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ORIGINAL PAPER

Augmentation of estrogen receptor-mediated transcription by steroid and xenobiotic receptor

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Abstract The estrogen receptor (ER) is a key regulator of proliferation and differentiation in breast cancer cells. In the present study, the effect of steroid and xenobiotic receptor (SXR) on 17β-estradiol (E_2)-induced transcription through ERα was studied. SXR augmented ER-mediated transcription in the presence of E_2 in MCF-7 breast cancer-derived cells and CV-1 fibroblast-derived cells. On the other hand, SXR alone did not affect the estrogen response element (ERE)-containing promoter activity in CV-1 cells. SXR did not directly bind to ERα or ERE in vitro, indicating that SXR may affect ER-mediated transcription by altering cofactor binding to ER. Although SXR did not alter the binding between ERα and p300/CBP interacting protein (p/CIP), it decreased the binding of a specific corepressor, silencing mediator of retinoid and thyroid hormone receptors (SMRT) to liganded ERα as assessed by mammalian two-hybrid, glutathione *S*-transferase pull-down, immunoprecipitation and newly developed Liquid Chemiluminescent DNA Pull-Down Assays. These results indicate that SXR augmented ER-mediated transcription by dissociating SMRT from ERα. Thus, the expression of SXR in breast cancer cells may alter the ER signaling, which may play crucial role for growth and differentiation of breast cancer cells.

Keywords Estrogen receptor · Steroid and xenobiotic receptor · Breast cancer · SMRT

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Introduction

Estrogen plays a major role in regulating proliferation and differentiation of epithelial and stromal cells in the normal mammary gland. It also plays a predominant role in growth and progression of breast cancer by interacting with estrogen receptor (ER)α [1–3], which then binds to a specific DNA sequence termed estrogen response element (ERE), or interact with other transcription factors [4]. Ligand binding induces a conformational change of the ER and recruits differential sets of coactivators or corepressors that determine biological activity by altering the magnitude of the transcriptional responses according to the physiological needs [5].

On the other hand, steroid and xenobiotic receptor (SXR) is highly expressed in the liver and small intestine and regulates the expression of cytochrome P450 monooxygenase 3A4 (CYP3A4) and P-glycoprotein (P-gp) that is encoded by multidrug resistance 1 (MDR1) gene [6–12]. SXR is also expressed in normal and neoplastic breast

tissue [13, 14]. SXR forms heterodimer with retinoid X receptor (RXR) on steroid and xenobiotic-response elements (SXREs), located in the promoter region of target genes. Unlike classical nuclear hormone receptors, SXR possesses wide ligand binding surface allowing to bind a variety of chemicals including steroids, prescribed drugs, and xenobiotics bind as ligands [1, 7, 8, 12, 15–18].

There is extensive evidence showing that ER-mediated transcription is modulated by other nuclear receptors (NRs) and transcription factors. Thyroid hormone receptor, peroxisome proliferator-activated receptor [19–23], aryl hydrocarbon receptor [24–26], and progesterone receptor (PgR) [27] have been shown to modulate ER activity in a series of cell lines including human breast and hepatic cell lines. Besides NRs, additional nuclear factors may also affect the ER-mediated gene regulation in breast cancer cells [3, 28].

Many coactivators such as steroid receptor coactivators (SRCs) and corepressors interact with ER and affect ER-mediated transcription [3, 21, 29]. Among corepressors, nuclear receptor corepressor (N-CoR) [30] and silencing mediator of retinoid and thyroid hormone receptors (SMRT) [31] have been implicated in mediating ER signaling. ER may be able to interact with these corepressors regardless of agonist or antagonist treatment in vitro [32, 33]. N-CoR and SMRT interact with ligand binding domain of NRs, part of which may overlap with binding site for coactivators. Thus, ER-mediated transcription may be partly suppressed by such competitive corepressor binding even with the presence of estrogen to "counterbalance" its activity [1]. On the other hand, we have previously reported that SMRT, but not N-CoR, suppressed SXR-mediated transcription on CYP3A4 promoter not only without ligand but also with ligand, rifampicin, through SMRT–SXR interaction in HepG2 cells [34]. SMRT and N-CoR are expressed in breast cancer cells and may play important roles [1, 35, 36]. Although interaction of N-CoR and/or SMRT with SXR in hepatocyte-derived cells has been reported [37, 38], the interaction of SXR and these corepressors, and their possible involvement on progression of breast cancer have not been clarified. In the present study, we demonstrated the augmentation of ERα-mediated transcription by SXR in MCF-7 cells through a novel mechanism, and SMRT is a key factor for this augmentation.

Results

SXR augmented ERα-mediated transcription through ERE

To examine the effect of SXR on ERα-mediated transcription through ERE, we performed a series of transient transfection-based reporter gene assays using MCF-7 and CV-1 cells. Cotransfection of SXR augmented the ERα-mediated transcription through ERE in both MCF-7 and CV-1 cells (Fig. 1a, b, respectively). This augmentation was observed in the presence of 17β-estradiol (E_2), in a SXR dose-dependent manner. Probably because SXR is endogenously expressed in

