-luciferase transactivation assay showed that ER β -expression enhanced transcriptional transactivation from an ERE (Fig. 1E). Both receptors are known to form hetero- and homodimers with each other and to colocalize in the cell nuclei of clinical breast tumors and cell lines (Powell *et al.* 2012). We showed, using Co-IP experiments after vehicle and E₂ treatment, that ER β and ER α formed heterodimers in both MCF7-ER β and T47D-ER β cells, with or without E₂ stimuli (Fig. 2), supporting a previous report (Pace *et al.* 1997).

ER β affected ER α expression in MCF7 but not in T47D cells

ER α has an ERE-containing promoter and can be regulated by both ERs (Castles *et al.* 1997, Donaghue *et al.* 1999). We assessed whether the levels of ER α changed upon the expression of ER β . In T47D cells, the levels of ER α were not affected by neither ER α itself nor ER β , as measured using

Figure 1

Characterization of ER β expression in breast cancer cell lines T47D and MCF7. Using lentivirus transduction ER β was expressed in duplicate (T47D) and triplicate (MCF7) mixed-cell populations. (A) ER β mRNA levels were increased in T47D-ER β and MCF7-ER β mixed-cell populations compared with cells transduced with only control vector, as measured by qPCR. ER β mRNA in parental and control cells of both cell lines were barely detectable (C_T values > 32). (B) ER β protein was readily detectable in T47D-ER β and MCF7-ER β cells, measured by western blot of FLAG IP. Cell lysates were subjected to immunoprecipitation (IP) with a FLAG antibody before blotting with ER β antibody. The expressed ER β band is at 1 kDa higher than

recombinant ER β (at 59 kDa, not shown), due to the 8-aa FLAG tag. FLAG antibody heavy chain is coeluted and cross-reacts with secondary antibody, as indicated. IP/WB was carried out in duplicate for each mixed-cell population, representative experiment shown. (C and D) Radioactive ligand-binding assay shows that ligand-binding capacity is enhanced by ER β expression in both cell lines, and remains high also when ER α is silenced in MCF7 cells. Efficacy of silencing is shown in Fig. 6C. (E) Luciferase ERE-transactivation assay in MCF7 cells confirms enhanced transactivation through ER β expression upon E₂ treatment. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2

ER β and ER α heterodimerize in T47D and MCF7 cells. Co-IPs using antibodies directed against ER α and FLAG (for ER β) show that the ERs form heterodimers when coexpressed, both in absence or presence of ligand, in (A) T47D and (B) MCF7 cells. Co-IPs were performed after 24-h treatment with vehicle or 10 nM E₂. ER α was detectable in Co-IPs of the FLAG-tagged ER β from both vehicle and E₂-treated T47D-ER β and MCF7-ER β cells. In the control cells where ER β was not expressed, no Co-IP bands were detected, supporting the specificity of the experiment. Gels were loaded with equal volumes of input lysate and immunoprecipitate. Light and heavy chain (26 and 54 kDa respectively) of FLAG antibody, FLAG-tagged ER β at 60 kDa and ER α at 64 kDa are indicated.

qPCR and immunoblotting 24 h after vehicle or 10-nM E₂ treatment, in cells with and without ER β (Fig. 3A, B and C). In MCF7 cells, ER β expression doubled the basal levels (under estrogen-depleted conditions) of ER α (Fig. 3A, D and E). Although ER α is usually repressed by E₂ treatment, this regulation can vary across experimental conditions and be affected by, e.g. seeding density of cells and cell synchronization protocols. Small E₂-mediated upregulation of ER α mRNA, as noted in Fig. 3A, has been reported in MCF7 cells previously (Pawlak & Wiebe 2007). ICI treatment promoted degradation of ER α protein in all cases, whereas, under the experimental conditions used here, ER α protein degradation by E₂ was minimal in all cells. We conclude that expression of ER β significantly affected the levels of ER α in MCF7 cells, but not in T47D cells.

ER β exerted genome-wide effects on gene regulation

We next examined the effect of ER β over the transcriptome. Using microarray analysis, we assessed changes both

at basal level (Control+Vehicle vs ER β +Vehicle) and after 24-h estrogen stimulation (Control+E₂ vs ER β +E₂) in biological and technical duplicates of both cell lines. The results, illustrated in Fig. 4A and Supplementary Table 1, see section on supplementary data given at the end of this article, indicate that ER β expression mediated an impact on both basal and E₂-mediated gene expression in both cell lines. A total of 302 genes were regulated in both T47D and MCF7 cells (Fig. 4B and Supplementary Fig. 2), including repression of aryl hydrocarbon receptor (*AHR*), the Wnt inhibitor Dickkopf 1 (*DKK1*), and transforming growth factor β 2 (*TGFB2*). The repression of both *TGFB2* and *DKK1* indicates the potential effects on proliferation (Zhou et al. 2010). Other genes were regulated in an opposite manner in the two cell lines: the cell-cycle progression and poor-prognosis gene *CCNA2* (cyclin A2; Yam et al. 2002) was upregulated in MCF7-ER β but downregulated in T47D-ER β cells and *PIK3R1* (p85, a subunit of phosphatidylinositol 3-kinases (PIK3)) that can contribute to non-genomic ER α signaling (Mendez et al. 2003, Moghadam et al. 2011) was downregulated in MCF7-ER β but upregulated in T47D-ER β cells.

