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

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Abstract

The expression of estrogen receptor α (ERα) in breast cancer identifies patients most likely to respond to endocrine treatment. The second ER, ERβ, is also expressed in breast tumors, but its function and therapeutic potential need further study. Although in vitro studies have established that ERβ opposes transcriptional and proliferative functions of ERα, several clinical studies report its correlation with proliferative markers and poorer prognosis. The data demonstrate that ERβ opposes ERα are primarily based on transient expression of ERβ. Here, we explored the functions of constitutively expressed ERβ in ERα-positive breast cancer lines MCF7 and T47D. We found that ERβ, under these conditions heterodimerized with ERα in the presence and absence of 17β-estradiol, and induced genome-wide transcriptional changes. Widespread anti-ERα signaling was, however, not observed and ERβ was not antiproliferative. Tamoxifen antagonized proliferation and ER-mediated gene regulation both in the presence and absence of ERβ.

In conclusion, ERβ’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 ERβ’s role in breast tumors that coexpress both receptors and supports an emerging bi-faceted role of ERβ.

Key Words
- estrogen receptor α
- estrogen receptor β
- breast cancer
- SERM
- gene expression

Introduction

The role of estrogen receptor α (ERα (ESR1)) as a biological marker and target in breast cancer therapy is clear. ERα antagonists, such as tamoxifen, or estrogen ablation using aromatase inhibitors are efficient therapeutic approaches in the treatment of ERα-positive breast cancer. A second ER, ERβ (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 ERβ 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). ERβ 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 ERb (Mosselman et al. 1996).

The function of ERb in breast cancer is not clearly understood (Leygue & Murphy 2013) and ERb 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 ERb for consistent mechanistic and functional studies (Holbeck et al. 2010), and mRNA levels in the tissues are also persistently low in comparison with ERa (Sayers et al. 2012). To explore its role, ERb thus has to be introduced exogenously. The majority of studies has used transient or inducible induction of ERb and indicates that ERb in ERa-positive breast cancer cells counteracts the proliferative and transcriptional functions of ERa (Lazenec 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 ERb expression to better outcomes (Omoto et al. 2001, Nakopoulou et al. 2004, Sugjura et al. 2007), others have correlated ERb 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 ERa-negative tumors, ERb expression has been correlated to a higher aneuploidy, indicating a more aggressive phenotype (Fuqua et al. 2003). The body of data correlating ERb with both anti-proliferative and proliferative parameters has led to the suggestions of a bi-faceted role for ERb (Leygue & Murphy 2013). One clinical study indicated that tamoxifen treatment of ERb-positive tumors may be beneficial: ERb 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 ERb may help us to improve the treatment for breast cancer patients.

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

Subjects and methods

Cell culture, ERb expression, and treatments

Duplicate T47D-ERb and T47D-control and triplicate MCF7-ERb and MCF7-control mixed-cell populations were generated by lentiviral transductions with CMV-driven, FLAG-tagged full-length ERb 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 ERb 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). 17β-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 ERa and ERb, 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 ERb. Tamoxifen (Sigma) at 1 μM 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 μg 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 (ERb and control respectively) and negative controls. The primer sequences are provided upon request. The gene expression was normalized to 18S rRNA, GAPDH, or ARHGIDIA 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: ERα 1:1000 (HC-20, Santa Cruz Biotechnology), ERβ 1:1000 (NBPA-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 (\(-\log 2(\text{Cy5/Cy3})\)) higher than 0.4. Regulations that were not detected on at least three out of four arrays were discarded. Over-representation/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 μg/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 ERβ expression in two models for the luminal subtype of breast tumors: the human epithelial ERα-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 ERα and estrogen for growth. Previous studies have characterized the ligand-activated gene regulation by ERα 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 ERβ 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 ERβ that forms heterodimer with ERα

We used lentivirus transduction followed by selection with blasticidin to generate T47D and MCF7 cells stably expressing full-length, FLAG-tagged ERβ in replicated mixed-cell populations, in duplicates for T47D and in triplicates for MCF7 cells. ERβ transcript and protein levels were significantly increased in the ERβ-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-ERβ cells contained more than double as many ligand-binding receptors as the T47D control cells (Fig. 1C), indicating an ERβ:ERα ratio of ~2:1. MCF7-ERβ cells also exhibited increased ligand binding after expression of ERβ, and siRNA of ERα visualized the contribution by ERβ (Fig. 1D). In MCF7 cells, an ERE-luciferase transactivation assay showed that ERβ-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 ERβ and ERα formed heterodimers in both MCF7-ERβ and T47D-ERβ cells, with or without E2 stimuli (Fig. 2), supporting a previous report (Pace et al. 1997).