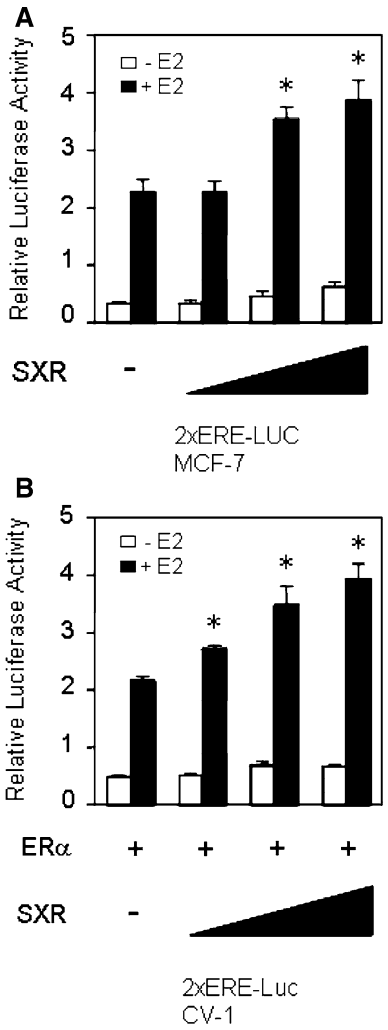

Fig. 1 SXR augmented ERα-mediated transcription in MCF-7 and CV-1 cells. **a** Increasing amount of expression vector encoding SXR (0, 0.04, 0.08, 0.20 μg) were cotransfected with 2xERE-LUC (0.4 μg) into MCF-7 cells. Cells were grown in the absence or presence of 10^{-8} M E_2. Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA) vs. SXR (−), E_2 (+) column. **b** Expression vector encoding ERα (0.04 μg) was cotransfected with increasing amount of SXR (0, 0.04, 0.08, 0.20 μg) and 2xERE-LUC (0.4 μg) into CV-1 cells. Cells were grown in the absence or presence of 10^{-8} M E_2. Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA) vs. ERα (+), SXR (−), E_2 (+) column. E2: 17β-estradiol

MCF-7 cells, the augmentation by small amount of SXR transfection was not seen in this cell type. Next, to examine whether ERα is required for the augmentation by SXR shown in Fig. 1, SXR was cotransfected with ERE-contained reporter vector into CV-1 cells, which do not express ER. As shown in Fig. 2, SXR alone did not activate transcription, even with E_2 or rifampicin. However, cotransfection of ERα effectively augmented the transcription in the presence of E_2 (Fig. 2a, b). These results indicate that SXR action through ERE requires ERα.

SXR did not bind directly to ERα in CV-1 cells

To examine whether SXR binds to ERα, we performed mammalian two-hybrid assays in CV-1 cells. The constructs used in the present study are shown in Fig. 3a. Cotransfection of Gal4-ERα-ligand binding domain (LBD) with VP16-blank did not show any transcriptional activation. As expected, Gal4-ERα-LBD with VP16-SRC-1-nuclear receptor binding domain (NBD-1) showed activation in the presence of E_2 (Fig. 3b). On the other hand, Gal4-ERα with VP16-SXR showed no change of activation (Fig. 3b). These results indicate that SXR did not bind to ERα in CV-1 cells. To examine further the ER-SXR binding in vitro,

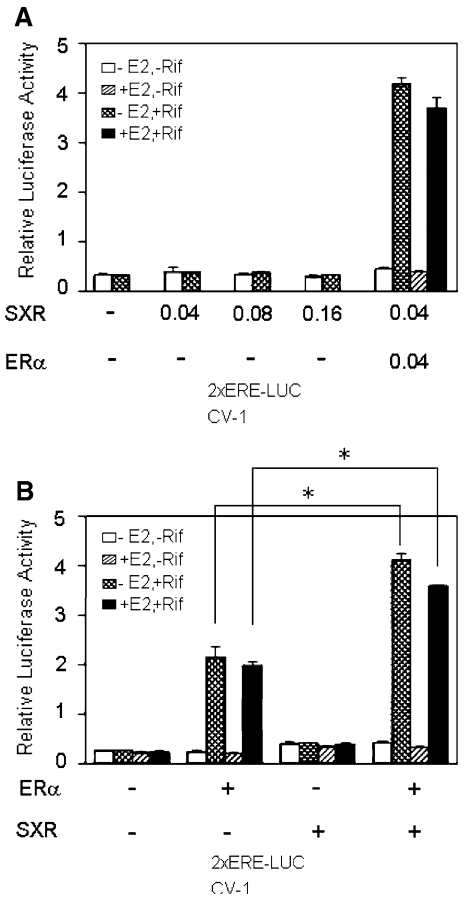

Fig. 2 SXR alone did not activate transcription through ERE in CV-1 cells. **a** Indicated amount (0, 0.04, 0.08, 0.16 μg) of expression vector encoding SXR or ERα (0.04 μg) were cotransfected with 2xERE-LUC (0.4 μg) into CV-1 cells. Cells were grown in the absence or presence of E_2 (10^{-8} M) and/or rifampicin (10^{-5} M). Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. **b** Expression vectors encoding ERα (0.04 μg) and SXR (0.08 μg) were cotransfected with 2xERE-LUC (0.4 μg) into CV-1 cells. Cells were grown in the absence or presence of E_2 (10^{-8} M) and/or rifampicin (10^{-5} M). Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA). E2: 17β-estradiol, Rif: rifampicin