To determine the signaling pathways that were affected by ER β , we analyzed for enrichment of predicted TF target genes. We found that putative targets of E2F and AP1 were significantly enriched among ER β -affected genes in both cell lines, whereas more *SMAD3*, *FOXJ2*, and *FOXO4* target genes were overrepresented in T47D cells and c-Myc, nuclear factor-Y (NFY), and Sp1 target genes predominated in the MCF7 cells (Table 1 and Supplementary Tables 3 and 4, see section on supplementary data given at the end of this article). Heatmaps representing gene expressions for putative target genes of c-MYC, the E2F family, transcription factor complex *AP-1*, *FOXO4*, *SMADs*, and *NFkB* across different samples are shown in Supplementary Fig. 3. Although ER β expression resulted in enhanced regulation of E2F target genes (Supplementary Fig. 3), many other AP1 target genes (including apolipoprotein D (*APOD*), *ANXA1*, and *SYNPO*, all previously reported as regulated by AP1 in the MCF7 cells, Dahlman-Wright et al. (2012)) were repressed in both cell lines along with the two AP1 factors c-Fos and c-Jun in the MCF7 cells (Supplementary Fig. 3C). In addition, c-Myc signaling which is imperative in tumor cell-cycle progression, and regulated by ER α in complex with NFY (Wang et al. 1999), was enhanced along with ER α , and NFY activity (Supplementary Fig. 3A) in MCF7-ER β cells.

In conclusion, ER β had major impacts on the transcriptome in both cell lines, not only with significant

Figure 3

ER β upregulates ER α in MCF7 but not in T47D cells. (A) Relative ER α mRNA levels as measured by qPCR in T47D and MCF7 cells after 24 h vehicle and E₂ treatment. Bar graphs illustrate the average and s.d. for all mixed-cell populations. ER α protein in T47D (B) and MCF7 (C) after 24-h treatment with

vehicle, 10 nM E₂ or 10 nM ICI. Two mixed-cell populations were analyzed for each condition and representative experiments are shown. Quantifications of all western blots are shown for T47D (D) and MCF7 (E). Band intensities were normalized to β -actin. * P < 0.05 and ** P < 0.01.

similarities but also with cell-specific characteristics. Several known ER α and ER β -affected TF modules, including AP1, E2F, c-Myc, TGF β , NF κ B, and FoxO4, were affected as a consequence of ER β expression in both cell lines.

Constitutively expressed ER β augmented ER α -signaling

Others and we have previously shown that transient expression of ER β opposes ER α -regulated transcription (Chang *et al.* 2006, Williams *et al.* 2008). Here, we explored what proportion of direct ER α targets was affected by stable ER β expression. We found that about one-third of genes that ER β affected in both cell lines (112 of the 302 genes, including *TGFB2*, *PIK3R1*, and *GATA3*) were identified as direct transcriptional targets of ER α using GRO-seq analysis (Hah *et al.* 2011), as illustrated in Supplementary Fig. 2. Further, using microarrays, to identify the genes that ER α regulated (24-h E₂ treatment) in each cell line, we compared how ER β affected these genes. In MCF7, 37% of

ER α -E₂ regulated genes (483 out of 1291, including ER α itself) were significantly affected by ER β expression, whereas in T47D cells only 9% of ER α -regulated genes (99 out of 1103 genes) were affected (Fig. 4C). Although the more extensive effects on ER α targets in MCF7-ER β cells are likely attributable to the increase in ER α , ER β expression that appeared to enhance ER α signaling for the majority of the affected genes in both cell lines (84% or 406 genes out of 483 in MCF7; and 75% or 73 out of 97 genes in T47D). The ER β -enhanced ER α -target genes included many well-known direct targets (*pS2* (*TFF1*), *PGR*, *GREB1*, and *MYBL1*), and affected functions such as cell proliferation (e.g. *BCL2*, *c-MYC*, and *IGFBP4*), cell migration/cell adhesion, and DNA repair in both cell lines (Supplementary Table 2, see section on supplementary data given at the end of this article). The ER β opposed ER α -E₂-induction of only 77 genes in MCF7-ER β cells and 24 in T47D cells (Supplementary Table 7). These genes include growth- and proliferation-associated *TGFB2*,

Figure 4

Large transcriptome effects of ER β in T47D and MCF7 cells. Microarrays were used to analyze changes in gene expression between cells with and without ER β , in the absence and presence of 10 nM E₂. Both biological (different mixed-cell populations) and technical replicates were used for each condition and cell line. (A) A heatmap based on hierarchical clustering of transcripts (rows) and samples (columns) illustrates the genome-wide

changes of both upregulated (red) and downregulated (blue) genes.

(B) Genes affected by ER β expression (in presence of E₂) in both T47D and MCF7 are illustrated using Venn diagram. (C) Comparison of genes regulated by ER α (after 24 h of 10-nM E₂ treatment) in parental cells with genes affected by ER β expression is illustrated by a Venn diagram for T47D and MCF7.

SLC3A2, *B4GALT1*, and *CCNA2* in T47D-ER β , and genes associated to cell migration and motion, cell death, and hypoxia (e.g. *IGF1R*, *THBS1*, *KLF10*, *AEN*, *CAV1*, and *PLOD2*) in the MCF7 cells. We conclude that while ER β did attenuate a small proportion of ER α gene-regulations, this was not its predominant action.

