Gene expression profiling reveals that the regulation of estrogen-responsive element-independent genes by 17 β -estradiol-estrogen receptor β is uncoupled from the induction of phenotypic changes in cell models

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Abstract

Estrogen hormone 17 β -estradiol (E_2) is involved in the physiology and pathology of many tissues. E_2 information is conveyed by the transcription factors estrogen receptors (ER) α and β that mediate a complex array of nuclear and non-nuclear events. The interaction of ER with specific DNA sequences, estrogen-responsive elements (EREs), constitutes a critical nuclear signaling pathway. In addition, E_2 -ER regulates transcription through interactions with transactors bound to their cognate regulatory elements on DNA, hence the ERE-independent signaling pathway. However, the relative importance of the ERE-independent pathway in E_2 -ER β signaling is unclear. To address this issue, we engineered an ERE-binding defective ER β mutant (ER β_{EBD}) by changing critical residues in the DNA-binding domain required for ERE binding. Biochemical and functional studies revealed that ER β_{EBD} signaled exclusively through the ERE-independent pathway. Using the adenovirus infected ER-negative cancer cell models, we found that although E_2 -ER β_{EBD} regulated the expression of a number of genes identified by microarrays, it was ineffective in altering cellular proliferation, motility, and death in contrast to E_2 -ER β . Our results indicate that genomic responses from the ERE-independent pathway to E_2 -ER β are not sufficient to alter the cellular phenotype. These findings suggest that the ERE-dependent pathway is a required signaling route for E_2 -ER β to induce cellular responses.

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Introduction

Estrogen hormones, particularly the main circulating estrogen, 17 β -estradiol (E_2), play important roles in the regulation of many tissue functions (Huang *et al.* 2005a, Deroo & Korach 2006). E_2 is also involved in the initiation and development of target tissue malignancies. The E_2 information is primarily conveyed by estrogen receptors (ER) α and β (Huang *et al.* 2005a, Deroo & Korach 2006). ERs are members of the conserved superfamily of hormone receptors and are ligand-activated transcription factors. The effects of E_2 -ER are exerted through a complex array of convergent and divergent signaling pathways that mediate genomic events involved in the regulation of mitogenesis, motogenesis, and apoptosis (Hall *et al.* 2001, Nilsson *et al.* 2001, Huang *et al.* 2005a). The interaction of E_2 -ER with specific DNA sequences, estrogen-responsive elements (EREs), constitutes a primary genomic signaling pathway (Hall *et al.* 2001, Nilsson *et al.* 2001, Huang *et al.* 2005a). The ERE-bound ER recruits

an ensemble of multi-subunit complexes responsible for the alteration of local chromatin structure and the interaction with the basal transcription machinery. The integrated effects of these complexes regulate transcription. This type of E_2 -ER-mediated signaling is referred to as the ERE-dependent signaling pathway (Hall *et al.* 2001, Nilsson *et al.* 2001, Huang *et al.* 2005a).

In addition, the E_2 -ER complex regulates the expression of E_2 -responsive genes through functional interactions with transcription factors bound to their cognate regulatory elements on DNA (Kushner *et al.* 2000, Safe 2001). In this DNA-dependent and ERE-independent signaling pathway, transcriptional responses are dependent upon the ER-subtype, promoter, and cell-context (Kushner *et al.* 2000, Safe 2001). However, the relative importance of the ERE-independent pathway in physiology and pathophysiology of E_2 -ER β signaling is unknown. We envisioned that a selective regulation of the ERE-independent genes would allow us to begin to address this issue. To accomplish this, we generated an ERE-binding defective ER β mutant (ER β_{EBD}) which

renders the receptor nonfunctional at the ERE-dependent pathway, while conserving the regulatory potential at the ERE-independent pathway. We used ER-negative cells as experimental models, with which exogenously introduced ERs were shown to regulate the expression of responsive genes (Licznar *et al.* 2003, Kian Tee *et al.* 2004, Stossi *et al.* 2004, Moggs *et al.* 2005, Monroe *et al.* 2005) and to induce phenotypic changes (Garcia *et al.* 1992, Jiang & Jordan 1992, Zajchowski *et al.* 1993, Lazennec & Katzenellenbogen 1999, Lazennec *et al.* 2001, Licznar *et al.* 2003). In adenovirus-infected cells, we found that genomic responses induced by ER β_{EBD} in response to a physiological level of E_2 are insufficient to alter cellular proliferation, death, or motility, in contrast to E_2 -ER β . These results imply that the ERE-dependent pathway is the required signaling route mediated by the E_2 -ER β complex.

Materials and methods

Generation of ER β DNA-binding defective mutant (ER β_{EBD})

The human ER β cDNAs that encode the 530 amino acid ER β have been described previously (Yi *et al.* 2002a). This ER β cDNA also contains sequences encoding an amino-terminal Flag epitope (Yi *et al.* 2002a). For the engineering of an ERE-binding defective ER β (ER β_{EBD}), we utilized an overlapping PCR with the ER β cDNA as the template and primers that contain amino acid substitutions to replace glutamic acid and glycine at positions 167 and 168 respectively with alanine residues in the first zinc finger of the DNA-binding domain (DBD) of the receptor.

Restriction and DNA modifying enzymes were obtained from New England Bio-Labs (Beverly, MA, USA) and Invitrogen.

Cell culture

The culturing of MDA-MB-231 and HeLa cells has been described previously (Yi *et al.* 2002a). U-2 OS cells derived from osteosarcoma were purchased from ATCC (Manassas, VA, USA). U-2 OS cells were grown in McCoy's 5 α medium supplemented with 10% fetal bovine serum (FBS, Invitrogen). In all experiments, the medium was changed every third day.

Transient transfections

Transient transfections for the simulated ERE-dependent and -independent pathways were accomplished as described previously (Yi *et al.* 2002a, Huang *et al.* 2004). The transfected cells were treated without or with 10^{-9} M of E_2 , 10^{-7} M of 4-hydroxyl-tamoxifen (4-OHT,

Sigma-Aldrich), 10^{-7} MICI 182 780 (Imperial Chemical Industries, Tocris Inc., Ballwin, MO, USA), and 10^{-8} M diarylpropionitrile (DPN, Tocris) for 24 or 40 h to assess the effects of ligands on ER-mediated transcriptional responses from the ERE-dependent or -independent signaling pathways respectively.

Generation of a recombinant adenovirus bearing an ER cDNA

Recombinant adenovirus bearing none, the cDNA of Flag-ER β or Flag-ER β_{EBD} were produced using the AdEasy-XL Adenoviral System (Stratagene, La Jolla, CA, USA) as described previously (Huang *et al.* 2005b). The purified viruses were titered using an Adeno-X Rapid Titer Kit (BD Biosciences, Palo Alto, CA, USA) to determine the multiplicity of infection (MOI).

Western blot (WB), electrophoretic mobility shift assay (EMSA), and immunocytochemistry (ICC)

Transfected or infected cells in a time-dependent manner were processed for WB, EMSA, and ICC as described previously (Muyan *et al.* 2001, Yi *et al.* 2002a). For WB, proteins were probed with horseradish peroxidase-conjugated monoclonal Flag antibody (M2-HRP, Sigma-Aldrich) using the ECL-Plus Western Blotting kit (Amersham-Pharmacia). The images were captured using PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). For ICC, we used an ER β -specific antibody (Zymed Laboratories, San Francisco, CA, USA) followed by a fluorescein conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

In situ E_2 binding and ERE competition assays

To assess the functionality of ER β species in transfected cells, we used the *in situ* E_2 binding assay and the *in situ* ERE competition assays as described previously (Huang *et al.* 2005b).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using Flag-M2 antibody-conjugated agarose beads (Sigma-Aldrich) as described previously (Huang *et al.* 2005b). The generation of a 366 bp PCR fragment indicates the specificity of PCRs.