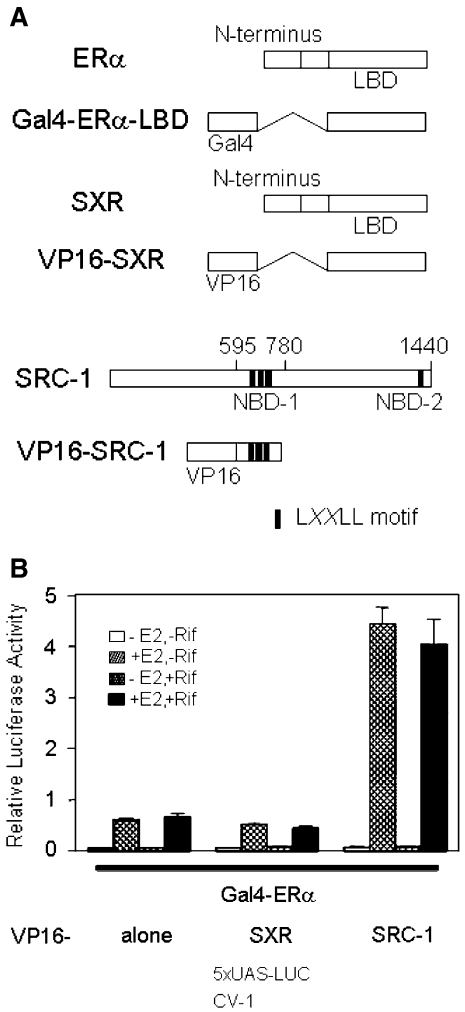

Fig. 3 SXR did not interact with ERα in CV-1 cells. **a** Schematic representative of expression vectors that were used in the present study. **b** Expression plasmids encoding Gal4-ERα-LBD (0.02 μg) and VP16-blank, VP16-SXR-LBD, or VP16-SRC-1-NBD-1 (0.1 μg) were cotransfected with 5xUAS-TK-LUC reporter plasmid (0.3 μg) into CV-1 cells. Cells were grown in the absence or presence of 10^{-8} M E_2 and/or 10^{-5} M rifampicin. Data represent means ± S.E.M. of experiments performed in triplicate. No significant difference was observed except for positive control. LBD: ligand binding domain

glutathione *S*-transferase (GST) pull-down studies were performed, and no such binding was seen (data not shown).

SXR did not bind to ERE or ER–ERE complex in vitro

To examine SXR binding to ERE in vitro, electrophoretic mobility shift assay (EMSA) was performed (Fig. 4). Although in vitro-translated ER**α** binds to ERE as a homodimer in the absence or presence of E_2 (lanes 2 and 3), SXR alone (lane 4), or SXR/RXR complex (lane 5) did not bind to ERE. Together with the data shown in Fig. 2, these results indicate that SXR does not bind directly to ERE to activate transcription. We also examined the interaction between full-length SXR and ER**α** on ERE (Fig. 4, lane 6). When both ER**α** and SXR were incubated together, no ER**α**/SXR heterodimer band was observed (lane 6). Similarly, no ER**α**/SXR/RXR complex band was observed (lane 7). These results indicate that SXR did not bind to ERα directly. We also performed EMSA using SXRE. SXR bound to SXRE as heterodimer with RXR (Fig. 4c). ERα did not bind to SXRE as a homodimer, or heterodimer with SXR (data not shown).

The interaction between ERα and coactivator was not increased by SXR

We then investigated the effect of SXR on the binding between ERα-LBD and p/CIP-NBD-1 using a mammalian two-hybrid assay in CV-1 cells (Fig. 5). Constructs used in

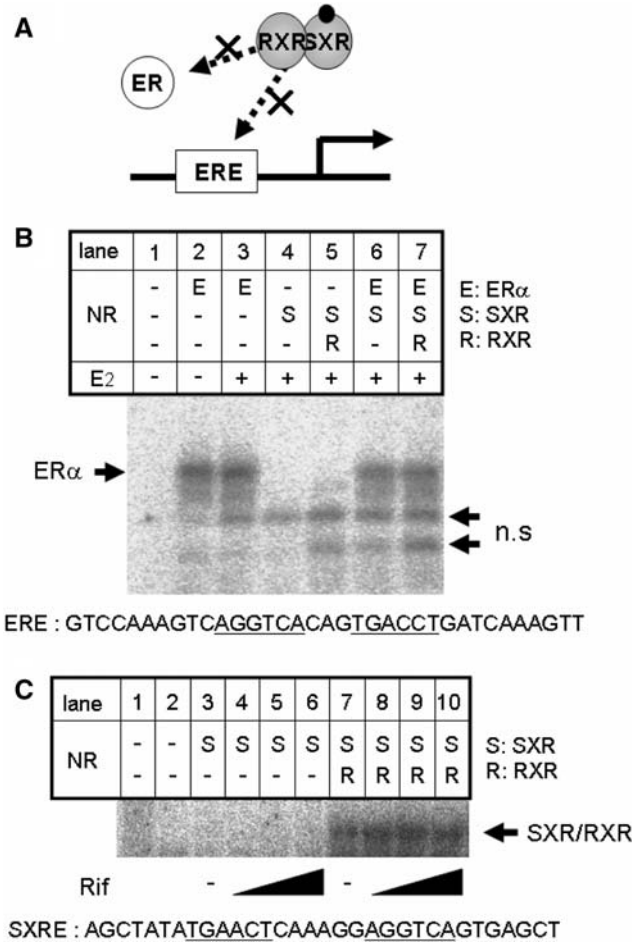

Fig. 4 SXR does not directly interact with ERα or ERE in vitro. **a** Schematic representative of EMSA experiment. **b** In vitro-translated ERα (1.5 μl), SXR or SXR/RXR (3 μl) were incubated with [^{32}P]-labeled consensus ERE oligonucleotide with or without E_2. Three independent sets of the same experiment yielded similar result. n.s.: nonspecific. **c** In vitro-translated SXR (1.5 μl) and RXR (1.5 μl) were incubated with [^{32}P]-labeled consensus SXRE oligonucleotide in the absence or increasing concentration (0, 10^{-7}, 10^{-6}, 10^{-5} M) of rifampicin. Three independent sets of the same experiment yielded similar results

