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ORIGINAL ARTICLE

Microarray analysis of altered gene expression in ERβ-overexpressing HEK293 cells

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Abstract Estrogen receptors (ERs), ERα and ERβ, mediate estrogen actions in a broad range of target tissues. With the introduction of microarray techniques, a significant understanding has been gained regarding the interplay between the ERα and ERβ in breast cancer cell lines. To gain a more comprehensive understanding of ERβ-dependent gene regulation independent of ERα, we performed microarray analysis on HEK293/mock and HEK293/ERβ cells. A total of 332 genes was identified as ERβ-upregulated genes and 210 identified as ERβ-downregulated genes. ERβ-induced and ERβ-repressed genes were involved in cell–cell signaling, morphogenesis, and cell proliferation. The ERβ repressive effect on genes related to proliferation was further studied by proliferation assays, where ERβ expression resulted in a significant decrease in cell proliferation. To identify primary ERβ target genes, we examined a number of ERβ-regulated genes using chromatin immunoprecipitation assays for regions bound by ERβ. Our results showed that ERβ recruitment was significant to regions associated with 12 genes (IL1RAP, TMSB4X, COLEC12, ENPP2, KLRC1, RERG, RGS16, TNNT2, CYR61, FER1L3, FAM108A1, and CYP4X1), suggesting that these genes are likely to be ERβ primary target genes. This study has provided novel information on the gene regulatory function of ERβ independent of ERα and identified a number of ERβ primary target genes. The results of Gene Ontology analysis and proliferation assays are consistent with an antiproliferative role of ERβ independent of ERα.

Keywords ERβ · Microarray · HEK293 · Target gene · Chromatin immunoprecipitation

Abbreviations

IL1RAP	Interleukin 1 receptor accessory protein
TMSB4X	Thymosin, beta 4, X-linked
COLEC12	Collectin sub-family member 12
ENPP2	Ectonucleotide pyrophosphatase/ phosphodiesterase 2
KLRC1	Killer cell lectin-like receptor subfamily C, member 1
RERG	RAS-like, estrogen-regulated, growth inhibitor
RGS16	Regulator of G-protein signaling 16
TNNT2	Troponin T-type 2
CYR61	Cysteine-rich, angiogenic inducer, 61
FER1L3	Fer-1-like 3, myoferlin
FAM108A1	Family with sequence similarity 108, member A1
CYP4X1	Cytochrome P450, family 4, subfamily X, polypeptide 1

Introduction

Estrogens, by binding to and activating two estrogen receptors (ERs), ERα and ERβ, play a critical role in the growth, development, and maintenance of a diverse range of tissues. ERα and ERβ belong to the superfamily of

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nuclear receptors and specifically to the family of steroid receptors that act as ligand-regulated transcription factors [1]. ERs regulate gene expression through distinct DNA response elements. The classical mechanism of estrogen signaling is through an estrogen response element (ERE). ER dimerizes and interacts with EREs in target gene promoters, followed by the recruitment of a variety of coregulators to alter chromatin structure and facilitate recruitment of the RNA polymerase II (Pol II) transcriptional machinery [2, 3]. Estrogen signaling also occurs through alternative mechanisms where liganded ERs are tethered to DNA via the association with other transcription factor complexes including Fos/Jun (binding to AP-1-responsive elements) [4] or SP-1 (binding to GC-rich SP-1 motifs) [5]. In addition, growth factor signaling or stimulation of other signaling pathways leads to activation of kinases that can phosphorylate and thereby activate ERs or associated coregulators in the absence of ligand [6].

ERα and ERβ have different biological functions, as indicated by their specific expression patterns and distinct phenotypes observed in ERα and ERβ knockout mice. There have been a number of studies in the past few years aimed at comprehensively unraveling the complete estrogen-regulated gene expression programs in cancer cells. These reports can be attributed to the introduction of microarrays for global gene expression profiling. To date, much of the work on identification of gene expression profiles has been focused on the role of ERα. A few studies have examined the role of ERβ in regulating global gene expression programs in ERα-expressing breast cancer cell lines in which ERβ was coexpressed [7–10]. To elucidate the gene regulatory function of ERβ independent of ERα, we assayed gene expression profiles in HEK293 cells stably expressing ERβ. These cells lack functional endogenous ERs [11]. We identify here a variety of genes with altered expression in response to ERβ. Using chromatin immunoprecipitation (ChIP) assays, we identify a number of genes as ERβ primary target genes.