Endogenous gene expression

MDA-MB-231 cells (100 000 cells/well) plated in six-well tissue culture plates in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses without or with 10^{-9} M E_2 for 48 h to

assess the effects of ER on the expression of the trefoil factor 1 (*TFF1*), complement factor 3 (*C3*), matrix metalloproteinase 1 (*MMPI*), and retinoic acid receptor α (*RARA*) genes.

The cells were also infected with recombinant adenoviruses in the absence of E₂ and maintained for 48 h, the time during which the synthesis of ERs reaches comparable levels (Fig. 1). The infected cells were then treated with 10⁻⁹ M E₂ for 6, 12, or 24 h to confirm the identities of genes determined by microarrays using

quantitative PCR (qPCR). At termination, the cells were collected and subjected to total RNA extraction using the RNeasy Mini Kit (Qiagen) for qPCR, which we used custom TaqMan Low-Density Arrays with proprietary primer and probe sequences (Applied Biosystems, Foster City, CA, USA). All qPCRs were carried out at the Functional Genomic Center of the University of Rochester, NY, USA. The expression of the actin β (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes was used as the control. The

Figure 1 Generation of an ERE-binding defective ERβ (ERβ_{EBD}). (A) ERβ_{EBD} was engineered by changing glutamic acid and glycine at positions 167 and 168 of the first zinc finger of the DNA-binding domain of the ERβ respectively to alanine residues. (B) The synthesis of ERβ species. MDA-MB-231 cells were transiently transfected with an expression vector bearing none (V), the ERβ or ERβ_{EBD} cDNA. Cell extracts (10 μg) were subjected to western blotting (WB) using a horseradish peroxidase-conjugated monoclonal Flag antibody. MW in kDa is indicated. The cell extracts (20 μg) of transfected cells were also subjected to electrophoretic mobility shift assay (EMSA) without (–) or with (+) a Flag antibody (Ab). ERE denotes unbound and ER-ERE indicates ER-bound radiolabeled ERE. Representative results from three independent experiments of WB and EMSA are shown. (C) The *in situ* ERE competition assay. MDA-MB-231 cells were transiently transfected with a reporter vector bearing one ERE upstream of the TATA box promoter driving the expression of *Firefly* luciferase cDNA and the expression plasmid for PPV, together with various concentrations of expression vector bearing the ERβ or ERβ_{EBD} cDNA. The cells were also co-transfected with a reporter plasmid bearing *Renilla* luciferase cDNA to monitor the transfection efficiency. The cells were then treated without (–E₂) or with (+E₂) 10⁻⁹ M E₂ for 24 h. The relative luciferase activity was presented as the percentage (%) change compared with control (PPV alone in the absence of E₂), which was set to 100%. The mean of three independent experiments performed in duplicate has been shown. S.E.M., which was less than 15% of the mean, is not shown for simplicity. (D) Chromatin immunoprecipitation (ChIP) assay. MDA-MB-231 cells were transiently transfected with an expression vector bearing the ERβ or ERβ_{EBD} cDNA together with a reporter bearing none (TATA) or one ERE TATA box promoter. The cells were treated without (–) with (+) 10⁻⁷ M E₂ for 1 h prior to ChIP using the Flag antibody-conjugated agarose beads. The size of the DNA fragment is indicated in bp. A representative image from three independent experiments is shown.

real-time RT-PCR amplifications were accomplished using an ABI Prism 7900HT Sequence Detection System with a TaqMan Low Density Array Upgrade (Applied Biosystems). Relative quantification analysis was performed using the comparative C_T method (Livak & Schmittgen 2001).

Cell proliferation

MDA-MB-231 cells (5000 cells/well) plated in 24-well tissue culture plates in phenol red-free DMEM containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for different durations of time. The cells were collected and counted using a hemacytometer (Hausser Scientific, Horsham, PA, USA).

Additionally, we used a colorimetric proliferation assay, the 3-(4,5 dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). The cells, plated and infected with adenoviruses as described for hemacytometric cell counting, were incubated with 200 μ l phenol red-free DMEM with 10% CD-FBS containing 60 μ M MTT (Invitrogen) for 2 h. The spent medium was removed and 200 μ l dimethyl sulfoxide (DMSO) was added to each well to dissolve the MTT formazan. The absorbance was measured using a microplate spectrophotometer (SpectraMax Plus; Molecular Devices, Sunnyvale, CA, USA) to estimate the cell number.

For U-2 OS cell proliferation, cells (5000 cells/well) were plated in 24-well tissue culture plates, pre-coated with poly-L-lysine (Sigma-Aldrich), in McCoy's 5 α medium containing 10% FBS, for 24 h. The cells were subsequently incubated with McCoy's 5 α medium containing 10% CD-FBS for an additional 24 h. They were then infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for different durations of time. We used Ad5-ER β at 40 MOI. At this concentration, the recombinant adenovirus synthesized a concentration of ER β that is dependent upon E_2 for functioning. Ad5-ER β_{EBD} was used at 50 MOI, which produced levels of receptor comparable to that of ER β . At the termination of an experiment, cells were subjected to cell counting and MTT assays.

Cell cycle analysis

MDA-MB-231 cells (50 000 cells/well) in six-well tissue culture plates were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for different durations. The cells were collected and pelleted. For fixing and permeabilization, the pelleted cells were resuspended in ethanol (70%) at 4 °C overnight. The cells were subsequently incubated with 1 mg/ml RNase A (Sigma-Aldrich) for 30 min followed by 20 μ g/ml propidium iodide (PI) (Sigma-Aldrich) for 10 min.

They were then subjected to fluorescence-activated cell sorting (FACS) using EPICS Elite (Coulter Corp., Miami, FL, USA).

Caspase 3/7 assay

MDA-MB-231 cells (12 500 cells/well) plated onto poly-L-lysine coated 96-well tissue culture black plates with clear bottom (BD Biosciences, Franklin Lakes, NJ, USA) in phenol red-free DMEM containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses, in the absence or presence of 10^{-9} M E_2 , for different lengths of time. The cells were then subjected to Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to the manufacturer's protocol. Fluorescence was then measured using a spectrophotometer.

Annexin V assay

To study apoptosis by examining the loss of cell membrane asymmetry as an indicator of middle stages of apoptosis, we used the Vybrant Apoptosis Assay Kit (Invitrogen). This assay is based on the specific recognition of phosphatidyl-serine (PS) by FITC-conjugated annexin V. MDA-MB-231 cells (100 000 cells/well) were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for different lengths of time. The cells were collected and subjected to annexin V assay, according to the instruction of the manufacturer, prior to FACS analysis.

TUNEL assay

To study the late stages of apoptosis by examining the fragmentation of genomic DNA (Korsmeyer 1999), MDA-MB-231 cells (25 000 cells/well) plated in poly-L-lysine-coated 48-well tissue culture plates in phenol red-free DMEM containing 10% CD-FBS for 24 h and infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for different lengths of time. The cells were then subjected to a TUNEL assay using the DeadEnd Fluorometric TUNEL System (Promega), according to the manufacturer's protocol. 4',6-diamino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA) was used to stain cell nuclei. The stained cells were imaged under a microscope with corresponding filters.

Wound-healing assay

MDA-MB-231 cells (200 000 cells/well in 12-well tissue culture plates) were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for 48 h for confluence. A wound was then created using a 1 ml pipette tip. The gap closure was photographed every 24 h. Due to the irregular shape of the edges of a

wound, five randomly selected cross edges were used to obtain a mean gap measure for wound healing.

Invasion assay

We used BD Matrigel Invasion Chambers (BD Biosciences, San Diego, CA, USA) for the invasion assay. MDA-MB-231 cells (100 000 cells/well) in six-well tissue culture plates were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E₂ for 48 h. The cells were then trypsinized and counted. The same number (25 000 cells/chamber) of cells was seeded on the upper section of the chamber, which contained phenol red-free DMEM without or with 10^{-9} M E₂. The lower section of the chamber contained phenol red-free DMEM supplemented with 10% CD-FBS and 30 μ g/ml fibronectin in the absence or presence of 10^{-9} M E₂. After 24 h incubation, the cells on the upper section of the membrane were removed using a cotton swab. The cells on the bottom of the chamber membrane were stained with the Diff-Quik Stain Set (Dade Behring, Newark, DE, USA), dried, and mounted onto a glass slide. The images were captured and the stained cells were counted from images.