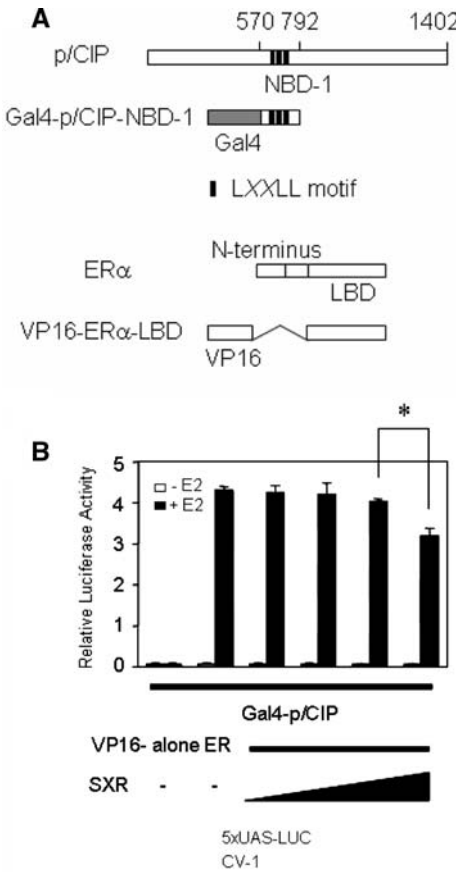

Fig. 5 The binding of ERα to p/CIP was not increased by SXR in CV-1 cells. **a** Schematic representative of expression vectors that were used in the present study. **b** Expression plasmids encoding Gal4-p/CIP-NBD-1 (0.02 μg) were cotransfected with VP16-ERα-LBD (0.1 μg), 5xUAS-TK-LUC reporter plasmid (0.2 μg) and increasing amounts of expression vector encoding SXR (0, 0.02, 0.04, 0.1, 0.2 μg) into CV-1 cells. Cells were grown in the absence or presence of 10^{-7} M E_2. Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. p/CIP: p300/CBP-interacting protein and NBD: nuclear receptor binding domain

this experiment are shown in Fig. 5a. Gal4-p300/CBP interacting protein (p/CIP)-NBD-1 with VP16-blank did not activate transcription either with or without E_2 (Fig. 5b, column 1). Transactivation by Gal4-p/CIP-NBD-1 and VP16-ERα-LBD was observed in the presence of E_2 (Fig. 5b, column 2). Activated transcription by Gal4-p/CIP-NBD-1 and VP16-ERα-LBD with E_2 was not significantly activated or suppressed by SXR at concentrations 0.02, 0.04, and 0.1 μg (columns 3–5). When 0.2 μg of SXR was cotransfected, the activation was slightly decreased (column 6). These results suggest that the effect of SXR on the binding of p/CIP to ERα-LBD is limited, which does not account for the augmentation of ERα-mediated transcription by SXR.

Overexpression of SMRT suppressed the augmentation of ER-mediated transcription by SXR

Finally, we studied the interaction between SMRT–ERα and SMRT–SXR, since we previously reported that SMRT, but not N-CoR, binds to SXR in the presence of its agonist rifampicin [34]. Additionally, binding between ERα and SMRT, regardless of the presence of E_2, by O'Malley's group using a GST pull-down study [32]. Fleming et al. also reported the binding between ERα and SMRT in the presence of E_2 using immunoprecipitation studies [39]. Cotransfection of ERα and SMRT, with an ERE-containing reporter revealed that overexpression of SMRT suppressed the augmentation of ER-mediated transcription by SXR (Fig. 6), in the presence of E_2, while N-CoR was not effective (data not shown). These results suggest that ERα and SXR may competitively bind to SMRT.

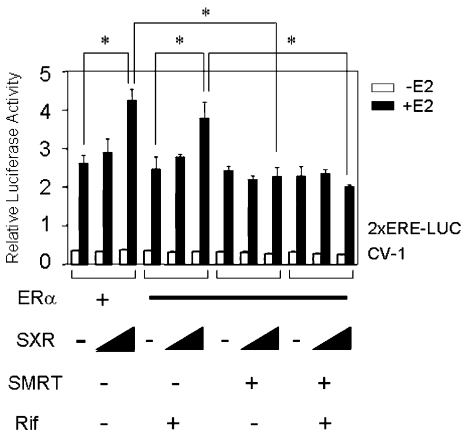

Fig. 6 Overexpression of SMRT suppressed the ER-mediated transcription augmented by SXR. Increasing amount (0, 0.04, 0.2 μg) of expression vector encoding SXR, ERα, and/or SMRT (0.25 μg) and 2xERE-LUC (0.2 μg) were cotransfected into CV-1 cells. Cells were grown in the absence or presence of 10^{-8} M E_2 and/or 10^{-5} M rifampicin. Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA). Rif: rifampicin

SMRT was dissociated from ERα by increasing amount of SXR

To confirm these observations, we tested the effect of SXR on the binding between ERα and SMRT using mammalian two-hybrid assays in CV-1 cells (Fig. 7). Constructs used in this study are shown in Fig. 7a. Gal4-ERα and VP16-alone induced a transcription probably through its own transactivation domain of ER. Cotransfection of VP16-SMRT-receptor interacting domain (RID) further activated the transcription in the presence of E_2 (Fig. 7b, column 3). The augmentation of transcription by VP16-SMRT-RID