Results

Stable expression of ERβ in HEK293 cells

To create a system for studying the function of ERβ independently of ERα, a clone mix stably expressing ERβ was established in HEK293 tet-on cells, which lack functional endogenous ERs [11]. The expression of ERβ protein in the presence of doxycycline (Dox) in the clone mix stably expressing ERβ, termed HEK293/ERβ, was verified by Western blotting. No detectable ERβ protein was expressed in a clone mix of HEK293 cells stably transfected with the empty vector, termed HEK293/mock, whereas high levels of ERβ protein were observed in the HEK293/ERβ cells (Fig. 1a).

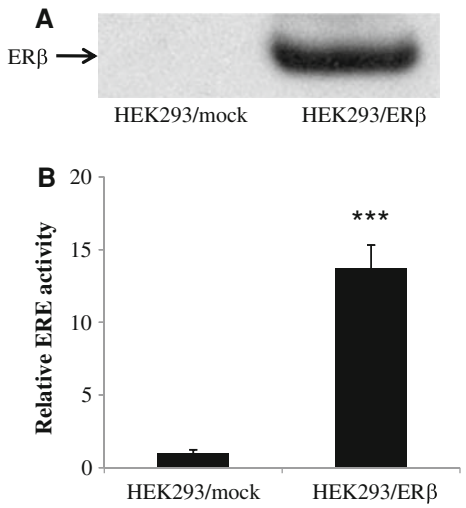


Fig. 1 Confirmation of functional ERβ expression in HEK293/ERβ cells. **a** HEK293/ERβ and HEK293/mock cells were cultured in the presence of Dox for 16 h. ERβ was expressed only in HEK293/ERβ cells as determined by Western blot analysis using the anti-Flag antibody M5. **b** Transfection of HEK293/ERβ and HEK293/mock tet-on cells with an ERE luciferase reporter shows E2 induction of ERE activity in HEK293/ERβ cells compared with the HEK293/mock cells. The *bars* represent mean ± SD, and the results are representative of three independent experiments, with the first sample set to 1. *** Significant difference ($P < 0.001$) as compared to HEK293/mock cells

To confirm the functionality of the expressed ERβ protein, the transcriptional activity of the protein was assayed on an ERE-driven reporter. As shown in Fig. 1b, overexpression of ERβ in HEK293/ERβ cells enhanced luciferase activity compared to HEK293/mock cells in the presence of E2, indicating that HEK293/ERβ cells express functional ERβ.

Identification of ERβ-regulated genes

To investigate global transcriptional modulation by ERβ, we performed microarray analysis on the HEK293/mock and HEK293/ERβ cells where both cell lines were treated with Dox for 16 h, and then treated with E2 for an additional 24 h. The GeneChip® Human Gene 1.0 ST Array (Affymetrix) that contains probes for 28,869 genes was used for this analysis. Modulation of gene expression by ERβ was determined by comparison of total RNA isolated from E2-treated HEK293/ERβ cells with E2-treated HEK293/mock cells. For applying a filter of $P < 0.05$ for significantly modulated gene expression and at least a 1.5-fold change in mean differential expression, a total of 332 genes was identified as ERβ-upregulated genes and 210 identified as ERβ-downregulated genes (the complete list of these regulated genes is provided as Supplementary

Table 1). Thirty-one and 30 genes, respectively, were increased or decreased more than twofold (Table 1). A subset of genes identified as regulated by ERβ in the microarray analysis was assayed using real-time PCR with samples from independent biological triplicates (Fig. 2a). The regulation observed for these genes in the microarray experiment could be confirmed for all tested genes. Confirmation was also performed using the same RNA that was used for the microarray analysis (Fig. 2b). The real-time PCR data have a high correlation with the microarray data, with a correlation coefficient of $r = 0.985$.

Gene Ontology analysis

To determine whether specific biological pathways are affected by ERβ expression, we used the *Expression Analysis Systematic Explorer* software (EASE) to annotate the 542 regulated genes according to the information provided by the Gene Ontology (GO) Consortium [12]. The GO database provided annotation for 48% (262 out of 542) of the regulated genes. Among the genes upregulated by ERβ, the 'cell–cell signaling' gene group was the most overrepresented one (EASE score 1.2e−007) followed by 'immune response,' 'morphogenesis,' and 'negative regulation of cell proliferation.' Overrepresented gene groups that were downregulated following the ERβ induction belonged to 'morphogenesis,' 'cell motility,' 'cell adhesion,' 'cell growth,' and 'cell proliferation.' Details of overrepresented gene groups are shown in Table 2.

Effect of ERβ expression on cell proliferation

The GO analysis suggested that ERβ activation may play a role in inhibition of cell proliferation. We next examined the effects of ERβ activation on cell proliferation using a WST-1 assay. HEK293/mock and HEK293/ERβ cells were cultured in the presence of Dox, and then treated with E2 for an additional 0, 24, and 72 h. As shown in Fig. 3, ERβ expression in HEK293 cells resulted in a significant decrease in cell proliferation as compared to HEK293/mock cells at 24 and 72 h.