Microarray analysis

To examine the effects of E₂ on endogenous gene expression mediated by ERs, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of E₂ for 48 h. The infected cells were then treated with 10^{-9} M E₂ for 6 h. This duration of E₂ treatment was expected to induce significant changes in the level of immediate/early gene expression, as observed with the responses to E₂ in ER α synthesizing breast cancer cell lines (Frasor *et al.* 2003). At termination, the cells were subjected to RNeasy Mini kit (Qiagen) for total RNA extraction. The processing of RNA for microarray analysis was carried out at the Functional Genomic Center of the University of Rochester, NY. cDNA synthesis and subsequent fragmentation and biotinylation of cDNA fragments were carried out using the Ovation kit (NuGEN, San Carlos, CA, USA) according to the manufacturer's procedure. The biotinylated cDNA fragments were then used for hybridization with microarrays. We used Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The arrays were scanned using the GeneChip Scanner 3000 7G. GeneChip Operating Software (GCOS, Affymetrix) was used for initial processing of the scanner data, including the generation of cel files. Array normalization for the Affymetrix signal method (GCOS) involves multiplying raw signals by a scaling factor such that the trimmed mean (excluding highest and lowest 2%) of all expression scores is 500 arbitrary units for every array.

Experimental sets for microarrays were replicated six independent times, executed on different days. Although we also conducted microarray data analysis based on probe sets that relied on earlier genome and transcriptome annotation (Gautier *et al.* 2004, Dai *et al.* 2005, Harbig *et al.* 2005) and posted at <http://dbb.urmc.rochester.edu/labs/muyan/ArrayAddendum.htm>, we present here the data analysis using reorganized and updated probe sets (Gautier *et al.* 2004, Dai *et al.* 2005, Harbig *et al.* 2005) based on the up-to-date genome, cDNA/EST clustering, and single nucleotide polymorphism information through web-based custom GeneChip library files (Chip Definition Files or CDFs, <http://arrayanalysis.mbn.med.umich.edu>) which increase accuracy and reduce false discovery rates (Dai *et al.* 2007). Following UniGene transformation, the data sets were subjected to the *N*-statistic test (Klebanov *et al.* 2006) in conjunction with the step-down Westfall & Young (1993) procedure controlling the familywise error rate (FWER) at a level of 0.05, which was reported here. Minimum Information About a Microarray Experiment (MIAME)-compliant microarray data for the six independent replicate studies have been submitted to the Gene Expression Omnibus (GEO) database (GEO 2004) with the accession number GSE9761.

Statistical analysis

Results were presented as the mean $\pm$ S.E.M. of, at least, three independent experiments. Student's *t*-test was employed for the comparison of means between two groups wherein *P* < 0.05 was considered significant.

Results

The functional characterization of the ERE-binding defective ER β (ER β _{EBD})

ERs recognize an ERE as a dimer mediated by a dimerization interface located in the ligand-binding domain and a weak interaction surface in the DBD (Parker 1998). The DBD of ER α contains two zinc finger-like modules that fold to form a single functional domain. Each DBD of the ER α dimer makes analogous contacts with one of the inverted motifs of ERE, which results in a rotationally symmetrical structure (Luisi *et al.* 1994). Distinct residues in a region of the first zinc finger module of DBD, the P-box, particularly glutamic acid and glycine at positions 203 and 204 respectively, determine the DNA-binding specificity critical for sequence discrimination (Green *et al.* 1988). The residues in the second zinc finger-like module, the D-box, are involved in the discrimination of half-site spacing through a protein–protein interaction between two ER monomers (Green *et al.* 1988). The

human ER α and ER β share a 97% amino acid identity in their DBDs with identical residues at the P- and D-boxes (Mosselman *et al.* 1996). This structural homology is reflected in the abilities of ERs to bind various ERE sequences with a similar specificity and affinity by interacting with the same nucleotides (Yi *et al.* 2002b).

Studies showed that the regulation of E₂ responsive genes through ERE-independent signaling involves a functional interaction between ERs and a transcription factor bound to its cognate response element on DNA through regions which also encompass the DBD of ERs (Kushner *et al.* 2000, Safe 2001). Changing the glutamic acid and glycine at positions 207 and 208 of the mouse ER α , and at positions 167 and 168 of human ER β respectively, at the P-box of the first zinc finger of the DBD hinders the ER-ERE interactions, while conserving the capacity to regulate transcription from the ERE-independent signaling pathway (Jakacka *et al.* 2001, Bjornstrom & Sjoberg 2002). Based on these observations, we engineered an ERE-binding defective ER β variant (ER β_{EBD}) bearing alanine residues at positions 167 and 168 to exclusively regulate the DNA-dependent and ERE-independent pathway (Fig. 1A).

The initial biochemical characterization of ER β_{EBD} was carried out in transiently transfected ER-negative cell models. Cell extracts, shown for MDA-MB-231 cells, were subjected to WB and EMSA. The detection of receptor proteins by WB using a Flag antibody (M2) directed to the Flag epitope at the amino terminal of the receptors indicated that the receptors were synthesized at similar levels (Fig. 1B, WB). EMSA, using a ³²P-end labeled DNA fragment bearing the consensus ERE, revealed that despite the comparable level of synthesis, ER β , but not ER β_{EBD} , interacted *in vitro* with ERE (Fig. 1B, EMSA).

To ensure that ER β_{EBD} is indeed defective in binding to ERE *in situ*, we used *in situ* ERE competition and ChIP assays. The *in situ* ERE competition assay is based on the ability of ER to compete for ERE binding with a designer activator, designated as PPV, which potently and constitutively induces transcription from an ERE-driven reporter construct (Huang *et al.* 2005b). The interference of the activator-mediated transcription by unliganded or liganded ERs is then taken as an indication of ER-ERE interaction. The reporter TATA box plasmid bearing none (TATA) or one ERE was co-transfected with an expression vector encoding the PPV cDNA into cell models, shown for MDA-MB-231 cells, in the absence or presence of varying amounts of an expression vector bearing an ER cDNA without or with a physiological concentration (10⁻⁹ M) of E₂ for 24 h. The normalized luciferase activity mediated by PPV alone in the absence of E₂ was set to 100%. Alterations in the reporter enzyme activity as a result of a co-transfected ER cDNA without or with E₂ are depicted as percentage (%) change compared with the activity induced by PPV alone. Previously, we

(Huang *et al.* 2005b) and others (Hall & McDonnell 1999) showed that ER β , in contrast to ER α , interacts with ERE *in situ* independent of E₂. Consistent with this finding, our results here also reveal that transfection with increasing concentrations of the expression vector bearing ER β , but not ER β_{EBD} , cDNA gradually decreased the luciferase activity induced by PPV independent of E₂ (Fig. 1C), without altering responses from the reporter plasmid bearing the TATA box promoter (data not shown).

A ChIP assay was also employed to corroborate our finding (Fig. 1D). The expression vectors were co-transfected with the reporter TATA box promoter vector bearing none (TATA) or one ERE into MDA-MB-231 cells. The cells were treated without or with a saturating concentration (10⁻⁷ M) of E₂ for 1 h, and processed for ChIP. We found that ER β , but not ER β_{EBD} or the parent vector (data not shown), produced a PCR product from cells co-transfected with the reporter vector bearing ERE irrespective of whether the cells were treated with E₂ as observed with the *in situ* ERE competition assay (Fig. 1C). Thus, these results collectively suggest that ER β_{EBD} does not interact with ERE *in situ* as well.