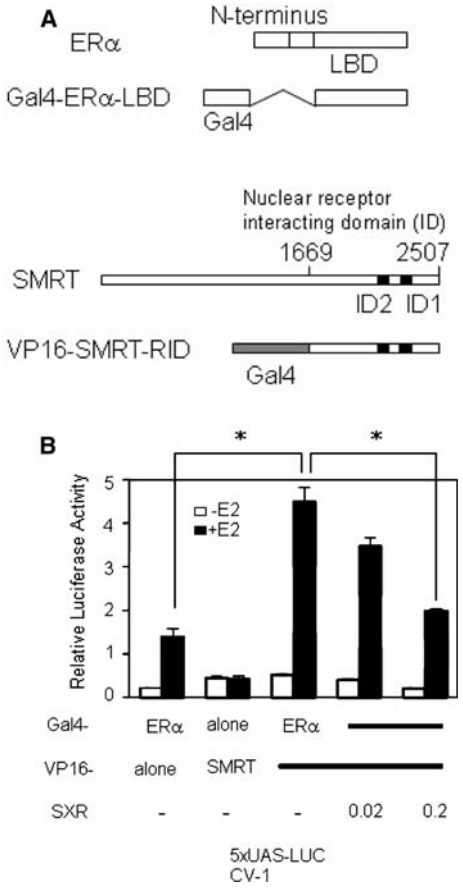

Fig. 7 The binding of ERα to SMRT was decreased by SXR in a receptor concentration-dependent manner in CV-1 cells. **a** Schematic representative of the constructs that were used in the present study. **b** Expression plasmids encoding Gal4-ERα-LBD (0.02 μg) were cotransfected with VP16-SMRT-RID (0.1 μg), 5xUAS-TK-LUC reporter plasmid (0.2 μg), and increasing amounts (0, 0.02, 0.2 μg) of expression vector encoding SXR into CV-1 cells. Cells were grown in the absence or presence of 10^{-8} M E_2. Total amounts of DNA for each well were balanced by adding an empty vector. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA). RID: receptor interacting domain

was decreased by increasing amount of SXR (columns 4 and 5). To examine further the effect of SXR on ERα–SMRT binding, GST pull-down study was performed (Fig. 8). Constructs used in this study are shown in Fig. 8a. As shown in Fig. 8b, GST-fused SMRT bound to ERα in the absence or presence of E_2, as reported by another group [32]. ERα was dissociated from SMRT when increasing amount of SXR was added. We also performed the GST pull-down study using GST-fused SMRT and [^{35}S]-labeled full-length SXR. Binding of SXR to SMRT was also decreased by increasing amount of ERα, but larger amount was required to dissociate SXR from SMRT (data not shown), indicating that binding affinity for SXR–SMRT is greater than that for ER–SMRT. These series of experiments clearly indicate that increasing amount of SXR competitively binds to SMRT to dissociate it from ERα. Although the present study and a previous study [32] have shown the interaction of SMRT with liganded ER, another study using EMSA showed that SMRT did not bind to ERα in the presence of agonist [39]. From our experience, we consider that EMSA may not be an ideal method to detect a weak protein–protein binding with DNA binding. Subtle protein–protein binding may be dissociated during electrophoresis through acrylamide gel. To conserve a weak protein–protein binding with DNA binding, we developed a novel assay to detect the DNA–protein binding without gel electrophoresis. As stated in the method, this assay is to detect the protein-bound DNA that is labeled with digoxigenin (DIG) by chemical luminescent reaction (Fig. 9a). In combination with GST pull-down method, protein–DNA complex bound to glutathione sepharose beads can be detected in the liquid. We named this method as "Liquid Chemiluminescent DNA Pull-Down Assay." GST-fused SMRT was incubated with ERα followed by incubation with DIG-labeled ERE. After several washing, DIG was immunologically detected with anti-DIG antibody that was conjugated with alkaline phosphatase. Finally, light activity was measured with a luminometer. As shown in Fig. 9b,

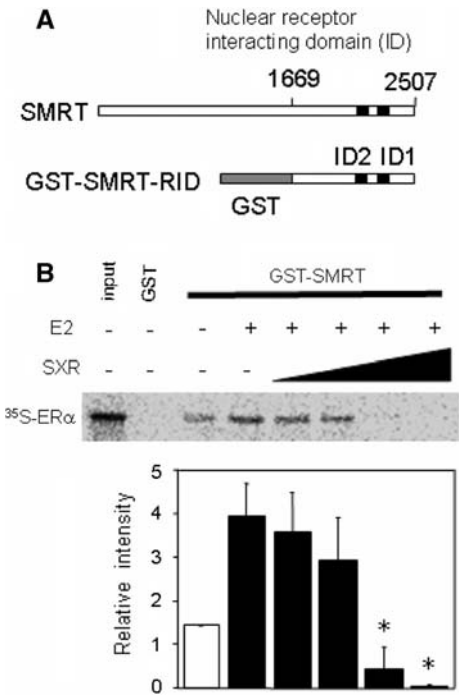

Fig. 8 The binding of ERα to SMRT was decreased by SXR in a receptor concentration-dependent manner in vitro. **a** Schematic representative of the constructs that were used in the present study. **b** GST-fused SMRT-RID was incubated with in vitro-translated [^{35}S]-methionine-labeled full-length ERα (5 μl) and increasing amount of in vitro-translated cold full-length SXR (0, 5, 10, 25, 50 μl) with or without 10^{-6} M E_2. Bound proteins were resolved by SDS-PAGE and detected by autoradiography. Negative control was GST alone. Input is 10% of total labeled protein used in other lanes. Representative results from three independent experiments. Kodak 1D Image Analysis Software was used to analyze quantitative data. *Statistically significant ($P < 0.01$ by ANOVA) vs. ERα (+), GST-SMRT (+), SXR (−), E_2 (+) column