Identification of ERβ target genes adjacent to ERβ-binding sites

Our group has recently pursued whole genome identification of ERβ binding regions in MCF7 cells by the ChIP-on-chip assay (unpublished data). We queried the ERβ binding sites and selected 20 binding sites that were within 50 kb upstream/downstream of the transcription start site of genes, which were found to be upregulated or downregulated by ERβ in HEK293 cells. Using the ChIP assay, we determined binding of ERβ to these sites in HEK293 cells. Binding of ERβ to these sites would indicate that they represent primary ERβ target genes.

As shown in Fig. 4a, ERβ was detected following immunoprecipitation and Western blotting when the anti-ERβ ligand-binding domain (LBD) antibody was used, but was not observed when ChIP was performed using control antibody IgG. Immunoprecipitated DNA from HEK293/ERβ cells was amplified by real-time PCR of 20 selected ERβ-binding regions. Among them, ERβ was recruited to DNA-binding regions associated with 12 genes (IL1RAP, TMSB4X, COLEC12, ENPP2, KLRC1, RERG, RGS16, TNNT2, CYR61, FER1L3, FAM108A1, and CYP4X1) (Fig. 4b). The mRNA expression levels of eight genes (COLEC12, ENPP2, KLRC1, RERG, RGS16, TNNT2, FAM108A1, and CYP4X1) were increased by ERβ expression, as shown in Supplementary Table 1. The residual four genes adjacent to ERβ binding sites, IL1RAP, TMSB4X, CYR61, and FER1L3, exhibited a decrease in expression levels induced by ERβ. It is noteworthy that six binding sites for ERβ are located within genes (COLEC12, ENPP2, CYP4X1, FER1L3, RERG, and IL1RAP), four are downstream (KLRC1, TNNT2, FAM108A1, and TMSB4X), and two are upstream of genes (RGS16 and CYR61).

Discussion

A significant understanding has been gained regarding the interplay between ERα and ERβ in breast cancer cell lines [7, 8, 10]. However, few reports exist that decipher the role of ERβ independently of ERα in the regulation of endogenous genes [13, 14]. Therefore, we developed cell lines stably expressing ERβ in HEK293 cells to determine ERβ-dependent gene regulation independently of ERα. HEK293 cells were used in this study because of their lack of endogenous ER expression and wide application in investigations of ER transactivation function and screening of compounds that bind to ER [15–17]. Our study demonstrates that ERβ modulates the expression of a variety of genes in HEK293 cells. Our data also suggest that ERβ inhibits cell proliferation independently of ERα. Using a ChIP assay, we identify 12 genes that likely represent ERβ direct target genes.

To confirm the microarray results, the expression level of a number of genes was measured with real-time PCR with samples from independently performed, triplicate experiments. Gene regulation for the selected genes showed very high concordance between microarray analysis and real-time PCR analysis. We identified a total of 332 genes as ERβ-upregulated genes and 210 as ERβ-downregulated genes, showing that ERβ has effects on gene expression profiles independently of ERα. Our findings are consistent with previous reports in human