QPCR analysis confirms ER α /ER β interplay

We confirmed the microarray data and dissected the manner in which ER β influences ER α signaling further using qPCR on the selected genes. Data for four genes where expression of ER β enhances ER α -E₂ regulation and four genes where ER β expression results in an opposing or divergent E₂ response are shown in Fig. 5.

The ERE-controlled target gene *PS2*, the anti-apoptotic *BCL2*, and the proliferative *MYC* were increased at both basal and E₂-regulated transcriptional levels by ER β in both cell lines. Similarly, ER β expression enhanced the regulation of the proliferative ion-channel *KCNK5* (potassium channel, subfamily K, member 5; Alvarez-Baron et al. 2011) and the cell-cycle gene *CCNA2* in MCF7 cells, but attenuated these regulations in T47D cells. ER β could also enhance ER α repression, as illustrated by *APOD* in both cell lines. On the other hand, ER β expression reversed the effect of ER α regulation for Claudin 1 (*CLDN1*) and attenuated the E₂-induction of the poor-prognosis gene Cathepsin D in both cell lines (all regulations shown in Fig. 5). Overall, the qPCR analysis confirmed the microarray data and illustrated

Table 1 Enrichment of transcription factor target genes in ER β gene expression profiles

TF	Motif	T47D-ERβ		MCF7-ERβ	
		<i>n</i>	<i>P</i> value	<i>n</i>	<i>P</i> value
Upregulated					
PRRX2	V\$S8_01	9	3.84×10^{-3}	7	9.36×10^{-1}
STAT5A	V\$STAT5A_03	9	3.97×10^{-3}	8	8.81×10^{-1}
GTF2A1	V\$TFIII_Q6	8	4.22×10^{-3}	5	9.60×10^{-1}
TFDP1	V\$E2F_Q6_01	8	4.89×10^{-3}	11	3.54×10^{-1}
FOXO4	TTGTTT_V\$FOXO4_01	37	4.98×10^{-3}	80	8.52×10^{-1}
EBF2	CCCNNGGGAR_V\$OLF1_01	10	4.99×10^{-3}	9	9.40×10^{-1}
ZEB1	CAGGTA_V\$AREB6_01	18	5.23×10^{-3}	21	9.95×10^{-1}
E2F1	V\$E2F1_Q3	8	7.38×10^{-3}	14	1.42×10^{-1}
IRF1	V\$IRF1_01	8	1.17×10^{-2}	4	9.96×10^{-1}
SREBF1	V\$SREBP1_Q6	8	1.28×10^{-2}	11	5.74×10^{-1}
NRF1	RCGCANGCGY_V\$NRF1_Q6	10	4.91×10^{-1}	43	7.84×10^{-2}
PAX3	CGTSACG_V\$PAX3_B	1	8.06×10^{-1}	10	6.68×10^{-2}
TFDP1	V\$E2F_Q4_01	5	1.34×10^{-1}	15	6.32×10^{-2}
STAT6	V\$STAT_01	3	6.13×10^{-1}	17	6.19×10^{-2}
E2F1	V\$E2F1_Q6_01	2	7.73×10^{-1}	17	2.40×10^{-2}
USF1	V\$USF_C	5	2.53×10^{-1}	20	2.10×10^{-2}
SP1	GGGCGGR_V\$SP1_Q6	40	1.52×10^{-1}	146	1.15×10^{-2}
AR	V\$AR_02	2	7.70×10^{-2}	6	5.29×10^{-3}
MYC	CACGTG_V\$MYC_Q2	12	5.34×10^{-1}	60	4.71×10^{-3}
NFYB	GATTGGY_V\$NFY_Q6_01	18	1.17×10^{-1}	77	1.76×10^{-5}
Downregulated					
RUNX1	V\$AML_Q6	9	1.20×10^{-3}	18	2.39×10^{-1}
IKZF1	V\$IK1_01	8	5.99×10^{-3}	18	3.00×10^{-1}
FOXA2	V\$HNF3B_01	7	7.32×10^{-3}	16	2.18×10^{-1}
STAT1/STAT2	V\$ISRE_01	7	7.32×10^{-3}	19	5.64×10^{-2}
SMAD3	V\$SMAD3_Q6	7	8.01×10^{-3}	16	2.38×10^{-1}
NKX6-1	V\$NKX61_01	7	8.75×10^{-3}	13	5.80×10^{-1}
HNF4A	V\$HNF4_01_B	7	1.07×10^{-2}	27	5.95×10^{-4}
POU3F2	V\$BRN2_01	7	1.16×10^{-2}	28	3.38×10^{-4}
SRF	V\$SRF_Q4	7	1.19×10^{-2}	25	3.70×10^{-3}
LHX3	YTAATTAA_V\$LHX3_01	6	1.23×10^{-2}	13	3.02×10^{-1}
TCF7	V\$TCF1P_Q6	1	9.11×10^{-1}	27	4.59×10^{-4}
MTF1	V\$MTF1_Q4	2	7.15×10^{-1}	28	4.02×10^{-4}
MAZ	GGGAGGRR_V\$MAZ_Q6	21	6.55×10^{-1}	163	2.51×10^{-4}
SOX9	V\$SOX9_B1	2	7.04×10^{-1}	29	1.19×10^{-4}
LEF1	CTTTGT_V\$LEF1_Q2	22	2.81×10^{-1}	146	1.12×10^{-4}
PAX2	V\$PAX2_02	5	1.18×10^{-1}	31	6.35×10^{-5}
FOXO4	TTGTTT_V\$FOXO4_01	24	1.86×10^{-1}	153	3.50×10^{-5}
NFATC1	V\$NFAT_Q4_01	21	3.00×10^{-1}	33	1.68×10^{-5}
TCF3	CAGGTG_V\$E12_Q6	28	1.94×10^{-1}	180	1.42×10^{-5}
CEBPB	V\$CEBPB_02	4	2.83×10^{-1}	34	8.94×10^{-6}