ERβ affected ERα expression in MCF7 but not in T47D cells

ERα 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 ERα changed upon the expression of ERβ. In T47D cells, the levels of ERα were not affected by neither ERα itself nor ERβ, as measured using...
ERβ exerted genome-wide effects on gene regulation

We next examined the effect of ERβ over the transcriptome. Using microarray analysis, we assessed changes both at basal level (Control+Vehicle vs ERβ+Vehicle) and after 24-h estrogen stimulation (Control+E2 vs ERβ+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 ERβ 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 β2 (TGFB2). The repression of both TGFβ2 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-ERβ but downregulated in T47D-ERβ cells and PIK3R1 (p85, a subunit of phosphatidylinositol 3-kinases (PIK3)) that can contribute to non-genomic ERα signaling (Mendez et al. 2003, Moghadam et al. 2011) was downregulated in MCF7-ERβ but upregulated in T47D-ERβ cells.

To determine the signaling pathways that were affected by ERβ, we analyzed for enrichment of predicted TF target genes. We found that putative targets of E2F and AP1 were significantly enriched among ERβ-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 NFκB across different samples are shown in Supplementary Fig. 3. Although ERβ 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, Dahlmann-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 ERα in complex with NFY (Wang et al. 1999), was enhanced along with ERα and NFY activity (Supplementary Fig. 3A) in MCF7-ERβ cells.

In conclusion, ERβ had major impacts on the transcriptome in both cell lines, not only with significant...
similarities but also with cell-specific characteristics. Several known ERα and ERβ-affected TF modules, including AP1, E2F, c-Myc, TGFβ, NFκB, and FoxO4, were affected as a consequence of ERβ expression in both cell lines. Constitutively expressed ERβ augmented ERα-signaling

Others and we have previously shown that transient expression of ERβ opposes ERα-regulated transcription (Chang et al. 2006, Williams et al. 2008). Here, we explored what proportion of direct ERα targets was affected by stable ERβ expression. We found that about one-third of genes that ERβ affected in both cell lines (112 of the 302 genes, including TGFB2, PIK3R1, and GATA3) were identified as direct transcriptional targets of ERα using GRO-seq analysis (Hah et al. 2011), as illustrated in Supplementary Fig. 2. Further, using microarrays, to identify the genes that ERα regulated (24-h E2 treatment) in each cell line, we compared how ERβ affected these genes. In MCF7, 37% of ERα–E2 regulated genes (483 out of 1291, including ERα itself) were significantly affected by ERβ expression, whereas in T47D cells only 9% of ERα-regulated genes (99 out of 1103 genes) were affected (Fig. 4C). Although the more extensive effects on ERα targets in MCF7-ERβ cells are likely attributable to the increase in ERα, ERβ expression that appeared to enhance ERα 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 ERβ-enhanced ERα-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 ERβ opposed ERα–E2-induction of only 77 genes in MCF7-ERβ cells and 24 in T47D cells (Supplementary Table 7). These genes include growth- and proliferation-associated TGFB2,
SLC3A2, B4GALT1, and CCNA2 in T47D-ERβ, 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 ERβ did attenuate a small proportion of ERα gene-regulations, this was not its predominant action.

**QPCR analysis confirms ERα/ERβ interplay**

We confirmed the microarray data and dissected the manner in which ERβ influences ERα signaling further using qPCR on the selected genes. Data for four genes where expression of ERβ enhances ERα-E₂ regulation and four genes where ERβ 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 ERβ in both cell lines. Similarly, ERβ 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. ERβ could also enhance ERα repression, as illustrated by APOD in both cell lines. On the other hand, ERβ expression reversed the effect of ERα 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 changes of both upregulated (red) and downregulated (blue) genes.

**Figure 4**

Large transcriptome effects of ERβ in T47D and MCF7 cells. Microarrays were used to analyze changes in gene expression between cells with and without ERβ, 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 ERβ expression (in presence of E₂) in both T47D and MCF7 are illustrated using Venn diagram. (C) Comparison of genes regulated by ERα (after 24 h of 10-nM E₂ treatment) in parental cells with genes affected by ERβ expression is illustrated by a Venn diagram for T47D and MCF7.
that ERβ augmented ERα-signaling for many critical genes, while mediating differential regulation on others.