Table 1 Genes regulated more than twofold by ERβ

Gene symbol	Gene name	Accession no.	FC
MYOM2	Myomesin (M-protein) 2, 165 kDa	NM_003970	6.30
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	NM_002615	4.85
GIP	Gastric inhibitory polypeptide	NM_004123	4.20
KU-MEL-3	KU-MEL-3	AY870321	3.43
OR4C12	Olfactory receptor, family 4, subfamily C, member 12	NM_001005270	2.70
TSSK1B	Testis-specific serine kinase 1B	NM_032028	2.65
TMDCII	tMDC II	NR_001448	2.48
HSPA1L	Heat shock 70 kDa protein 1-like	NM_005527	2.44
SELENBP1	Selenium-binding protein 1	NM_003944	2.39
TPRX1	Tetra-peptide repeat homeobox 1	NM_198479	2.39
OR4B1	Olfactory receptor, family 4, subfamily B, member 1	NM_001005470	2.37
CYSLTR2	Cysteinyl leukotriene receptor 2	NM_020377	2.35
APOL5	Apolipoprotein L, 5	NM_030642	2.32
OR4C15	Olfactory receptor, family 4, subfamily C, member 15	NM_001001920	2.30
ITGA8	Integrin, alpha 8	NM_003638	2.28
HSPB8	Heat shock 22 kDa protein 8	NM_014365	2.25
PGDS	Prostaglandin D2 synthase, hematopoietic	NM_014485	2.21
ICAM4	Intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)	NM_001544	2.21
COLEC12	Collectin sub-family member 12	NM_030781	2.20
CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit	NM_000719	2.20
AMPD1	Adenosine monophosphate deaminase 1 (isoform M)	NM_000036	2.18
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	NM_177534	2.12
NCKAP1L	NCK-associated protein 1-like	NM_005337	2.12
DKKL1	Dickkopf-like 1 (soggy)	NM_014419	2.08
OR4D11	Olfactory receptor, family 4, subfamily D, member 11	NM_001004706	2.08
CFH	Complement factor H	NM_000186	2.07
RXFP1	Relaxin/insulin-like family peptide receptor 1	NM_021634	2.07
RPTN	Repetin	uc001ezs.1	2.05
OR4S2	Olfactory receptor, family 4, subfamily S, member 2	NM_001004059	2.04
TSC22D3	TSC22 domain family, member 3	NM_198057	2.02
LOC158572	Hypothetical LOC158572	AK056746	2.00
HIST1H3I	Histone cluster 1, H3i	NM_003533	0.15
OR4C16	Olfactory receptor, family 4, subfamily C, member 16	NM_001004701	0.15
UNQ6125	Hypothetical LOC442092	NM_001030078	0.21
TLR4	Toll-like receptor 4	NM_138554	0.24
AMBN	Ameloblastin (enamel matrix protein)	NM_016519	0.25
HIST1H1A	Histone cluster 1, H1a	NM_005325	0.27
PEG10	Paternally expressed 10	NM_001040152	0.28
CDH6	Cadherin 6, type 2, K-cadherin (fetal kidney)	NM_004932	0.32
IAH1	Isoamyl acetate-hydrolyzing esterase 1 homolog (*Saccharomyces cerevisiae*)	NM_001039613	0.34
ECHDC3	Enoyl Coenzyme A hydratase domain containing 3	NM_024693	0.35
CDH19	Cadherin 19, type 2	NM_021153	0.36
SYT14L	Synaptotagmin XIV-like	NM_001014372	0.36
TMSB4X	Thymosin, beta 4, X-linked	NM_021109	0.39
LSAMP	Limbic system-associated membrane protein	NM_002338	0.41
EMP1	Epithelial membrane protein 1	NM_001423	0.42
GPR50	G protein-coupled receptor 50	NM_004224	0.42