Transcription factors whose predicted targets were overrepresented upon expression of ER β in the presence of E₂. *n* indicates number of genes. The list is ranked according to ascending *P* value, starting from the top for T47D-ER β and from the bottom for MCF7-ER β , and redundant instances and motifs for unknown factors are removed. Overrepresentation is considered significant if *P* < 0.05, italics indicate no significance.

that ER β augmented ER α -signaling for many critical genes, while mediating differential regulation on others.

Silencing of ERs and receptor-selective ligand treatment confirm ER β 's contribution

Discriminating the effects of ER α - and ER β -mediated regulation was complex because ER β upregulated ER α in the MCF7 cells, and E₂-treatment downregulated ER β expression in both cell lines (Supplementary Fig. 5A, see

section on [supplementary data](#) given at the end of this article). We therefore attempted siRNA treatment to investigate receptor-specific gene regulations. Silencing of the endogenous ER α in MCF7 cells was effective, reducing its mRNA levels by 80–90% (Fig. 6A), and ablating detectable protein expression (Fig. 6C) along with reduction of downstream target genes *PS2*, *CCNA2*, and *BCL2* (exemplified in Fig. 6D). As ER β is stably expressed from a CMV promoter, its transcript levels could only be mildly reduced by siRNA (by 30% using

Figure 5

Gene-specific transcriptional effects by ER β . Known ER α -target genes are affected by ER β expression in absence and presence of E₂. The regulation of pS2, KCNK5, MYC, BCL2, Claudin 1, APOD, Cathepsin D, and CCNA2 is

double transfections, Fig. 6B). This relatively slight silencing still resulted in the reduction of the target genes *PS2* (Fig. 6D), *CCNA2*, and *BCL2*. Using the ER β -selective ligand KB101471 or E₂, in combination with siER α , we demonstrated ER β homodimer transactivation of pS2 (Fig. 6E). The same experiment in control (no ER β) cells ablated pS2 regulation (Fig. 6E). Tamoxifen treatment reduced ER β -mediated gene regulations (Supplementary Fig. 5D). In conclusion, we distinguish gene-specific effects of both ERs that can be quenched upon silencing of the receptors.

ER β expression affected genes associated with proliferation, apoptosis, and adhesion

After establishing that, under the conditions analyzed ER β enhanced rather than attenuated ER α signaling for most target genes, we proceeded to explore the biological outcome that stable expression of ER β generated in these cells. Enrichment analyses of Gene Ontology biological processes, presented in Tables 2 and 3, and Supplementary Tables 5 and 6, see section on supplementary data given at the end of this article, indicated that ER β -affected genes involved in the response to E₂, cell adhesion, apoptosis, proliferation, transcription, and inflammatory response in both cell lines. Functional studies were carried out to determine the effect of stably expressed ER β on proliferation, apoptosis, and migration.

illustrated by bar graphs. Statistically significant differences between ER β and non-ER β expressing cells are indicated by asterisks. The significance of changes by E₂ treatment alone is not indicated for simplicity.

Stable ER β expression did not reduce proliferation

To determine the effect of ER β on cellular proliferation, we subjected cell lines grown in full-serum medium for cell counting over a period of 6–8 days after seeding. The T47D-ER β cells grew at a pace similar to both the control mixes and the parental cell line, while MCF7-ER β cells grew faster than both control and parental cells (Fig. 7A). Thus, ER β did not exhibit anti-proliferative properties when stably expressed in our experiment. Next, we evaluated the effect of ER β under serum-starved conditions, and after treatment with E₂, tamoxifen, or ICI. Using MTS assays, we again observed that the expression of ER β did not significantly change the proliferation of T47D (Fig. 7B). ER β did, however, slightly reduce the E₂-dependent induction of cell growth. The growth inhibitory effect of tamoxifen or ICI was similar in the ER β -expressing cells as in the control cells. The MCF7-ER β cells showed an increased level of proliferation also in estrogen-depleted media, and an enhanced response to E₂ (Fig. 7B). This growth was also reduced by the addition of tamoxifen or ICI. Control experiments were carried out to verify that control-transduced cells behaved similarly to parental cell lines and to previous experiments using a synchronizing protocol (10 nM ICI for 24 h) before E₂ treatment (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The effect of ER β expression on the proliferative phenotype of

Figure 6

Receptor-specific effects on gene transcription identified through receptors silencing and SERM treatments in MCF7 cells. siRNA targeting the ERs and subsequent ligand treatments with E₂ (10 nM), ER β -selective ligand KB101471 (0.5 nM), and ER antagonist tamoxifen (1 μ M) was used to identify the contribution by each receptor. QPCR measurements of relative mRNA levels of (A) ER α and (B) ER β in MCF7-ER β cells indicate effectiveness of RNA silencing.