To ensure that ER β_{EBD} is functional only in the ERE-independent pathway, the expression vectors were transiently transfected into ER-negative MDA-MB-231, HeLa, or U-2 OS cells. The cells were also co-transfected with a reporter vector containing a promoter driving the expression of the firefly luciferase cDNA as the reporter enzyme. Promoters were derived from the estrogen-responsive *TFF1*, *pS2* and *C3* genes that contain ERE sequences, thereby modeling the ERE-dependent signaling pathway (Yi *et al.* 2002a, Huang *et al.* 2004). The normalized activity from each reporter construct was compared with the parent expression vector (V) in the absence of E₂, with the latter value set to 1. Results showed that the ER β_{EBD} had no effect on luciferase activity in the absence or presence of 10⁻⁹ M E₂ from either the *TFF1* or the *C3* gene promoter in all cell lines tested, shown MDA-MB-231 and HeLa cells (Fig. 2A). In contrast, ER β increased the reporter enzyme activity in response to E₂ from both the promoters in all cell lines.

To confirm that the transregulatory function of ER β_{EBD} is restricted to the ERE-independent signaling pathway, we used a reporter construct that bears the proximal *MMP1* or *RARA* gene promoter (Huang *et al.* 2004). The functional interaction of ERs with the Jun/Fos family of proteins bound to an activator protein-1 (AP1) element in the proximal promoter of the *MMP1* gene provides the basis for the responsiveness to ERs in a ligand- and cell-type dependent manner in experimental systems (Webb *et al.* 1995, Paech *et al.* 1997, Webb *et al.* 1999, Kushner *et al.* 2000). Similarly, the interaction of ER with specificity protein-1 (SP1) bound to GC boxes of the proximal promoter is critical for the

ligand-mediated regulation of the *RARA* gene (Rishi *et al.* 1995, Sun *et al.* 1998, Safe 2001). Transiently transfected MDA-MB-231, HeLa or U-2 OS cells were incubated without or with 10⁻⁹ M E₂, 10⁻⁸ M of 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), 10⁻⁷ M of 4-hydroxytamoxifen (4-OHT), and 10⁻⁷ MICI 182 780 (Imperial Chemical Industries). DPN is an ER β -potency selective estrogenic compound (Harrington *et al.* 2003). Results revealed that while the parent vector had no effect on transcription in the absence or presence of ligands in all cell lines tested, ER β and ER β_{EBD} mediated the effects of ligands on the reporter enzyme activity similarly independent of promoter and cell type (Fig. 2B). In MDA-MB-231 cells, only DNP and 4-OHT enhanced both ER β - and ER β_{EBD} -induced responses from the *RARA* promoter construct, while E₂ or ICI had no effect on the reporter enzyme activity mediated by either ER. In contrast to MDA-MB-231 cells, ERs expressed in HeLa cells enhanced the reporter enzyme activity from the *MMP1* promoter in response to only 4-OHT. In these cell lines, ER β or ER β_{EBD} had no effect on the transcriptional responses from the *RARA* gene promoter in the absence or presence of ligands. In U2-OS cells, on the other hand, E₂ and DPN repressed the reporter enzyme activity mediated by ER β and ER β_{EBD} from both the *MMP1* and the *RARA* promoter constructs, whereas 4-OHT and ICI augmented responses to both the receptors.

While the transcriptional responses mediated by ER species varied depending upon the nature of ligand, promoter, and cell context, our findings clearly indicate that ER β_{EBD} mirrored the effect of ER β on the reporter enzyme activity from reporter constructs emulating the ERE-independent signaling pathway. Our results suggest that ER β_{EBD} retains its transregulatory function in the ERE-independent pathway, despite the fact that it does not bind to ERE and, consequently, is nonfunctional in the ERE-dependent signaling pathway.

Regulation of endogenous gene expression by E₂-ER β s

The experimental reporter systems we used here contain minimal promoter sequences responsive to E₂-ER signaling. While transient transfection into mammalian cells has been a valuable tool for the understanding of action mechanisms of transactors, nucleosome deposition onto the non-replicating reporter vector displays an incompletely organized nucleosome array in contrast to the replicative cellular chromatin (Archer *et al.* 1992, Lee & Archer 1994, Pennie *et al.* 1995). This results in a more accessible chromatin of the transfected DNA that affects basal and transactor-regulated transcription compared with the responses observed with cellular chromatin (Archer *et al.* 1992, Lee & Archer 1994, Pennie *et al.* 1995).

Moreover, the temporal regulation of endogenous E₂ target gene expression is the result of integrated effects of transcription factors that bind to cognate responsive elements within the regulatory regions. The combinatorial effects of transactors and ER ultimately determine the magnitude and/or direction of the endogenous gene expression (Nunez *et al.* 1989, Vyhldal *et al.* 2000). Consistent with heterologous expression systems, as we have demonstrated here (Fig. 2), the presence of an ERE in the *TFF1* gene promoter provides the endogenous responsiveness to E₂-ER signaling (Nunez *et al.* 1989). The regulation of the *RARA* gene expression is, on the other hand, attributed to ER-SPI interactions in experimental systems (Rishi *et al.* 1995, Sun *et al.* 1998, Safe 2001), as we also showed here (Fig. 2) and previously (Li *et al.* 2004). However, a recent genomics approach identified a long-distance regulatory module that contains a functional ERE responsible for the regulation of the *RARA* gene expression in breast cancer cell models expressing endogenous ER (Laganiere *et al.* 2005).

To ensure that ER β_{EBD} can indeed discriminately regulate endogenous gene expression, we used MDA-MB-231 cells as a model within which exogenously expressed ERs were shown to induce gene expressions that affect phenotypic characteristics (Garcia *et al.* 1992, Jiang & Jordan 1992, Zajchowski *et al.* 1993, Lazennec & Katzenellenbogen 1999, Lazennec *et al.* 2001, Licznar *et al.* 2003, Moggs *et al.* 2005). We also used recombinant adenoviruses for an efficient gene delivery (Huang *et al.* 2005b).

Recent studies showed that augmented levels of unliganded ER α lead to aberrant gene expression and an altered phenotype through mechanisms that are distinct from those mediated by E₂ (Fowler *et al.* 2004, 2006). To circumvent this potential problem, we used various concentrations of the recombinant adenovirus bearing ER β cDNA (Ad5-ER β) to obtain an optimal MOI that leads to a level of receptor synthesis which requires E₂ to regulate gene expression and cellular growth. We found that MDA-MB-231 cells infected with Ad5-ER β at 600 MOI synthesize a concentration of ER β that is strictly dependent upon E₂ for function (data not shown). The recombinant adenovirus expressing ER β_{EBD} cDNA (ER β_{EBD}) was used at 900 MOI producing comparable levels of receptor to that of ER β (see also Fig. 3B). We therefore used 900 MOI of the parent recombinant adenovirus (Ad5), 600 MOI of Ad5-ER β , which was brought to 900 MOI by supplementing with 300 MOI of Ad5, and 900 MOI of ER β_{EBD} in subsequent experiments.

The synthesis of functional receptor protein was examined by ICC, WB, E₂ binding, and EMSA assays in a time-dependent manner in infected MDA-MB-231 cells. ICC revealed that receptor proteins became detectable in the nuclei of infected cells as a function of time such that at 48 h post-infection nearly all cells showed staining for

ER β and ER β_{EBD} (Fig. 3A). This was a reflection of the level of ER synthesis as assessed by WB, which showed that ER β and ER β_{EBD} were synthesized comparably in a time-dependent manner (Fig. 3B). The *in situ* E_2 binding assay further showed that 3H - E_2 was similarly retained in cells that synthesize ER β and ER β_{EBD} but not the parent recombinant adenovirus (Fig. 3C). The co-incubation of cells with 4-OHT prevented the retention of radiolabeled E_2 . This indicates that 3H - E_2 was specifically retained by ERs in cells. These results demonstrate that MDA-MB-231 cells infected with recombinant adenoviruses synthesize comparable amounts of receptor proteins capable of binding with E_2 . Moreover, EMSA further revealed that ER β_{EBD} did not interact with ERE, while ER β effectively bound to ERE (Fig. 3D).