**Silencing of ERs and receptor-selective ligand treatment confirm ERβ’s contribution**

Discriminating the effects of ERα- and ERβ-mediated regulation was complex because ERβ upregulated ERs in the MCF7 cells, and E2-treatment downregulated ERβ 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 ERα 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 ERβ is stably expressed from a CMV promoter, its transcript levels could only be mildly reduced by siRNA (by 30% using
Endocrine-Related Cancer
P Jonsson et al. Bi-faceted role of ER\(\beta\) in breast cancer

Stable ER\(\beta\) expression did not reduce proliferation

To determine the effect of ER\(\beta\) 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\(\beta\) cells grew at a pace similar to both the control mixes and the parental cell line, while MCF7-ER\(\beta\) cells grew faster than both control and parental cells (Fig. 7A). Thus, ER\(\beta\) did not exhibit anti-proliferative properties when stably expressed in our experiment. Next, we evaluated the effect of ER\(\beta\) under serum-starved conditions, and after treatment with E\(2\), tamoxifen, or ICI. Using MTS assays, we again observed that the expression of ER\(\beta\) did not significantly change the proliferation of T47D (Fig. 7B). ER\(\beta\) did, however, slightly reduce the E\(2\)-dependent induction of cell growth. The growth inhibitory effect of tamoxifen or ICI was similar in the ER\(\beta\)-expressing cells as in the control cells. The MCF7-ER\(\beta\) cells showed an increased level of proliferation also in estrogen-depleted media, and an enhanced response to E\(2\) (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 E\(2\) treatment (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The effect of ER\(\beta\) expression on the proliferative phenotype of

Figure 5
Gene-specific transcriptional effects by ER\(\beta\). Known ER\(\alpha\)-target genes are affected by ER\(\beta\) expression in absence and presence of E\(2\). 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\(\beta\)-selective ligand KB101471 or E\(2\), in combination with siER\(\alpha\), we demonstrated ER\(\beta\) homodimer transactivation of pS2 (Fig. 6E). The same experiment in control (no ER\(\beta\)) cells ablated pS2 regulation (Fig. 6E). Tamoxifen treatment reduced ER\(\beta\)-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\(\beta\) expression affected genes associated with proliferation, apoptosis, and adhesion

After establishing that, under the conditions analyzed ER\(\beta\) enhanced rather than attenuated ER\(\alpha\) signaling for most target genes, we proceeded to explore the biological outcome that stable expression of ER\(\beta\) 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\(\beta\)-affected genes involved in the response to E\(2\), 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\(\beta\) on proliferation, apoptosis, and migration.
the two cell lines reflected the changes observed at the transcriptome level.

As the level of ERα was doubled in the MCF7-ERβ 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 ERα in MCF7-ERβ cells brought down its level to one fifth, yielding twofold less ERα levels compared with the MCF7 control cells. As expected, this silencing significantly reversed the increased basal and E2-induced proliferation of the MCF7-ERβ cells. ERβ alone could thus not sustain the elevated proliferation. The slight silencing of ERβ that we accomplished did not significantly affect proliferation. We conclude that ERβ did not mediate anti-proliferative events, and that the increased proliferation noted in the MCF7-ERβ cells is, at least partly, attributed to increased ERα levels.

**ERβ expression did not affect apoptosis**

Both ERα and ERβ 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 ERβ (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 ERβ, E2, and the DNA-damaging agent cisplatin. We did observe increased PARP levels...
overall by ERβ expression, but the fraction of cleaved PARP remained unchanged (data not shown). These experiments, thus, did not support an effect on apoptosis in MCF7-ERβ cells.

**ERβ expression reduced migration**

ERβ 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-ERβ and MCF7-ERβ 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 ERβ on migration in control cells, and the corresponding impact of ERβ. Our experiments showed that in T47D control cells, E2 treatment significantly stimulated ERβ-mediated migration (Fig. 7D), confirming previous studies (Li et al. 2010b). When ERβ was expressed, however, E2 no longer mediated a significantly increased migration. This aligns with the observed gene regulations in T47D cells where ERβ repressed migratory CLDN1 expression and attenuated the ERα-induced TGFβ (TGFβ2). In MCF7 cells, on the other hand, migration was not affected by either ERα or ERβ (Fig. 7D). The ability of the assay to detect effects on migration in MCF7-ERβ cells was, however, occluded by increased proliferation under serum-starved conditions upon expression of ERβ. In conclusion, ERβ appears to have the capacity to attenuate E2-stimulated migration in T47D cells.