(C) Measurements of ER α protein levels using western blot confirm effective ER α silencing in MCF7-ER β . (D) Both ER α and ER β silencing in MCF7-ER β cells affect the regulation of direct target gene pS2. (E) Silencing of ER α and treatment with vehicle, E₂ (10 nM) or ER β -specific agonist KB101471 (0.5 nM) in MCF7 control and MCF7-ER β cells reveals ER β regulation of pS2. (A, B and D) Average of triplicate experiments. * P <0.05, ** P <0.01 and *** P <0.001.

the two cell lines reflected the changes observed at the transcriptome level.

As the level of ER α was doubled in the MCF7-ER β cells, we speculated that this might be the primary driving event for the increased growth noted in these cells. We silenced both ERs, and investigated their respective impact on proliferation using the MTS assay (Fig. 7C). Silencing of ER α in MCF7-ER β cells brought down its level to one fifth, yielding twofold less ER α levels compared with the MCF7 control cells. As expected, this silencing significantly reversed the increased basal and E₂-induced proliferation of the MCF7-ER β cells. ER β alone could thus not sustain the elevated proliferation. The slight silencing of ER β that we accomplished did not significantly affect proliferation. We conclude that ER β did not mediate anti-proliferative events, and that the increased proliferation noted in the

MCF7-ER β cells is, at least partly, attributed to increased ER α levels.

ER β expression did not affect apoptosis

Both ER α and ER β have previously been shown to induce or sensitize different type of cells to apoptosis (Song *et al.* 2001, Helguero *et al.* 2005, Lewis *et al.* 2005, Hodges-Gallagher *et al.* 2008, Edvardsson *et al.* 2011, Cotrim *et al.* 2013, Hussain *et al.* 2012). Apoptosis-associated genes were also overrepresented among genes affected by ER β (Table 2 and Supplementary Tables 5 and 6). To assess for altered susceptibility to apoptotic events, we examined PARP cleavage in MCF7 cells in presence and absence of ER β , E₂, and the DNA-damaging agent cisplatin. We did observe increased PARP levels

Table 2 Enriched biological processes in ER β gene expression profiles

Term	T47D-ER β		MCF7-ER β	
	<i>n</i>	<i>P</i> value	<i>n</i>	<i>P</i> value
Positive regulation of apoptosis	19	2.72×10^{-9}	32	5.08×10^{-9}
Regulation of cell proliferation	16	1.54×10^{-8}	33	1.24×10^{-12}
Response to estradiol stimulus	14	3.61×10^{-8}	27	6.71×10^{-11}
Cell adhesion	31	6.49×10^{-8}	71	5.79×10^{-12}
Collagen fibril organization	7	4.94×10^{-7}	6	2.40×10^{-3}
Branching morphogenesis of a tube	7	1.37×10^{-6}	5	2.10×10^{-2}
Cellular response to insulin stimulus	5	8.24×10^{-3}	31	2.74×10^{-11}
Response to drug	21	1.62×10^{-5}	56	1.45×10^{-11}
Interferon- γ -mediated signaling pathway	5	5.71×10^{-3}	22	4.68×10^{-13}
Phosphorylation	13	5.86×10^{-2}	63	2.48×10^{-13}
Axon guidance	17	1.11×10^{-5}	50	4.99×10^{-15}
Blood coagulation	20	4.13×10^{-5}	66	3.35×10^{-17}
Positive regulation of transcription from RNA polymerase II promoter	25	6.88×10^{-6}	81	1.01×10^{-19}
Cell cycle	21	3.58×10^{-4}	113	3.87×10^{-41}

Gene Ontology functions that were overrepresented among genes affected by ER β in both cell lines in the absence of ligand. The list is ranked according to ascending *P* value, starting from the top for T47D-ER β and from the bottom for MCF7-ER β . Redundant terms omitted, *n* indicates number of genes. Overrepresentation is considered significant at *P* < 0.05. Italics indicate no significance.

overall by ER β expression, but the fraction of cleaved PARP remained unchanged (data not shown). These experiments, thus, did not support an effect on apoptosis in MCF7-ER β cells.

ER β expression reduced migration

ER β has been implicated as a repressor of migration in breast cancer cells (Lindberg *et al.* 2010, Lam *et al.* 2012, Thomas *et al.* 2012) and of invasiveness in the models of inflammatory breast cancer (Ohshiro *et al.* 2012). The gene expression profiles of T47D-ER β and MCF7-ER β cells suggested altered ability of the cells to migrate, as indicated by the enrichment of related biological processes (Table 3 and Supplementary Table 5). We performed a wound-healing assay to assess the influence of ER α + E₂ on migration in control cells, and the corresponding impact of ER β . Our experiments showed that in T47D control cells, E₂ treatment significantly stimulated ER α -mediated migration (Fig. 7D), confirming previous studies (Li *et al.* 2010b). When ER β was expressed, however, E₂ no longer mediated a significantly increased migration. This aligns with the observed gene regulations in T47D cells where ER β repressed migratory *CLDN1* expression and attenuated the ER α induced TGF β (*TGFB2*). In MCF7 cells, on the other hand, migration was not affected by either ER α or ER β (Fig. 7D). The ability of the assay to detect effects on migration in MCF7-ER β cells was, however, occluded by increased proliferation under serum-starved conditions upon expression of ER β . In conclusion, ER β

appears to have the capacity to attenuate E₂-stimulated migration in T47D cells.