Based on these observations, we anticipated that ER β_{EBD} would not regulate endogenous gene expression mediated by the ERE-dependent pathway. Our studies using qPCR revealed that ER β only in the presence of E_2 effectively induced the expression of estrogen-responsive genes mediated by ER-ERE interactions, exemplified by the *TFF1* and *C3* genes, while ER β_{EBD} had no effect on the expression of these genes (Fig. 4A). We also observed that E_2 -ER β , but not E_2 -ER β_{EBD} , activated the endogenous *RARA* gene expression. This is consistent with the finding that the regulation of the *RARA* gene expression is dependent on ER-ERE interactions (Laganiere *et al.* 2005), which is clearly in contrast to the response from the reporter system we observed here and reported previously (Rishi *et al.* 1995, Sun *et al.* 1998, Safe 2001). On the other hand, both ERs in the presence of E_2 repressed the expression of the *MMP1* gene that models an ERE-independent signaling-regulated gene.

To further verify the functionality of ER β_{EBD} and to preliminarily identify the genes mediated by the ERE-independent pathway, we used a global gene expression profiling approach. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of E_2 for 48 h. At this time of post-infection, nearly all cells synthesized receptor proteins at levels that were maximal and comparable (Fig. 3). The cells were then treated with 10^{-9} M E_2 for 6 h, which is expected to induce significant changes in the level of transcription of immediate/early estrogen responsive genes (Frasor *et al.* 2003).

Expression profiling revealed that the genes regulated by ER β and ER β_{EBD} are involved in a broad range of function including metabolism, signal transduction, proliferation, apoptosis, and motility (Tables 1 and 2). As summarized in Fig. 4B, E_2 -ER β significantly altered the expression of 41 genes, whereas E_2 -ER β_{EBD} regulated the expression of 12 genes, 10 of which were also modified by E_2 -ER β . The majority of the identified genes were suppressed by both E_2 -ER β and E_2 -ER β_{EBD} . E_2 -ER β enhanced the expression of 12 responsive genes, whereas E_2 -ER β_{EBD} augmented the expression of three genes.

We further verified the expression of a subset of the identified genes with qPCR. Infected MDA-MB-231 cells were maintained for 48 h in the absence of E_2 (Fig. 4C). The cells were then treated without or with 10^{-9} M E_2 for 6, 12, and 24 h. Total RNA was subjected to qPCR. As observed with microarrays, only ER β in the presence of E_2 induced the expression of the cyclin-dependent kinase inhibitor (*CDKN1A*, WAF1 p21, CIP1) and lysyl oxidase-like 4 (*LOX4*) genes. The expression of the follistatin (*FST*) gene was indeed repressed by only E_2 -ER β_{EBD} .

E_2 -ER β and E_2 -ER β_{EBD} activated the cysteine-rich secretory protein LCCL domain containing 2 (*CRISPLD2*) and thromboxane A2 receptor (*TBXA2R*) gene expressions. On the other hand, both receptors repressed the expression of the *MMP1*, (*SERPINB2*) serpin peptidase inhibitor, clade member 2, hyaluronan synthase 2 (*HAS2*), and heparin-binding EGF-like growth factor (*HBEGF*) genes in the presence of E_2 .

Thus, ER β_{EBD} is capable of modulating the expression of endogenous genes in response to E_2 .

Differential effects of E_2 -ER β and E_2 -ER β_{EBD} on cell growth

To examine whether the genomic responses mediated by E_2 -ER β_{EBD} participate in the regulation of cellular growth, we used proliferation assays. Infected MDA-MB-231 cells were grown for various durations of time in the absence or presence of 10^{-9} M E_2 . The cells were then subjected to cell counting (Fig. 5A) and MTT assay (Fig. 5B). Results revealed that ER β or ER β_{EBD} had no effect on cellular growth in the absence of E_2 (data not shown). On the other hand, E_2 treatment of the

Figure 2 Transcriptional responses to ER β s from heterologous reporter systems. (A) Responses from the ERE-dependent signaling pathway. MDA-MB-231 or HeLa cells were transiently transfected with an expression vector bearing none (V), ER β , or ER β_{EBD} cDNA together with a reporter vector bearing the *TFF1* or *C3* gene promoter in the absence or presence of 10^{-9} M E_2 for 24 h. Normalized luciferase values represented as fold changes compared with the luciferase values obtained with control vector in absence of E_2 , which was set to 1. The mean $\pm$ S.E.M. of three independent experiments performed in duplicate are shown. (B) The transactivation ability of ER β_{EBD} in simulated ERE-independent signaling pathways. MDA-MB-231, HeLa, or U-2 OS cells were transiently transfected with an expression vector bearing ER β or ER β_{EBD} cDNA together with reporter vector bearing the *MMP1* gene or the *RARA* gene proximal promoter. The cells were treated without or with 10^{-9} M E_2 , 10^{-8} DPN, 10^{-7} M of 4-OHT or ICI for 40 h. Normalized luciferase values are represented as fold change compared with the luciferase values obtained without ligand. Results are the mean $\pm$ S.E.M. of three independent experiments performed in triplicate.

infected cells synthesizing ERβ, but not ERβ_{EBD}, decreased the cell number as a function of time. These findings indicate that the E₂-ERβ_{EBD} signaling has no discernable effect on cell proliferation.

Moreover, despite the comparable synthesis as assessed by WB (Fig. 5C) and the *in situ* E₂ binding assay (data not shown), the ERE-binding defective ERβ_{EBD} (Fig. 5D) had no effect on cellular growth in the absence or presence of E₂ in infected U-2 OS cells in contrast to E₂-ERβ, which effectively suppressed the proliferation (Fig. 5E), as observed with MDA-MB-231 cells.

These results suggest that the ERE-independent pathway does not play a critical role in E₂-ERβ signaling to mediate cellular growth.

Effects of E₂-ERβ and E₂-ERβ_{EBD} on cell cycle

The absence of an effect of E₂-ERβ_{EBD} on cellular growth predicts that the ERE-independent pathway mediated by E₂-ERβ is also insufficient to alter cell cycle distribution.

To address this prediction, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of 10⁻⁹ M E₂ for various durations of time. The cells were then subjected to FACS. The kinetic analysis of histograms (Supplemental data Fig. 1A see Supplementary data in the online version of the Journal of Molecular Endocrinology at <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>), summarized as the percentile of cells in G1 phase (Fig. 6A), revealed that ERβ, only in the presence of E₂, increased the number of cells in the G1 phase and decreased the cell number accumulated in the S and G2 phases (data not shown). On the other hand, ERβ_{EBD} had little effect on cell cycle distribution whether or not the cells were treated with E₂. Thus, it appears that E₂ signaling conveyed by ERβ through the ERE-independent signaling pathway is insufficient to alter cell cycle distribution.

Since the effects of ERβ on the ERE-independent signaling are also dependent on the nature of ligand, we wanted to examine whether ER agonists or antagonists differentially affect cellular growth mediated by ERβ_{EBD}. The infected MDA-MB-231 cells were treated without or with 10⁻⁹ M E₂, 10⁻⁸ M DPN, 10⁻⁷ M of 4-OHT or ICI (data not shown) for 48 h. The cells were also co-treated with 10⁻⁹ M E₂ and 10⁻⁷ M of 4-OHT (or ICI, data not shown), or 10⁻⁸ M DPN together with 10⁻⁷ M of 4-OHT (or ICI, data not shown) for 48 h. These concentrations of ligands are optimally effective to alter the transcription in response to ERβ (Fig. 2 and data not shown). The cells

Figure 3 Functional ER synthesis in infected model cells.