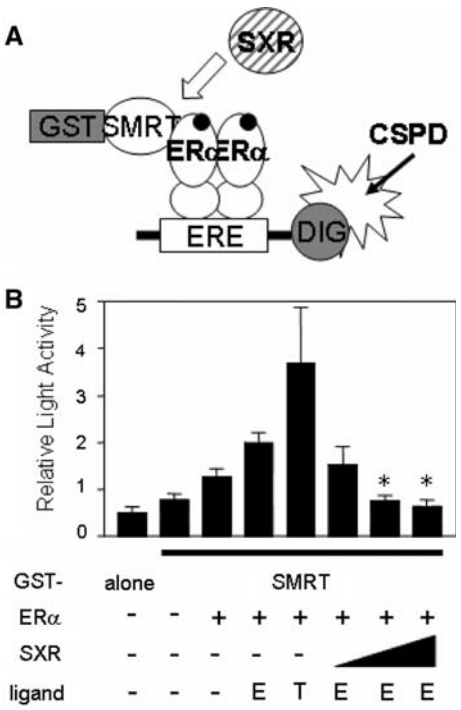

Fig. 9 The binding of ERα to SMRT was decreased by SXR in a receptor concentration-dependent manner in the presence of ERE in vitro. **a** Schematic representative of the method of Liquid Chemiluminescent DNA Pull-Down Assays. **b** Representative data for Liquid Chemiluminescent DNA Pull-Down Assays. In vitro-translated ERα was incubated with DIG-labeled ERE oligonucleotide. GST-SMRT was incubated with ERα that bound to DIG-labeled ERE and/or in vitro-translated SXR (0, 5, 10, 15 μl) in the presence or absence of E_2 (E) or TAM (T) (10^{-7} M). After detection reaction, light activities were measured with a luminometer. Data represent means ± S.E.M. of experiments performed in triplicate. *Statistically significant ($P < 0.01$ by ANOVA) vs. GST-SMRT, ERα (+), SXR (−) column. E: 17β-estradiol, T: tamoxifen, DIG: digoxigenin

while GST alone (column 1) or GST–SMRT (column 2) without ERα with labeled ERE showed little activity, GST–SMRT–ERα–ERE showed greater activity (columns 3 and 4). Tamoxifen (TAM) induced the activity (column 5), indicating that the binding between ERα and SMRT may be increased, as previously reported [40–43]. Addition of SXR suppressed light activities in a dose-dependent manner (columns 6–8). These results indicate that increasing amount of SXR dissociated SMRT from ERα in the presence of ERE in vitro. These results were compatible to the results of mammalian two-hybrid assay and GST pull-down studies.

SXR dissociated SMRT from ERα in cells in a dose-dependent manner

In addition to directly interacting in GST pull-down assays, SXR and SMRT were found to interact in cell extracts of HEK-293 cells where SMRT, ERα, and SXR were over-expressed. Using these total cell lysates, the binding of ERα with SMRT was detected in the presence of E_2 (Fig. 10). Addition of SXR resulted in the dissociation of SMRT from ERα in a dose-dependent manner. These results are compatible to the result of mammalian two-hybrid, GST pull-down and Liquid Chemiluminescent DNA Pull-Down assays.

SXR augmented the expression of PgR mRNA in MCF-7 cells

To confirm the effect of SXR on the estrogen action in MCF-7 cells, we performed realtime quantitative RT-PCR studies using an estrogen target gene, PgR (Fig. 11). The expression of PgR mRNA was augmented by increasing amount of SXR in a dose-dependent manner. These results are comparable with reporter gene assay results showing that increasing amount of SXR augmented ER-mediated transcription through ERE.

Discussion

In the present study, we have shown the augmentation of ERα-mediated transcription by SXR through ERE in the presence of E_2 using reporter gene assays. This finding was confirmed by realtime quantitative RT-PCR studies showing the augmentation of estrogen-induced intrinsic PgR mRNA expression by SXR in MCF-7 cells. Additional series of experiments have shown that the mechanism of this augmentation may be dissociation of SMRT from ERα by SXR.