Table 1 continued

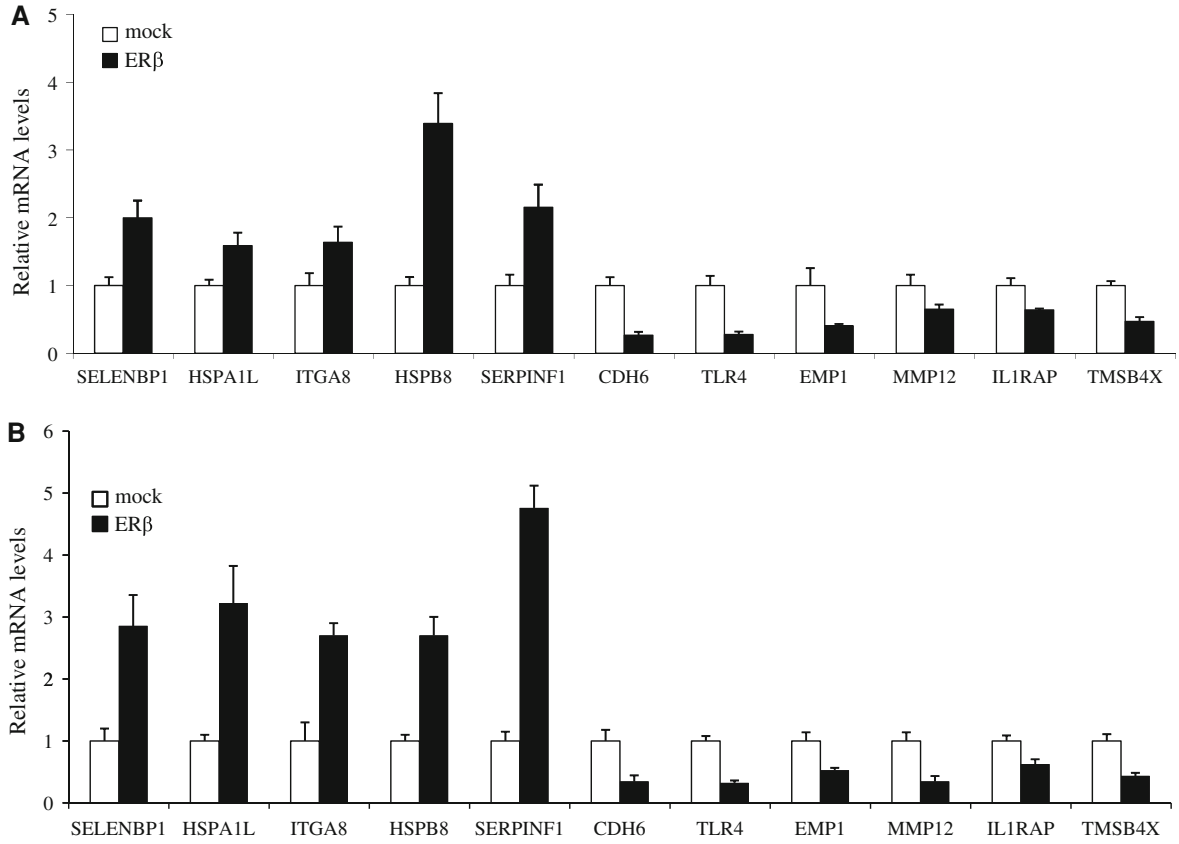
Gene symbol	Gene name	Accession no.	FC
C8orf4	Chromosome 8 open reading frame 4	NM_020130	0.43
NMUR2	Neuromedin U receptor 2	NM_020167	0.43
FER1L3	fer-1-like 3, myoferlin (*Caenorhabditis elegans*)	NM_133337	0.43
TFPI2	Tissue factor pathway inhibitor 2	NM_006528	0.43
PLD5	Phospholipase D family, member 5	NM_152666	0.45
KLHL4	Kelch-like 4 (Drosophila)	NM_057162	0.45
TMEM132D	Transmembrane protein 132D	NM_133448	0.46
LRRC7	Leucine rich repeat containing 7	NM_020794	0.46
KRTAP19-5	Keratin associated protein 19-5	NM_181611	0.47
DUSP10	Dual specificity phosphatase 10	NM_144728	0.47
SNORD116-4	Small nucleolar RNA, C/D box 116-4	NR_003319	0.47
MMP12	Matrix metallopeptidase 12 (macrophage elastase)	NM_002426	0.48
LOC284067	Hypothetical LOC284067	BC070324	0.49
DKK1	Dickkopf homolog 1 (*Xenopus laevis*)	NM_012242	0.49

FC fold change

osteoblastic cells [13] and Hs578T cells [14], in which a unique set of genes was found to be regulated solely by ERβ. We further compared our results with the study by Secreto et al. [14]. Of 95 genes found to be regulated by ER$\beta \geq$ 2-fold by Secreto et al. [14], 3 genes (PTGER4, ENPP2, and DKK1) are confirmed in this study. Interestingly, one of the genes (ENPP2) is identified as a primary ERβ target gene in this study. For one gene (PTGS2), we show downregulation by ERβ, whereas Secreto et al. report upregulation by ERβ. The reasons for the limited similarity of ERβ-regulated genes identified by the two studies could include the use of different cell lines (HEK293 versus Hs578T breast cancer cells), different levels of ERβ in the two cell lines, differences in number of genes on the array (28,869 genes in our study versus 8,700 genes in the study be Secreto et al.), and the different cut-off (1.5-fold vs. 2-fold). Despite these differences, both studies report inhibition of cell proliferation by ERβ expression independently of ERα, suggesting a similar function of ERβ in different cell types. It has also been reported that genes specifically regulated by ERβ, in the presence of ERα, in two different breast cancer cell lines showed limited similarity [10].

Classification of genes based on GO terms is a powerful bioinformatics tool suited for the analysis of DNA microarray data. Analysis of GO annotation allows one to identify families of genes in expression profiles that may play significant roles in specific molecular or biological processes [12]. Our results show that the types of genes affected by ERβ expression are involved in different functions, including cell–cell signaling, morphogenesis, cell motility, cell adhesion, cell growth, and cell proliferation. We identified a significant enrichment of genes from the cell proliferation-associated functional category within the population of genes downregulated by ERβ. ERβ downregulates 12 transcripts involved in cell proliferation (EMP1, CYR61, PTN, CCNA1, OSMR, PYY, SPOCK, NEDD9, SNK, SYCP2, PPP1R15A, and CRL3). EMP1 has been reported to be an estrogen-regulated gene in primary osteoblasts and a putative ERE was identified in the promoter region of this gene [18]. Cyr61 was found to be estrogen upregulated via ERα in MCF7 cells and was suggested to have a role in the progression of breast cancer and may be involved in estrogen-mediated tumor development [19]. Our finding that the Cyr61 expression level is decreased by ERβ is in line with the results of Williams et al. [10] where they found that ERβ opposes ERα at the transcriptome level. Furthermore, ERβ upregulates six genes involved in negative regulation of cell proliferation (IL6, GML, PRKR, DEC1, BTG4, and RERG). Finlin et al. [20] reported that RERG mRNA expression is induced rapidly in MCF7 cells stimulated by estrogen and overexpression of RERG results in inhibition of cell growth. The effect of ERβ expression on cell proliferation was investigated by a proliferation assay. The results showed that ERβ expression in HEK293 cells resulted in a significant decrease in cell proliferation as compared to HEK293/mock cells (Fig. 3). This finding is in line with previously published data, where overexpression of ERβ inhibited the proliferation of breast cancer cells [10, 14, 21, 22]. Taken together, our data suggest an antiproliferative role of ERβ independently of ERα.