(A) MDA-MB-231 cells were infected with the parent recombinant adenovirus (Ad5) at MOI 900, a recombinant adenovirus bearing ERβ cDNA (ERβ) at 600 MOI together with 300 MOI Ad5 to equalize the total adenovirus titer, or 900 MOI for virus bearing ERβ_{EBD} cDNA. The intracellular localization of receptor proteins was examined by immunocytochemistry. Infected cells as a function of time (shown at 18 and 48 h post-infection) were probed with an ERβ-specific antibody followed by a fluorescein-conjugated secondary antibody (FITC). DAPI was used to stain nuclei. (B) Cell extracts (10 μg) of infected cells at indicated times were subjected to WB using the horseradish peroxidase conjugated monoclonal Flag antibody. Molecular weight in kDa is indicated. *Indicates extracts of the parent adenovirus infected cells at 96 h. The image is a representative of three independent experiments. (C) *In situ* E₂ binding assay. Infected MDA-MB-231 cells, maintained for various durations of time as indicated, were incubated in a medium containing 10⁻⁷ M of ³H-E₂ for 1 h. The radioactivity retained in cells was quantified. That ³H-E₂ was specifically retained by ERs in cells was assessed by the co-incubation of cells with 10⁻⁶ M of 4-OHT. The graph represents the mean ± S.E.M. of three independent experiments performed in duplicate. (D) Cell extracts (20 μg) of infected cells for the indicated times were subjected to EMSA in the absence (-) or presence (+) of the anti-flag antibody. *Denotes extracts of the parent adenovirus infected cells at 72 h. ER-ERE indicates the radiolabeled ERE-bound ERs. Unbound radiolabeled ERE is not shown for simplicity. A representative image from three independent experiments is shown.

Figure 4 Effects of ERs on endogenous gene expression. (A) MDA-MB-231 cells were infected with recombinant adenoviruses in the absence (–) or presence (+) of 10^{-9} M E₂ for 48 h. Total RNA from infected cells was subjected to qPCR for the expression of the *TFF1*, *C3*, *MMP1* and *RARA* genes. (B) Summary of genes identified with microarrays. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of E₂ for 48 h. The cells were then treated with 10^{-9} M E₂ for 6 h. Total RNA was subjected to arrays. Results are the mean of six independent determinations. (C) The verification of subset of genes identified with arrays. The infected MDA-MB-231 cells were maintained in the absence of E₂ for 48 h. The cells were then treated without or with 10^{-9} M E₂ for 6, 12, or 24 h. Total RNA was subjected to qPCR. Results, which are the mean $\pm$ S.E.M. of four independent determinations in quadruplicate, are depicted as fold change compared with the level of gene expression in cells infected with the parent recombinant adenovirus (Ad5) in the absence of E₂ at 6 h of E₂ treatment. The expression of the *LOXL4*, *CDKN1A*, *FST*, *CRISPLD2*, *TBXA2R*, *SERPINB2*, *MMP1*, *HAS2*, and *HBEGF* genes are shown.

Table 1 Genes mediated by 17β-estradiol (E₂)-estrogen receptor β (ERβ)

	Symbol	Fold change	Process
Gene name			
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	−3·70	Anti-apoptosis
Matrix metallopeptidase 1 (interstitial collagenase)	MMP1	−3·57	Collagen catabolism, proteolysis
Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	EDG1	−2·33	Signal transduction
Hyaluronan synthase 2	HAS2	−2·33	Matrix formation
Gap junction protein, β2, 26 kDa (connexin 26)	GJB2	−2·27	Cell–cell signaling
Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	MMP3	−2·17	Collagen catabolism, proteolysis
Hypothetical protein LOC285628	LOC285628	−2·13	Unknown
Plasminogen activator, urokinase receptor	PLAUR	−1·75	Cell motility, signaling, chemotaxis
Rho GTPase activating protein 18	ARHGAP18	−1·75	Signal transduction
Transforming growth factor, β2	TGFB2	−1·76	Proliferation, apoptosis, migration
CDNA FLJ25677 fis, clone TST04054	–	−1·69	Unknown
Heparin-binding EGF-like growth factor	HBEGF	−1·64	Signal transduction
Adenosine A2b receptor	ADORA2B	−1·59	Signal transduction
KIAA1609 protein	KIAA1609	−1·56	Unknown
Dickkopf homolog 1 (<i>Xenopus laevis</i>)	DKK1	−1·56	Signal transduction
Forkhead box Q1	FOXQ1	−1·49	Transcription
Adhesion molecule with Ig-like domain 2	AMIGO2	−1·47	Cell adhesion
Dual specificity phosphatase 5	DUSP5	−1·45	Protein dephosphorylation
G-protein-coupled receptor	GPRC5A	−1·43	Signal transduction
CD44 antigen	CD44	−1·35	Cell adhesion
Interleukin 18 (interferon-γ-inducing factor)	IL18	−1·35	Signal transduction
Connective tissue growth factor	CTGF	−1·32	Signal transduction
Follicular lymphoma variant translocation 1	FVT1	−1·27	Metabolism
Baculoviral IAP repeat-containing 2	BIRC2	−1·27	Apoptosis
Immediate early response 2	IER2	−1·25	Mitogenesis
Myeloid-associated differentiation marker	MYADM	−1·22	Transport vehicle biogenesis
UDP-glucose ceramide glucosyltransferase	UGCG	−1·22	Glycosphingolipid biosynthesis
Fucosyltransferase 1	FUT1	−1·20	Glycosphingolipid biosynthesis
Ectodermal-neural cortex	ENC1	1·42	Organ development, apoptosis
Kruppel-like factor 10	KLF10	1·68	Cell proliferation, cell–cell signaling
Olfactomedin-like 3	OLFML3	1·69	Matrix-related functions
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	1·85	Cell cycle
Hypothetical protein LOC283551	LOC283551	1·90	Unknown
3'-Phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	1·92	Nucleic acid metabolism
Pim-1 oncogene	PIM1	2·41	Cell proliferation, apoptosis
Cdk5 and Abl enzyme substrate 2	CABLES2	2·44	Cell division
Transcribed locus	–	2·58	Unknown
Cysteine-rich secretory protein LCCL domain containing 2	CRISPLD2	2·80	Signal transduction
Thromboxane A2 receptor	TBXA2R	3·16	Signal transduction
SLIT and NTRK-like family, member 4	SLITRK4	5·65	Axonogenesis
Lysyl oxidase-like 4	LOXL4	24·99	Ion binding

were then subjected to FACS (Supplemental data, Fig. 1B <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5>). The treatment of the infected cells synthesizing ERβ with DPN repressed the cell number accumulated in the G1 phase comparable to that observed with the E₂ treatment, while the compound had no effect on the growth of cells infected with Ad5 or Ad5-ERβ_{EBD}. Furthermore, the ER antagonist 4-OHT (or ICI, data not shown) alone did not affect cell cycle distribution in cells synthesizing either ERβ species, but it effectively blocked the effect of E₂ or DPN on G1 phase mediated solely by ERβ. The effects of ligand-ERβ on cell cycle distribution were mirrored in cellular growth, which was suppressed only by agonists (Supplemental data,

Fig. 1C <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>).

Regulation of apoptosis by E₂-ERβs

Since cellular growth encompasses cell proliferation and death, the inability of E₂-ERβ_{EBD} to affect cell growth suggests that the ERE-independent pathway participates minimally in inducing apoptosis, which is a complex, multiple-step event that culminates in cell death (Korsmeyer 1999). To address this issue, MDA-MB-231 cells were infected in the absence or presence of 10^{−9} M E₂ for different lengths of time. The cells were then subjected to caspase 3/7, annexin V, and

Table 2 Genes mediated by 17β-estradiol (E₂)-estrogen receptor β_{EBD} (ERβ_{EBD})

Gene name	Gene symbol	Fold change	Process
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	−4.76	Anti-apoptosis
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	−3.70	Collagen catabolism, proteolysis
Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	MMP3	−2.56	Collagen catabolism, proteolysis
Hyaluronan synthase 2	HAS2	−2.13	Matrix formation
Follistatin	FST	−2.08	Signal transduction
Heparin-binding EGF-like growth factor	HBEGF	−1.72	Signal transduction
Interleukin 18 (interferon-γ-inducing factor)	IL18	−1.59	Signal transduction
Dickkopf homolog 1 (<i>Xenopus laevis</i>)	DKK1	−1.49	Signal transduction
G-protein-coupled receptor	GPRC5A	−1.35	Signal transduction
Thromboxane A2 receptor	TBXA2R	1.73	Signal transduction
PDZ and LIM domain 1 (elfin)	PDLIM1	1.98	Signal transduction, cytoskeletal reorganization
Cysteine-rich secretory protein LCCL domain containing 2	CRISPLD2	4.04	Signal transduction

TUNEL assays, which assess early-, mid-, and late-stages of apoptosis respectively.