**Discussion**

In this study, we aimed to further our understanding of ERβ’s role in ERα-positive breast cancer. We generated

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**Table 2** Enriched biological processes in ERβ gene expression profiles

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<tr>
<th>Term</th>
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<td>66</td>
<td>3.35 × 10^-17</td>
</tr>
<tr>
<td>Positive regulation of transcription from RNA</td>
<td>25</td>
<td>6.88 × 10^-6</td>
<td>81</td>
<td>1.01 × 10^-19</td>
</tr>
<tr>
<td>polymerase II promoter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell cycle</td>
<td>21</td>
<td>3.58 × 10^-4</td>
<td>113</td>
<td>3.87 × 10^-41</td>
</tr>
</tbody>
</table>

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

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**Table 3** Enriched biological processes among ERβ-regulated genes in both cell lines

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

Overrepresentation analysis based on Gene Ontology gene sets for the common (both MCF7-ERβ and T47D-ERβ) differentially expressed genes. n indicates number of genes. Ranking according to P value.
T47D and MCF7 cell lines coexpressing the two ERs constitutively, each in replicated mixed-cell populations. Previous studies have indicated that transiently expressed ER\(_b\) opposes ER\(_a\) signaling and corresponding proliferative function (as referenced above). After assessing constitutive expression at mRNA and protein levels (Fig. 1), we showed that ER\(_b\) formed heterodimer with ER\(_a\) (Fig. 2) and induced large transcriptomic changes in both cell lines (Fig. 4). Our gene-expression analysis showed that general ER\(_a\) signaling was not attenuated by stably expressed ER\(_b\) (Figs 4 and 5). Enrichment analysis indicated ER\(_b\)'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\(_b\) in breast cancer cell lines opposes ER\(_a\) gene regulations in a genome-wide manner and thereby reduces proliferation.

The enrichment analysis indicated that ER\(_b\)-affected genes were involved in proliferation, and that key cell-cycle driving genes, including ER\(_a\), CCNA2, and c-MYC, were upregulated in the MCF7-ER\(_b\) cells. Also in T47D-ER\(_b\), 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

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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-ERβ cells (Supplementary Fig. 5B) and the repressive E2F7 was upregulated in T47D-ERβ cells, contributing to the more extensive effects noted in MCF7 cells. The E2F1 is a known target of ERα, regulated in tandem with AP1 in MCF7 cells (Dahlman-Wright et al. 2012) and constitutes an essential part of ERα-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 ERβ-mediated signaling (Zhao et al. 2010, Dahlman-Wright et al. 2012), and 60% of ERβ–chromatin binding sites have been found to contain both ERE- and AP1-like sites (Zhao et al. 2010). Functional analysis demonstrated that ERβ did not affect overall proliferation in T47D-ERβ cells, whereas in MCF7-ERβ 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 ERα. Overexpression of ERα in MCF7 cells has previously been shown to increase cell proliferation (Zajchowski et al. 1993, Tolhurst et al. 2011). When we silenced ERα in MCF7-ERβ cells, the cells reduced their proliferation to the levels of the control cells. However, as the reduced level of ERα was twofold lower than control cells, we cannot exclude that ERβ contributes to a growth advantage.

The reasons for the divergent results of ERβ 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 ERβ cells could enrich for cells that can proliferate in the presence of ERβ, thereby obscuring anti-proliferative properties. We initially noted, within a week of transduction, that ERβ opposed ERα signaling in the T47D-ERβ cells, as reported previously (Williams et al. 2008). However, as the cells adapted to ERβ expression this was reverted to a pro-ERα activity for many target genes in both T47D and MCF7 cells. Whereas the selection may favor cells that can proliferate in the presence of ERβ 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 ERβ 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 ERβ. We need to also consider the impact of cofactor squelching during transient expression (Meyer et al. 1989). When significant levels of ERβ are introduced, ERβ will compete for interaction with many of the same cofactors that ERα also requires. This, by itself, can result in an attenuation of the ERα-mediated gene regulation and proliferation. When ERβ 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 ERβ is not anti-proliferative when stably expressed in breast cancer cells aligns with the clinical findings where ERβ 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 ERβ 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 ERα were not explored in detail. In triple-negative breast cancer cell lines, several studies have reported a lack of anti-proliferative effects by ERβ (Tonetti et al. 2003, Hou et al. 2004, Rousseau et al. 2004). We suggest that our study offers a potential mechanistic model for ERβ’s role in a subset of breast tumors. Our result does not preclude that ERβ can possess anti-proliferative abilities in other cells, in other circumstances, or when activated de novo.