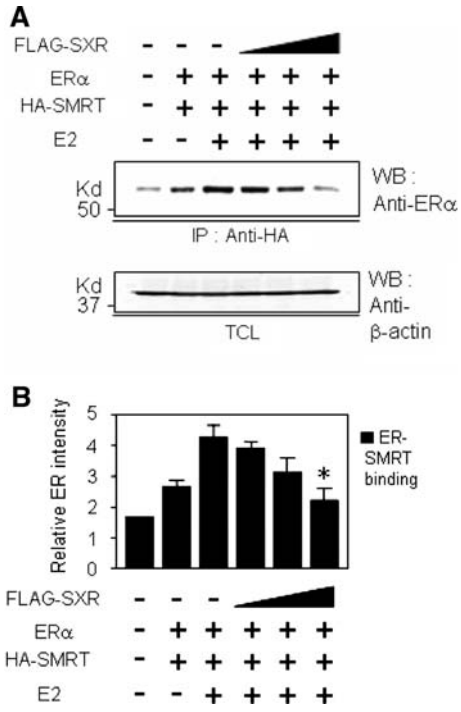

Fig. 10 SXR dissociated SMRT from ERα in cells in a receptor concentration-dependent manner. **a** Increasing amount (0, 0.1, 1.0, 2.0 μg) of expression vector encoding FLAG-SXR and HA-SMRT (0.2 μg) were cotransfected with ERα (0.1 μg) into HEK-293 cells. Total amounts of DNA for each well were balanced by adding an empty vector. Cells were grown in the absence or presence of E_2 (10^{-8} M) for 40 min. Following cell lysis, equal amounts of cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody and immunoprecipitates were analyzed by western blotting (WB) with anti-ERα. Representative blots from four replicates are shown. The positions of standard molecular masses (kDa) are indicated at the left. *β*-actin is used as an internal control. TCL; total cell lysate. **b** Histograms of the relative intensity of dissociated ERα from SMRT by addition of SXR. Intensity values of bands are expressed as a percentage of the values of the intensity without transfection and E_2 treatment. Results are means ± S.E.M. of four individual experiments. *Statistically significant ($P < 0.01$ by ANOVA) vs. column 3

Initially, we hypothesized four possible mechanisms inducing the augmentation of ERα-mediated transcription by SXR; (i) the interaction of SXR with ERE, (ii) the interaction of SXR with ERα, (iii) the increase in coactivator interaction with ERα by SXR, and, (iv) the dissociation of corepressor from ERα by SXR. Of note, we confirmed that the expression levels of ERα and SMRT were not changed using realtime RT-PCR studies (data not shown). Although SXR augmented transcription through ERE in the presence of ERα in both MCF-7 and CV-1 cells, it did not activate the transcription through ERE when ERα was not cotransfected (Fig. 2), indicating that ERα is necessary in this augmentation. Using EMSA, we further confirmed that SXR does not bind to the ERE (Fig. 4b). These results indicate that the augmentation by SXR is not

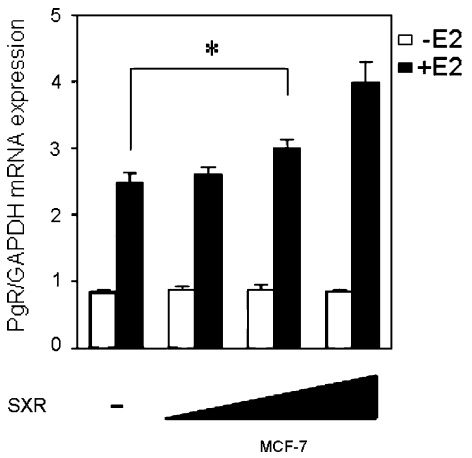

Fig. 11 Expression of PgR mRNA was augmented by SXR in MCF-7 cells. MCF-7 cells were cultured in the 6-well plate and transfected with increasing amounts of expression vector encoding SXR (0, 0.1, 0.2, 0.5 μg). Cells were incubated in the absence or presence of 10^{-8} M E_2 for 12 h. cDNAs were obtained from total RNAs from each plate with reverse transcriptase and random decamer. Realtime quantitative RT-PCR was performed with primers indicated in Materials and methods section. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Each experiment was performed at least three times. *Statistically significant ($P < 0.05$ by ANOVA) vs. SXR (−) column

exerted through direct biding of SXR to ERE. On the other hand, previous reports have shown that ERα may interact with other nuclear receptors that do not always bind to DNA. For example, Min et al. reported that constitutive androstane receptor (CAR) inhibits ERα activity without binding to ERE in HepG2, hepatoma-derived clonal cells, due to squelching the limiting amount of p160 coactivators [44]. In the present study, we found that SXR does not bind directly to ERα using mammalian two-hybrid assays (Fig. 3), GST pull-down study (data not shown) and EMSA (Fig. 4). These results indicate that SXR does not bind to ERα directly.

Next, we examined the effect of SXR on the interaction of cofactors with ERα. Since pCIP/TRAM-1/SRC-3 is highly expressed in MCF-7 cells [42], we used p/CIP constructs in our mammalian two-hybrid studies. Increasing amount of SXR did not increase the binding of ERα to p/CIP in CV-1 cells. It was rather dissociated, indicating that p/CIP is not involved in the augmentation by SXR. Finally, we studied the binding between ERα and corepressors, since previous studies have shown the interaction between ERα and SMRT even in the presence of E_2 [32, 34, 39]. We focused on the role of SMRT on the augmentation of ERα action by SXR. Cotransfection of overdose of SMRT with ERα resulted in the suppression of transcription that was augmented by SXR (Fig. 6). Using mammalian two-hybrid studies, SXR repressed the transcription induced by Gal4-ERα and VP16-SMRT in a dose-dependent manner (Fig. 7b). In vitro-translated SXR effectively decreases the binding between ERα and SMRT in GST pull-down studies (Fig. 8b). These results are consistent with the hypothesis that SXR dissociates SMRT from ERα to augment ERα-mediated transcription. However, the binding between ERα and SMRT was not detected in the absence or presence of E_2 using EMSA (data not shown). This discrepancy may be due to the weak binding between ERα and SMRT, which may be disrupted during passing through the acryl amide gel. Thus, we developed a novel in vitro binding assay, Liquid Chemiluminescent DNA Pull-Down Assay (Fig. 9). Since all procedures are performed in solution, the effect of electrophoresis in an acryl amide gel can be excluded. Using this novel assay, we have demonstrated that SMRT was dissociated from ERα that bound to ERE (Fig. 9b). We further confirmed that SXR dissociated the SMRT from ERα in the presence of E_2 in HEK-293 cells using immunoprecipitation assay (Fig. 10). These results are compatible to those of mammalian two-hybrid assays (Fig. 7b) and GST pull-down studies (Fig. 8b).