We found that ERβ_{EBD} had no effect on the activity of caspase 3/7 whether or not the cells were treated with E₂, while ERβ in the presence of E₂ increased the enzyme activities compared with cells infected with Ad5 (Fig. 6B).

Similar results were obtained with annexin V staining of infected cells (Fig. 6C and Supplemental data, Fig. 2A, see Supplementary data in the online version of the Journal of Molecular Endocrinology at <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>). The number of apoptotic cells in a population of cells infected with a recombinant adenovirus bearing a receptor cDNA was compared with the number of apoptotic cells infected with the parent adenovirus in the absence of E₂ as a function time. We observed that the unliganded or E₂-bound ERβ_{EBD} had no effect on the cell population stained with annexin V, while ERβ in response to E₂ increased the number of stained cells.

We corroborated our results with a TUNEL assay by incorporating FITC-conjugated nucleotides into the fragmented genomic DNA (Gorczyca *et al.* 1998). Infected MDA-MB-231 cells were incubated without or with 10^{−9} M E₂ for various durations of time. The cells were then subjected to the TUNEL assay. Results, depicted as the number of cells stained with TUNEL (Supplemental data, Fig. 2B <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>) and summarized as a function of time (Fig. 6D), showed that E₂-ERβ induced the fragmentation of genomic DNA of the infected cells. In contrast, ERβ_{EBD} had no discernable effect.

Collectively, these results indicate that ERβ, but not ERβ_{EBD}, induced apoptosis in infected cells treated with E₂. These results suggest that the ERE-dependent

pathway is the critical E₂-ERβ signaling pathway for the induction of apoptosis.

Differential effects of ERβ and ERβ_{EBD} on cell migration and invasion

Studies showed that ER-positive breast cancer cell models and ER-negative cell lines expressing ectopically introduced ER cDNA are less motile and invasive than the parent cells (Garcia *et al.* 1997, Lazennec *et al.* 2001).

To examine whether the ERE-independent pathway participates in the anti-motogenic effect of E₂-ERβ signaling, we used wound-healing and invasion assays. MDA-MB-231 cells infected with recombinant adenoviruses in the absence or presence of 10^{−9} M E₂ were grown for 48 h to allow the cells to reach near confluence. A wound was then created and the rate of wound closure was assessed. The results showed that while ERβ_{EBD} had little effect on wound closure in the absence or presence of E₂, ERβ without E₂ delayed the wound healing, which was further delayed by the presence of E₂ (Fig. 6E and Supplemental data, Fig. 2C <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>).

We also utilized an invasion assay that assesses the capacity of cells to migrate through a reconstituted basement membrane to independently verify our findings. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of 10^{−9} M E₂ for 48 h. The cells were then collected, and an equal number of cells were seeded on the reconstituted basement membrane of invasion chambers in the absence or presence of E₂. After 24 h incubation, cells on the bottom of the chamber membrane were stained and imaged

Figure 5 Effects of ER β s on cell growth. (A and B) Infected MDA-MB-231 cells were maintained in the absence (data not shown) or presence of 10^{-9} M E_2 . At 48, 96, and 144 h post-infection, the cells were subjected to (A) cell counting or (B) MTT assay. The graphs represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. (C and D) U-2 OS cells were infected with Ad5 (50 MOI), Ad-5ER β (40 MOI) supplemented with 10 MOI of Ad5), or Ad5-ER β_{EBD} (50 MOI) for 48 h. Cell extracts were subjected to (C) WB and (D) EMSA. Molecular weight in kDa is shown. Free (unbound) and ER-bound ERE are indicated. Representative images from three independent experiments are shown. (E) Infected U-2 OS cells were maintained in the absence or presence of 10^{-9} M E_2 . At 144 h post-infection, the cells were subjected to cell counting. The mean $\pm$ S.E.M. indicates three independent experiments performed in duplicate. *Denotes a significant effect compared with the parent adenovirus (Ad5).

(Supplemental data, Fig. 2D <http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/>). A quantitative analysis of images revealed that ER β only in response to E_2 , but not E_2 -ER β_{EBD} , reduced invasive cell number (Fig. 6F).

These results collectively suggest that the ERE-independent pathway mediated by E_2 -ER β signaling plays a minimal role in cellular motility.

Discussion

The integrated effects of the ERE-dependent and ERE-independent nuclear E_2 -ER signaling are critical for the homeodynamic regulation of estrogen-responsive tissue functions. However, the relative importance of each

pathway in physiology and pathophysiology of E_2 signaling is unclear. Using an ERE-binding mutant of ER β (ER β_{EBD}), which acts primarily through the ERE-independent pathway, we show here that genomic responses to E_2 -ER β_{EBD} are not sufficient to alter phenotypic characteristics of model cells.

The elements of the nuclear action of ERs involve a multi-step regulation in which ERs are converted from an inactive form to a transcriptionally active state mediated by a conformational change upon E_2 binding (Parker 1998). The interaction of E_2 -ER with an ERE constitutes the initial step in the ERE-dependent signaling in which the gene expression is cyclically regulated (Metivier *et al.* 2003). In the ERE-independent pathway, E_2 -ER functionally interacts with transcription factors bound to their cognate regulatory

Figure 6 Effects of ERs on cell cycle, death, and motility. (A) To examine the effects of ERs on cell cycle distribution, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence (data not shown) or presence of 10^{-9} M E_2 for 24, 48, or 96 h. The cells were then subjected to FACS. Results showing the percent of cells in G1 phase as a function of time are the mean $\pm$ S.E.M. of four independent experiments. (B) Infected MDA-MB-231 cells in the absence or presence of 10^{-9} M E_2 as a function of time as indicated were subjected to a caspase 3/7 assay. The fluorescence was measured using a fluorometer. Results are the mean $\pm$ S.E.M. of three independent experiments. (C and D) To examine the effects of ERs on cell death, infected MDA-MB-231 cells at indicated times were subjected to (C) an annexin V assay using FACS or (D) a TUNEL assay. Results, which are the mean $\pm$ S.E.M. of three independent experiments, are summarized as the number of cells bound to annexin V or as the number of cells that incorporated FITC-conjugated dUTP into the fragmented DNA (TUNEL). (E) To assess the role of the ERE-independent signaling pathway in cellular motility, infected MDA-MB-231 cells were incubated in the absence or presence of 10^{-9} M E_2 for 48 h to allow cells to reach near 100% confluence. A wound was then created and images were captured at 0 h and at every 24 h thereafter. Results, which are the mean $\pm$ S.E.M. of three independent experiments performed in duplicate, are summarized as the wound closure at 96 h relative to 0 h. (F) To examine the effects of ERs on cellular invasiveness, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of 10^{-9} M E_2 for 48 h. The cells were collected and counted. The same number of cells from each experimental group was then seeded on the upper section of the invasion chamber. After 24 h incubation, those cells with invasive capabilities on the bottom of chamber membrane were counted. Results, which represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate, are percent change compared with cells infected with the parent adenovirus in the absence of E_2 , which is set to 100%. *Indicates a significant effect compared with the parent adenovirus (Ad5).

elements on DNA to modulate gene expression (Kushner *et al.* 2000, Safe 2001). This interaction involves the stabilization of transfactor binding to DNA and/or recruitment of co-regulators to the complex (Kushner *et al.* 2000, Safe 2001). Estrogen regulation of the *MMP1* and the insulin-like growth factor-1 (*IGF1*) genes is, for example, characterized by a functional interaction between the members of the AP1 family proteins and ER (Umayahara *et al.* 1994, Kushner *et al.* 2000). The modulation of the *FOS* (cellular oncogene fos) and the progesterone receptor (*PGR*) gene expressions, on the other hand, are dependent on an the interaction between ER and SP1 (Duan *et al.* 1998, Schultz *et al.* 2003). Similarly, ER interactions with the signal transducers and activators of transcription (STATs) family proteins provides a mechanism for the estrogen responsiveness of the β -casein (*CSN2*) gene (Bjornstrom *et al.* 2001).