Discussion

In this study, we aimed to further our understanding of ER β 's role in ER α -positive breast cancer. We generated

Table 3 Enriched biological processes among ER β -regulated genes in both cell lines

Term	<i>n</i>	<i>P</i> value
Blood coagulation	20	2.99×10^{-9}
Response to estradiol stimulus	11	2.86×10^{-8}
Regulation of cell proliferation	12	3.30×10^{-8}
Cholesterol biosynthetic process	7	4.67×10^{-8}
Kidney development	9	3.66×10^{-7}
Positive regulation of apoptosis	12	5.21×10^{-7}
Axon guidance	14	6.00×10^{-7}
Epithelial cell differentiation	7	1.03×10^{-6}
Metabolic process	43	1.08×10^{-6}
Sterol biosynthetic process	5	4.19×10^{-6}
Collagen fibril organization	5	6.83×10^{-6}
Leukocyte migration	8	7.13×10^{-6}
Regulation of catalytic activity	13	1.08×10^{-5}
Blood vessel development	6	1.40×10^{-5}
Bone development	4	1.89×10^{-5}
Negative regulation of mitosis	3	2.91×10^{-5}
Response to hypoxia	10	3.17×10^{-5}
Anti-apoptosis	10	3.40×10^{-5}
<i>In utero</i> embryonic development	10	3.40×10^{-5}
Lens fiber cell development	3	4.34×10^{-5}

Overrepresentation analysis based on Gene Ontology gene sets for the common (both MCF7-ER β and T47D-ER β) differentially expressed genes. *n* indicates number of genes. Ranking according to *P* value.

Figure 7

ER β does not repress proliferation in T47D and MCF7 cells. Proliferation was measured using both cell counting and MTS assay under varying conditions. (A) T47D cells and MCF7 cells, with and without ER β expression, were counted over 6 or 8 days of growth in full-serum medium. Duplicate (T47D) and triplicate (MCF7) mixed-cell populations were analyzed, and parental MCF7 cells were included. Each measurement was performed in duplicate or triplicates, and data for one representative mixed-cell population is shown. Proliferation of (B) T47D cells and MCF7 cells, with and without ER β expression, were measured using MTS assay. Cells were grown in estrogen-depleted medium and treated with vehicle, 10 nM E₂,

10 nM E₂ + 1 μ M tamoxifen, or 10 nM E₂ + 1 μ M ICI for 3–6 days. The average for all mixed-cell populations (two for T47D, each repeated twice, three for MCF7, each repeated four times) is shown in figure. (C) Proliferation was measured after silencing of each receptor using MTS assay. Each bar illustrates the average for two of the mixed-cell populations, each performed in four replicates and measurement made in technical triplicates. (D) The influence of ER β on migration was measured using wound-healing assay for T47D and MCF7 cells with and without ER β , after 24-h E₂ (10 nM) or vehicle treatment. * P < 0.05, ** P < 0.01 and *** P < 0.001.

T47D and MCF7 cell lines coexpressing the two ERs constitutively, each in replicated mixed-cell populations. Previous studies have indicated that transiently expressed ER β opposes ER α signaling and corresponding proliferative function (as referenced above). After assessing constitutive expression at mRNA and protein levels (Fig. 1), we showed that ER β formed heterodimer with ER α (Fig. 2) and induced large transcriptomic changes in both cell lines (Fig. 4). Our gene-expression analysis showed that general ER α signaling was not attenuated by stably expressed ER β (Figs 4 and 5). Enrichment analysis indicated ER β 's involvement in previously reported processes, including proliferation, DNA repair, adhesion,

and modulation of the inflammatory response (Tables 2 and 3). Collectively, however, our results diverge from the body of data, including our own, that suggests that ER β in breast cancer cell lines opposes ER α gene regulations in a genome-wide manner and thereby reduces proliferation.

The enrichment analysis indicated that ER β -affected genes were involved in proliferation, and that key cell-cycle driving genes, including *ER α* , *CCNA2*, and *c-MYC*, were upregulated in the MCF7-ER β cells. Also in T47D-ER β , proliferative genes were upregulated but a concurrent downregulation of other pro-proliferative factors was also observed (e.g. *CCNA2*). E2F targets were increased in both cell lines and the E2F family plays crucial roles in the

control of cell cycle and tumor progression. The increased E2F activity (Supplementary Fig. 3B) may be attributed to the downregulation of the repressing member, E2F4, in both cell lines. In addition, the activating E2F1 was upregulated in MCF7-ER β cells (Supplementary Fig. 5B) and the repressive E2F7 was upregulated in T47D-ER β cells, contributing to the more extensive effects noted in MCF7 cells. The E2F1 is a known target of ER α , regulated in tandem with AP1 in MCF7 cells (Dahlman-Wright *et al.* 2012) and constitutes an essential part of ER α -mediated cellular proliferation of breast cancer cells (Dubik & Shiu 1992, Stender *et al.* 2007, Dahlman-Wright *et al.* 2012). AP1 also has a central role in ER β -mediated signaling (Zhao *et al.* 2010, Dahlman-Wright *et al.* 2012), and 60% of ER β -chromatin binding sites have been found to contain both ERE- and AP1-like sites (Zhao *et al.* 2010). Functional analysis demonstrated that ER β did not affect overall proliferation in T47D-ER β cells, whereas in MCF7-ER β cells an increased proliferation was recorded (Fig. 7A and B). This was consistently observed in the independent mixed-cell populations. We primarily attributed the increased proliferation in MCF7 cells to the twofold increased levels of ER α . Overexpression of ER α in MCF7 cells has previously been shown to increase cell proliferation (Zajchowski *et al.* 1993, Tolhurst *et al.* 2011). When we silenced ER α in MCF7-ER β cells, the cells reduced their proliferation to the levels of the control cells. However, as the reduced level of ER α was twofold lower than control cells, we cannot exclude that ER β contributes to a growth advantage.