In this study, we showed that ERβ was recruited to DNA-binding regions associated with 12 genes, suggesting that these genes are primary ERβ target genes. It is noteworthy



FC by real-time PCR	2.9	3.2	2.7	2.7	4.7	-2.9	-3.1	-1.9	-2.9	-1.6	-2.3
FC by microarray	2.4	2.4	2.3	2.2	4.8	-3.1	-4.2	-2.4	-2.1	-1.5	-2.2

Fig. 2 Confirmation of ERβ regulated genes derived from microarray analysis by real-time PCR. **a** The HEK293/ERβ and HEK293/mock cell lines were cultured in the presence of 1 μg/ml Dox and treated with 10 nM E2 for 24 h. These samples are from independent experiments than those used for the microarray analysis. Total RNA was isolated, reverse transcribed, and amplified with primers specific for the indicated genes. Values are expressed as mean ± SD and relative to the mean values in HEK293/mock cells. *SELENBP1* selenium binding protein 1, *HSPA1L* heat shock 70 kDa protein 1-like, *ITGA8* integrin, alpha 8, *HSPB8* heat shock 22 kDa protein 8, *SERPINF1* serpin peptidase inhibitor, *CDH6* cadherin 6, *TLR4* toll-like receptor 4, *EMP1* epithelial membrane protein 1, *MMP12* matrix metallopeptidase 12, *IL1RAP* interleukin 1 receptor accessory protein, *TMSB4X* thymosin, beta 4, X-linked. **b** These samples represent the same RNA as used for the microarray analysis. FC from real-time PCR and microarray data, respectively, are presented as numbers below the bars. Values are expressed as mean ± SD and relative to the mean values in HEK293/mock cells

Table 2 The most ERβ regulated biological gene groups

Differential expression	Biological processes	Genes	EASE score
ERβ upregulated	Cell–cell signaling	25	1.2e−007
	Immune response	23	6.1e−005
	Morphogenesis	28	0.00042
	Negative regulation of cell proliferation	6	0.028
ERβ downregulated	Morphogenesis	21	0.00015
	Cell motility	8	0.012
	Cell adhesion	11	0.014
	Cell growth	4	0.059
	Cell proliferation	12	0.20

that these ERβ DNA-binding regions are not just located in the proximal promoters of the genes. ERβ DNA-binding regions are also mapped to intronic or distal locations. This is consistent with previous studies showing that most ERα binding sites were found at significant distances from transcription start sites [23, 24]. It has been suggested that these distal ERα-binding sites play an important role in estrogen-mediated regulation, as they could be physically associated with promoter–proximal regions. Previous studies have identified the consensus ERE in DNA as the most frequent motif found in ER target genes [23, 25]. To determine whether the identified ERβ target genes harbored ERE motif within their ERβ binding regions, we conducted a perfect ERE (AGGTCANNNTGACCT, Ref. [23]) search

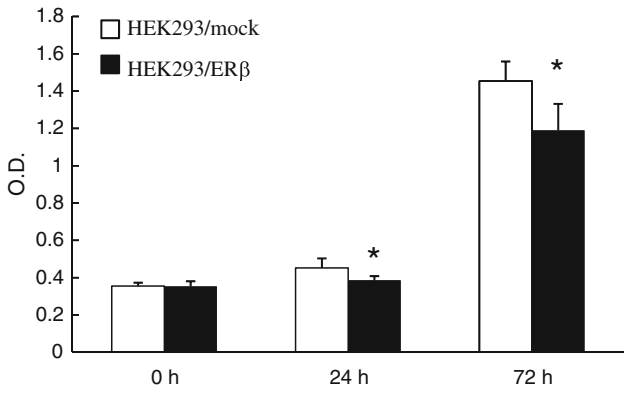


Fig. 3 Expression of ERβ reduces cell proliferation. HEK293/ERβ and HEK293/mock cell lines were cultured in the presence of 1 μg/ml Dox and treated with 10 nM E2 for 0, 24, and 72 h. Cell proliferation was determined by the WST-1 assay. The data are the average of six replicates ± SD. * Significant difference ($P < 0.05$) as compared to time-matched HEK293/mock cells