In the ERE-independent signaling, ER establishes interactions with transfactors through regions that also encompass the DBD, while the integrated effects of the amino and carboxyl termini are responsible for the gene expression (Kushner *et al.* 2000, Safe 2001, Bjornstrom & Sjoberg 2002, Cheung *et al.* 2005). Studies also demonstrated that the prevention of ER-ERE interactions by changing critical residues in the DBD of the mouse ER α and the human ER β prevent the transcription from the ERE-dependent pathway, while conserving the regulatory capacity of the receptors in simulated ERE-independent pathways (Jakacka *et al.* 2001, Bjornstrom & Sjoberg 2004). Consistent with these findings, we also show here that ER β_{EBD} , which had no effect on gene expression from the ERE-dependent pathway, mimics the effect of ER β on transcriptional responses from simulated ERE-independent signaling pathways in a ligand, promoter, and cell context-dependent manner. Although the underlying mechanisms are unclear, distinct conformational changes induced by a specific ligand (Nettles *et al.* 2007) could alter the extent of interaction with a transfactor specific to each gene, thereby altering the transcription output. Additionally, cellular differences in the concentration of transfactors and/or co-regulatory proteins (McKenna *et al.* 1999) could contribute to differential effects of ligand-ERs on responses from promoter constructs.

Extending these findings, our microarray and qPCR studies further indicate that ER β_{EBD} in the presence of E_2 regulates the expression of a number of genes, the majority of which (10 out of 12) are similarly modulated by E_2 -ER β in infected MDA-MB-231 cells (Tables 1 and 2). Although the nature of the regulatory elements critical for the E_2 responsiveness of the identified genes are unknown and presently being investigated, these genes are also targets for various cytokines and growth factors that utilize transfactors involved in cross-talk

with ERs. Growth factor-mediated expression of the *MMP1* and *MMP3* genes, for example, involves the AP1 proteins (Buttice *et al.* 1996, Aho *et al.* 1997). Similarly, while the basal expression of the *HAS2* gene is primarily controlled by the SP family of proteins (Monslow *et al.* 2006), the responsiveness of the gene to endocrine and paracrine signaling is mediated by STAT and the NF κ B transcription factors (Pasonen-Seppanen *et al.* 2003, Saavalainen *et al.* 2005). The protein products of these E_2 -ER β_{EBD} -responsive genes play critical roles in cell growth, death, and migration. Matrix metallo-peptidases (MMPs), for example, are a large family of endopeptidases responsible for tissue remodeling and degradation of the extracellular matrix (ECM) that includes collagens, elastins, gelatin, laminin, and matrix glycoproteins (Duffy *et al.* 2000). MMP1 is involved in the degradation of collagen, while MMP3 participates in the degradation of many ECM substrates including glycoproteins, laminin, fibronectin, and collagen (Duffy *et al.* 2000). Increased *MMP1* and *MMP3* gene expressions are associated with advanced stages of breast cancer and are involved in the development of metastasis (Duffy *et al.* 2000). Similarly, aberrant synthesis of hyaluronan (HA), the major glycosaminoglycan found in the ECM, is implicated in a diverse range of ECM-mediated processes including cellular proliferation, adhesion, and migration (Gotte & Yip 2006). HA is synthesized by the hyaluronan synthase family of transmembrane glycosyltransferases that are encoded by the corresponding *HAS1*, -2, -3a, and -3b genes (Gotte & Yip 2006). Preferential and high expression of the *HAS2* gene appears to be critical for invasiveness of breast cancer cells, including MDA-MB-231 cells (Gotte & Yip 2006). Studies also indicate that an aberrant expression of the *SERPINB2* (Andreassen *et al.* 1997), *HBEGF* (Ito *et al.* 2001), *DKK1* (Forget *et al.* 2007), *GPRC5A* (Nagahata *et al.* 2005), and *TBXA2R* (Watkins *et al.* 2005) genes alters the proliferation and motility of breast cancer cells.

Considering the ability of ER β_{EBD} to alter gene expression, the absence of an effect of ER β_{EBD} on the phenotypic characteristics of model cells is surprising. Studies indicate that the suppression of *HAS2* (Udabage *et al.* 2005, Li *et al.* 2007) or *MMP1* (Wyatt *et al.* 2005) synthesis by a silencing RNA approach decreases the growth and invasiveness of MDA-MB-231 cells. One explanation is that the extent of transcriptional alterations mediated by E_2 -ER β_{EBD} and subsequent changes in RNA levels are not sufficient to modify the corresponding protein concentrations in cells, in contrast to silencing RNA approaches. This is unlikely since the magnitude of E_2 -ER β -mediated transcriptional responses was comparable to those modulated by E_2 -ER β_{EBD} (Fig. 3 and Tables 1 and 2), while only E_2 -ER β induced alterations in cellular phenotypes. An alternative explanation is that the

integrated regulation of genes involved in a signaling cascade through both ERE-dependent and ERE-independent pathways is crucial for the manifestation of phenotypic alterations in response to E₂-ER signaling. The interaction of CD44 with HA, for example, induces signaling events that promote anchorage-independent cell growth and migration, thereby increasing metastatic spread (Gotte & Yip 2006). Importantly, post-transcriptionally mediated repression of HAS2 leads to a concomitant decrease in its cell surface receptor CD44 transcript (Udabage *et al.* 2005). A similar mechanism is also suggested for the members of MMP proteins (Wyatt *et al.* 2005). Thus, the concomitant regulation of the *HAS2* and the *CD44* gene expression, or genes involved in the MMP signaling cascade, by E₂-ER β , could be the underlying reason for the efficacy of the receptor to induce phenotypic alterations, in contrast to E₂-ER β _{EBD} which decreased only the *HAS2* gene expression without an effect on cellular phenotype.

Transcript profiling of infected MDA-MB-231 cells synthesizing an ERE-binding defective mouse ER α indicated that E₂ alters the expression of a number of genes involved in various cellular functions (Glidewell-Kenney *et al.* 2005). However, out of the 29 identified genes, the expression of only the *HAS2* and *SERPINE2* genes coincides with the altered gene expression reported here. Since transcriptional responses from ERE-independent signaling pathways in experimental systems are also dependent upon ER-subtype, a discordant gene regulation could be critical for differences in subtype-mediated cellular responses.

Augmented synthesis of ER α , as observed in atypical hyperplasia and *in situ* carcinoma of breast, causes an E₂-independent, tamoxifen-insensitive proliferation through aberrant gene expressions (Fowler *et al.* 2004, 2006). Our studies presented here were designed to assess the role of the ERE-independent signaling pathway mediated by ER β to induce cellular responses at concentrations that strictly depend upon E₂ for function. The circumvention of E₂-dependency by increased ER β levels could contribute to the characteristics of target tissue malignancies through a selective expansion, or altered expression, of target genes mediated by the ERE-independent signaling pathway.

In summary, our results show that genomic responses mediated by the ERE-binding defective ER β mutant in response to E₂ are insufficient to alter the phenotypic characteristics of model cell lines. These results imply that the ERE-dependent pathway is necessary for E₂-ER β to regulate growth of the responsive cells. Studies that aim to target specifically ERE-bearing genes through the use of designer transcription factors are underway to address this issue.

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