Aligning with our results that stably expressed ERβ did not reduce proliferation in ERα-positive breast cancer cells, we did not observe extensive attenuation of ERα’s transcriptional regulation in our experiments. On the contrary, we noted an enhancement of ERα target gene regulation when ERβ 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, Papoutsi et al. 2009, Zhao et al. 2010, Grober et al. 2011), but conflicting data have been reported on whether ERβ opposes or enhances ERα’s regulation (Matthews et al. 2006, Williams et al. 2008, Papoutsi et al. 2009, Grober et al. 2011, Wu et al. 2011). Our data indicate that ERβ enhances the ERα–E2 response for the most coregulated genes, in both cell lines. Of note is that ERα levels were induced a twofold increase by ERβ in MCF7 cells, which
contributed to an enhanced E\textsubscript{2} response in these cells. ER\textsubscript{x} regulation was, however, attenuated by ER\textsubscript{b} for a small proportion of target genes in both cell lines (Fig. 5). In T47D cells, further, the ER\textsubscript{x} levels were unchanged, but the regulations of known ER\textsubscript{x}-targets were still enhanced. Our data align with a previous suggestion that the ER\textsubscript{x}–ER\textsubscript{b} heterodimer largely acts in a manner similar to the ER\textsubscript{x} homodimer (Li et al. 2004). It is likely that the type of regulation depends on whether ER\textsubscript{b} 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\textsubscript{b}-binding sites were regions where ER\textsubscript{x} 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\textsubscript{b}-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\textsubscript{x} is known to activate AP1 sites (Dahman-Wright et al. 2012), whereas ER\textsubscript{b} 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\textsubscript{b}. Further, we note that genes with Sp1 motifs in their promoters were upregulated in MCF7-ER\textsubscript{b} cells, correlating with findings of ER\textsubscript{b} activity in osteosarcoma cell line U2OS (Vivar et al. 2010). That one of the few genes where ER\textsubscript{x} opposed ER\textsubscript{b} 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\textsubscript{x}. 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\textsubscript{x} and ER\textsubscript{b} homodimers.

We compared our gene expression dataset to the recently published data from MCF7 cells also engineered to express ER\textsubscript{b} stably (Grober et al. 2011, Wu et al. 2011) as well as our previous study of inducible ER\textsubscript{b} expression in T47D cells (Williams et al. 2008). Whereas the overlap of transcriptional changes between transient and stable ER\textsubscript{b} expression in T47D cells was low (10% of the transient-ER\textsubscript{b} 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\textsubscript{b} 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 E\textsubscript{2}-induced migration was opposed in T47D cells, that the MCF7-ER\textsubscript{b}-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\textsubscript{b}. ER\textsubscript{b} also affected several cell-adhesion genes in both cell lines, including repression of TGFB2 and, in MCF7-ER\textsubscript{b} cells, repression of the TGFB-induced KLF10, which has been demonstrated as an ER\textsubscript{b}-specific target in U2OS cells (Hawse et al. 2008). These properties are in line with the evidence that ER\textsubscript{b} has a role in cellular adhesion and TGF\beta 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\beta in cellular proliferation, cellular migration, and cancer metastasis (Tong et al. 2002, Buck & Knabbe 2006, Bierie & Moses 2009, Goto et al. 2011), this aspect of ER\textsubscript{b} regulation merits further investigation.

Our study suggests that ER\textsubscript{x} signaling and estrogen-induced proliferation can be unaffected or enhanced by ER\textsubscript{b}, 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-ligated ER\textsubscript{x} (Hall & McDonnell 1999) was maintained or enhanced in the presence of ER\textsubscript{b} in both cell lines (Supplementary Fig. 5C). Several studies have concluded that tamoxifen is an antagonist to ER\textsubscript{b} (Pettersson et al. 2000), but other has suggested that ER\textsubscript{x}-positive tumor cells become resistant to tamoxifen when ER\textsubscript{b} is expressed (Hopp et al. 2004). Our data support that patients with ER\textsubscript{x}/ER\textsubscript{b}-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\textsubscript{b}. Our analysis shows that ER\textsubscript{b} can enhance rather than oppose ER\textsubscript{x}-signaling, and this knowledge aids in the understanding of the role that ER\textsubscript{b} mediates in some ER\textsubscript{x}/ER\textsubscript{b}-positive breast cancer cells. Our study supports that the better survival noted in tamoxifen-treated patients with ER\textsubscript{b}-positive tumors is because tamoxifen antagonizes both ER\textsubscript{x} and ER\textsubscript{b} signaling. However, as also beneficial functions appeared mediated by ER\textsubscript{b}, 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 ERβ expression impacts tumor metastasis in ERα-positive breast cancer cells. As ERβ is a highly druggable target, a better understanding of its function is critical. Currently, only ERα is utilized in the clinic and our study supports the accumulating data that ERβ 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.

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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.

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