using CONSITE program. We discovered that 8 out of 12 genes harbored putative full EREs with the TF-score cut-off greater or equal 80% (Supplementary Table 2). Among the identified ERβ target genes, FER1L3, a gene predicted to encode a transmembrane protein, has been verified to be an E2-sensitive target gene in MCF7 cells [26] The ERα binding site was detected within FER1L3, and estradiol-enhanced SRC-3 and pol II association were also detected for this site. In this study, we identified an ERβ binding site within FER1L3, suggesting that this gene is bound by both ERα and ERβ. Indeed, the ChIP-on-chip approach has demonstrated a high degree of overlap between the regions identified as bound by ERα and ERβ, respectively [24].

In summary, this study has provided novel information on the gene regulatory function of ERβ independently of ERα and identified a number of novel ERβ primary target genes. Our data also suggest an antiproliferative role of ERβ independently of ERα.

Materials and methods

Generation of tetracycline-inducible stable cell lines

HEK293 tet-on cells were purchased from Clontech (Palo Alto, CA, USA) and maintained in DMEM (Invitrogen, Stockholm, Sweden) supplemented with 5% fetal calf

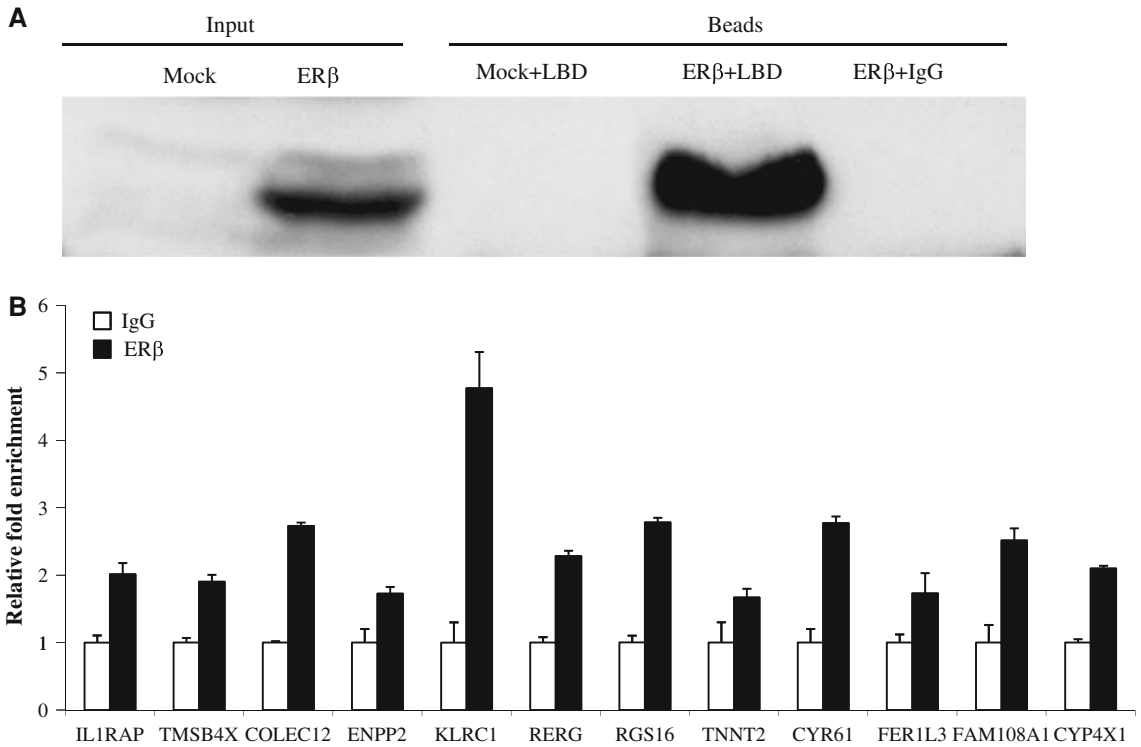


Fig. 4 ChIP followed by real-time PCR for detection of ERβ DNA-binding regions. **a** HEK293/ERβ and HEK293/mock cell lines were cultured in the presence of 1 μg/ml Dox and treated with 10 nM E2 for 45 min. The cells were progressed for ChIP analysis as described in "Materials and methods" using the ERβ LBD antibody or a nonspecific control antibody (IgG = normal rabbit IgG). Precipitated fractions (Beads) after immunoprecipitation were analyzed by Western blotting using the anti-Flag antibody M5. Input corresponds to sample prior to IP. **b** Chip of ERβ using the ERβ LBD antibody and real-time PCR of 12 ERβ binding regions in HEK293/ERβ cells. The real-time PCR data are presented as fold enrichment by ERβ LBD antibody compared to normal rabbit IgG control. The data are the average of three replicates ± SD