The reasons for the divergent results of ER β in terms of proliferation can be several. We note that most studies concluding anti-proliferative abilities have been performed using transient transfection or inducible systems, and we propose two factors that may contribute to the divergent results: selection of cells with proliferative advantage under stable conditions and cofactor squelching during transient conditions. The selection of stably expressing ER β cells could enrich for cells that can proliferate in the presence of ER β , thereby obscuring anti-proliferative properties. We initially noted, within a week of transduction, that ER β opposed ER α signaling in the T47D-ER β cells, as reported previously (Williams *et al.* 2008). However, as the cells adapted to ER β expression this was reverted to a pro-ER α activity for many target genes in both T47D and MCF7 cells. Whereas the selection may favor cells that can proliferate in the presence of ER β in a nonphysiological manner, the fact that we observed the same results in each of the mixed-cell populations analyzed, and did not note an anti-proliferative effect in

either cell population, indicates that this is not an infrequent characteristic. Possibly, similar characteristics may evolve in clinical tumors. As we have used the same system and selection procedure and found ER β to be anti-proliferative in colon cancer cells (Hartman *et al.* 2009, Edvardsson *et al.* 2011), we know that this approach can be used to detect anti-proliferative functions of ER β . We need to also consider the impact of cofactor squelching during transient expression (Meyer *et al.* 1989). When significant levels of ER β are introduced, ER β will compete for interaction with many of the same cofactors that ER α also requires. This, by itself, can result in an attenuation of the ER α -mediated gene regulation and proliferation. When ER β is stably expressed, on the other hand, the cells can adapt and reach a steady-state level of needed factors. Cells with stable expression may therefore evade the squelching effect. Our result that ER β is not anti-proliferative when stably expressed in breast cancer cells aligns with the clinical findings where ER β *in vivo* often does not correlate with an anti-proliferative phenotype (as referenced in 'Introduction' section and reviewed by Leygue & Murphy (2013)). Although analysis of stable ER β expression in MCF7 cells was recently reported (Grober *et al.* 2011, Wu *et al.* 2011) and a reduced proliferative response to E₂ was noted (Grober *et al.* 2011), effects on basal proliferation or interplay with ER α were not explored in detail. In triple-negative breast cancer cell lines, several studies have reported a lack of anti-proliferative effects by ER β (Tonetti *et al.* 2003, Hou *et al.* 2004, Rousseau *et al.* 2004). We suggest that our study offers a potential mechanistic model for ER β 's role in a subset of breast tumors. Our result does not preclude that ER β can possess anti-proliferative abilities in other cells, in other circumstances, or when activated *de novo*.

Aligning with our results that stably expressed ER β did not reduce proliferation in ER α -positive breast cancer cells, we did not observe extensive attenuation of ER α 's transcriptional regulation in our experiments. On the contrary, we noted an enhancement of ER α target gene regulation when ER β was expressed in both cell lines (Fig. 5). It is established that homo- and heterodimers of both ERs bind at the promoter of, e.g. pS2 (Matthews *et al.* 2006, Papoutsis *et al.* 2009, Zhao *et al.* 2010, Grober *et al.* 2011), but conflicting data have been reported on whether ER β opposes or enhances ER α 's regulation (Matthews *et al.* 2006, Williams *et al.* 2008, Papoutsis *et al.* 2009, Grober *et al.* 2011, Wu *et al.* 2011). Our data indicate that ER β enhances the ER α -E₂ response for the most coregulated genes, in both cell lines. Of note is that ER α levels were induced a twofold increase by ER β in MCF7 cells, which

contributed to an enhanced E₂ response in these cells. ER α regulation was, however, attenuated by ER β for a small proportion of target genes in both cell lines (Fig. 5). In T47D cells, further, the ER α levels were unchanged, but the regulations of known ER α -targets were still enhanced. Our data align with a previous suggestion that the ER α –ER β heterodimer largely acts in a manner similar to the ER α homodimer (Li et al. 2004). It is likely that the type of regulation depends on whether ER β binds as homo- or heterodimer, and whether it binds to *cis*-regulatory sequences at ERE sites or tethers with AP1 or Sp1 transcription modules.