serum (FCS) and 1% penicillin/streptomycin (Invitrogen, Stockholm, Sweden). The cells were transfected with either the parental plasmid (pBI-EGFP) to generate a mock cell line or a plasmid (pBI-EGFP-ERβ), expressing a Flag-tagged human ERβ using the Lipofectamine 2000 Reagent (Invitrogen, Stockholm, Sweden). Cells were selected in the presence of 5 μg/ml blasticidin for 10 days. ERβ protein levels were determined by Western blot analysis.

Transient transfection and luciferase assays

Transient transfection was performed essentially as previously described [27]. In brief, cells were seeded in 24-well plates and grown in DMEM supplemented with 5% FCS for 24 h before transfection. The cells were cotransfected with the ERE-TK-Luc reporter plasmid and the pRL-TK control plasmid, which contains a Renilla luciferase gene, to normalize for transfection efficiency. Cells were transfected using Lipofectamine 2000 (Invitrogen, Stockholm, Sweden). Five hours after transfection, the medium was changed to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FCS. Dox was added simultaneously. After a 16-h incubation with Dox, the cells were treated with 10 nM E2 for 24 h before harvesting and performing luciferase assay (Biothema, Dalarö, Sweden).

Western blot analysis

Cells were seeded in 100-mm dishes and treated with Dox for 16 h in order to induce ERβ expression. Nuclear extracts were prepared as described in Ref. [28]. An aliquot of 30 μg of protein extract was analyzed by Western blot using anti-FLAG M5 monoclonal antibody (Sigma-Aldrich, Stockholm, Sweden) and visualized using an ECL kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

RNA isolation and real-time PCR

HEK293/ERβ and HEK293/mock cells were cultured in 6-well cell culture dishes in DMEM-FBS media and stimulated with Dox for 16 h and subsequently treated with 10 nM E2 for 24 h in triplicate. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed as described previously [29]. Primer pairs for 11 genes are given detailed in Supplementary Table 3.

Cell proliferation assay

Cell proliferation was evaluated using the WST-1 proliferation assay as specified by the manufacturer (Roche Applied Science, Mannheim, Germany). The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The formazan dye produced by viable cells can be quantified by measuring the absorbance of the dye solution at a wavelength of 450 nm. To perform the experiments, HEK293/ERβ and HEK293/mock cells were plated at a density of 2000 cells/well in 96-well microplates, in normal growth medium supplemented with Dox. After a 16-h incubation, 10 nM E2 was added to six replicate wells and cultured for 0, 24, and 72 h. After treatment, 10 μl/well WST-1 reagent was added and incubated for 1 h at 37°C, after which the absorbance at 450 nm was measured with a microplate reader. The experiments were performed in triplicate.

ChIP

Cells were seeded in 150 mm dishes and grown in the presence of Dox for 16 h. Cells were treated with 10 nM E2 for 45 min and ChIP was performed as previously described [30]. The anti-ERβ rabbit polyclonal antibody LBD [31] was used to perform ChIP for ERβ. Immunoprecipitated DNA was amplified by real-time PCR using Platinum SYBR green quantitative PCR supermix uracil DNA glycosylase (Invitrogen, Stockholm, Sweden). We considered that enrichment was significant when fold enrichment by ERβ LBD antibody compared to normal rabbit IgG control was higher than 1.5. Primer pairs for 12 genes are given detailed in Supplementary Table 4.

Microarray analysis

Triplicate RNA samples were prepared as described above. GeneChip® Human Gene 1.0 ST Arrays (Affymetrix) that contain probes for 28,869 genes were used for microarray analysis. Target synthesis and hybridizations were performed by the Affymetrix core facility (NOVUM, Karolinska Institutet, Huddinge, Sweden) according to standard protocols. For statistical analysis of significantly changed genes, the probe logarithmic intensity error method was employed.

Classification into GO functional groups [32] and analysis of overrepresented themes were performed using the EASE package [33]. The complete human transcriptome was used for calculation of the expected frequencies in the overrepresentation analysis, and a GO theme was considered overrepresented if the calculated EASE score was below 0.3.

To search for the presence of ERE motifs within the ERβ binding regions, the CONSITE software was used [34].

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