Three groups have compared the DNA-binding sites of both receptors in MCF7 cells at a genomic scale (Charn et al. 2010, Zhao et al. 2010, Grober et al. 2011). They found that 33–73% of ER β -binding sites were regions where ER α homodimer can also bind, and that the majority of binding sites contained full or half EREs. Zhao et al. (2010) found that significant enrichment of AP1 and Forkhead motifs, and that 60% of ER β -binding sites contained both ERE- and AP1-like sites. Thus, the two ERs can bind many common regions that are possibly dominated by full EREs, whereas sites unique to each ER might be enriched for other type of motifs. ER α is known to activate AP1 sites (Dahlman-Wright et al. 2012), whereas ER β has been reported both to repress (Paech et al. 1997) and activate (Cheung et al. 2005) such transcription. Our data support that AP1 targets, with E2F1 as one exception, were repressed by ER β . Further, we note that genes with Sp1 motifs in their promoters were upregulated in MCF7-ER β cells, correlating with findings of ER β activity in osteosarcoma cell line U2OS (Vivar et al. 2010). That one of the few genes where ER β opposed ER α in both cell lines, Cathepsin D, is regulated in complex with Sp1 (Cavailles et al. 1993, Krishnan et al. 1994, Foekens et al. 1999), may indicate that its regulation via Sp1 motifs is different from that of ER α . Global ChIP–reChIP would need to be carried out to fully characterize genomic binding of the ER heterodimer, and to differentiate it from common binding by ER α and ER β homodimers.

We compared our gene expression dataset to the recently published data from MCF7 cells also engineered to express ER β stably (Grober et al. 2011, Wu et al. 2011) as well as our previous study of inducible ER β expression in T47D cells (Williams et al. 2008). Whereas the overlap of transcriptional changes between transient and stable ER β expression in T47D cells was low (10% of the transient-ER β regulated genes were observed), comparison between studies of stable expression in MCF7 cells rendered a

relatively large overlap. Here, 37 and 34%, respectively, of genes previously identified as regulated by ER β expression were changed in our study (Supplementary Fig. 4, see section on supplementary data given at the end of this article), supporting the generality of these data.

We further observed that E₂-induced migration was opposed in T47D cells, that the MCF7-ER β -expressing cells adhered to each other more strongly than the control cells, and that migration and proliferation assays were more dependent on cellular confluency when the cells expressed ER β . ER β also affected several cell-adhesion genes in both cell lines, including repression of *TGFB2* and, in MCF7-ER β cells, repression of the TGF β -induced KLF10, which has been demonstrated as an ER β -specific target in U2OS cells (Hawse et al. 2008). These properties are in line with the evidence that ER β has a role in cellular adhesion and TGF β signaling (Chang et al. 2006, Alonso-Magdalena et al. 2009, Lindberg et al. 2010, Thomas et al. 2012). Owing to the dual roles of TGF β in cellular proliferation, cellular migration, and cancer metastasis (Tong et al. 2002, Buck & Knabbe 2006, Brier & Moses 2009, Goto et al. 2011), this aspect of ER β regulation merits further investigation.

Our study suggests that ER α signaling and estrogen-induced proliferation can be unaffected or enhanced by ER β , and that tamoxifen or ICI treatment is still able to block these functions (Fig. 7B and Supplementary Fig. 5D). In addition, previously described upregulation of SPINK4 by tamoxifen-liganded ER α (Hall & McDonnell 1999) was maintained or enhanced in the presence of ER β in both cell lines (Supplementary Fig. 5C). Several studies have concluded that tamoxifen is an antagonist to ER β (Pettersson et al. 2000), but other has suggested that ER α -positive tumor cells become resistant to tamoxifen when ER β is expressed (Hopp et al. 2004). Our data support that patients with ER α /ER β -positive tumors may benefit from tamoxifen or fulvestrant treatment, in line with the clinical evidence presented by Honma et al. (2008).

In conclusion, we present the evidence that breast cancer cells are able to continue proliferating and thrive while stably expressing significant levels of ER β . Our analysis shows that ER β can enhance rather than oppose ER α -signaling, and this knowledge aids in the understanding of the role that ER β mediates in some ER α /ER β -positive breast cancer cells. Our study supports that the better survival noted in tamoxifen-treated patients with ER β -positive tumors is because tamoxifen antagonizes both ER α and ER β signaling. However, as also beneficial functions appeared mediated by ER β , e.g. repression of Cathepsin D and migration, the optimal treatment

approach will need to be carefully evaluated. *In vivo* studies are needed to investigate how stable ER β expression impacts tumor metastasis in ER α -positive breast cancer cells. As ER β is a highly druggable target, a better understanding of its function is critical. Currently, only ER α is utilized in the clinic and our study supports the accumulating data that ER β is a promising target for breast cancer therapy.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0444>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by faculty start-up funding from the University of Houston, the National Cancer Institute of the National Institutes of Health under Award Number R01CA172437 and Texas Emerging Technology Fund (300-9-1958). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

Author contribution statement

P Jonsson and A Katchy carried out experiments and data analysis. P Jonsson and C Williams designed the experiments and wrote the manuscript. C Williams supervised P Jonsson and A Katchy and initiated the study.

Acknowledgements

The authors thank Drs Anders Ström and Jan-Åke Gustafsson for the generous sharing of cell lines, lentivirus, reagents, and advice; Drs Prasennjit Dey, Christoforos Thomas, and Trang Vu for experimental assistance and advice, and Drs Jan-Åke Gustafsson and Margaret Warner for commenting on the manuscript (all at Department of Biology and Biochemistry, Center for Nuclear Receptors and Cell Signaling, University of Houston); Dr Stefan Nilsson (KaroBio, Sweden) for the generous gift of ER β -selective ligand KB101471; and Dr Karin Edvardsson (Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Sweden) for experimental assistance and advice.

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Received in final form 9 October 2013

Accepted 4 November 2013

Made available online as an Accepted Preprint

5 November 2013