Finally, we investigated the effect of SXR on endogenous E_2-target gene in MCF-7 cells using realtime quantitative RT-PCR studies (Fig. 11). The expression of PgR mRNA was augmented by the increasing amount of SXR.

These five independent experiments clearly showed that increasing amount of SXR dissociated SMRT from ERα resulting in the augmentation of ER-mediated transcription. Further, we confirmed that ERα-mediated transcription was suppressed when SMRT was overexpressed (Fig. 6). This idea is also compatible to the previous report showing that the expression patterns of ER-regulated genes are altered in SMRT deficient cells [45].

The biology of breast cancer is very complex, but there is no doubt that estrogen plays a central role [2]. About 70% of breast cancer patients are ERα positive upon initial diagnosis, and in the majority of those cancers, ERα status serves as a valuable predictive marker for probable response to anti-estrogen therapy [1–3]. On the other hand, to date, the expression of SXR in breast cancer cells has not been considered as a critical factor for prognosis. However, as shown in the present study, expression of SXR can augment the E_2 activities or E_2 sensitivities in breast cancer cells. Furthermore, we observed that the TAM activated the SXR-mediated transcription, through CYP3A4 and MDR1 promoter in MCF-7 cells in a ligand- and SXR dose-dependent manner [46]. We also reported that intrinsic CYP3A4 and MDR1 expressions were induced by TAM approximately 1.5-fold in MCF-7 cells [46]. Together with the previous studies, differential SXR expression may alter the metabolism or sensitivity to TAM, and effect or sensitivity to E_2. Thus, during pathological diagnosis of breast

cancer, in addition to examination of estrogen and progesterone receptors, histochemical or quantitative examination of SXR could be useful to select appropriate therapies or to clarify the nature of breast cancers. Trials to examine SXR expression in surgically removed breast tumor tissues are currently underway.

In conclusion, we have shown that SXR augmented the E_2-mediated transcription through ERE of ER-regulating genes by dissociating SMRT in MCF-7 breast cancer cells. Thus, SXR may be useful for a diagnostic classification or prognosis prediction of breast cancers as well as ER or PgR.

Materials and methods

Chemicals and plasmids

17β-estradiol (E_2), 4-hydroxy tamoxifen (TAM), and rifampicin were purchased from Sigma (St. Louis, MO, USA). pCMV-ERα and 2xERE-luciferase (LUC) reporters were described previously [47]. 5x upstream activating sequence (UAS)-thymidine kinase minimum promoter (TK)-LUC was kindly provided by Dr. A. N. Hollenberg [48–50]. Human SXR in pCDG1, RXRβ/pcAMP, VP16-SXR-LBD (amino acids 107–434), VP16-SRC-1-NBD-1 (amino acids 595–780), mouse N-CoR in pCEP4, and human SMRT in pCMX have been described previously [34, 38, 51, 52]. VP16-SMRT-RID (amino acids 1669–2507) and VP16-N-CoR-RID (amino acids 1579–2454) were described previously [34]. Gal4-p/CIP-NBD-1 (amino acids 570–792) was made by insertion of polymerase chain reaction-generated p/CIP-NBD-1 fragment into *Sal*I and *Xba*I sites of pM vector (CLONTECH, Palo Alto, CA). Gal4-ERα-LBD and VP16-ERα-LBD were constructed by ligating the LBD of human ERα (amino acids 302–595) into the pM or VP16 (CLONTECH) vectors, respectively. GST-SMRT-RID was constructed by inserting polymerase chain reaction-generated fragments of human SMRT (amino acids 2098–2507, containing receptor-interacting motifs, (I/L)*XX*II,) into the GST plasmid [48, 49, 53]. HA-tagged-SMRT/pcDNA3 was constructed by inserting polymerase chain reaction-generated fragments of human SMRT (amino acids 2098–2507) into *BamH*I and *EcoR*I sites of pcDNA3 (Invitrogen, Carlsbad, CA), which contains HA-tag. FLAG-tagged-SXR/pcDNA3 was constructed by inserting polymerase chain reaction-generated fragments of full length human SXR into *EcoR*I and *Xho*I sites of FLAG-tag-inserted pcDNA3.

Transient cotransfection experiments

MCF-7 cells were grown in RPMI 1640 Medium with 10% fetal bovine serum. CV-1 fibroblast-derived cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum. The serum was stripped of hormones by constant mixing with 5% (w/v) AG1-X8 resin (Bio-Rad, Hercules, CA) and powdered charcoal before ultrafiltration. Cells were maintained without antibiotics and phenol red. Approximately 1×10^5 cells/cm^2 were transiently transfected using the calcium phosphate coprecipitation method [54] or LipofectoAmine 2000 (Invitrogen) in 12- or 24-well plates. CMV-β-galactosidase plasmid was cotransfected as an internal control. In some samples, empty expression vectors were added to equalize total transfected plasmid concentration. Cells were grown for 24 h in the absence or presence of ligands and then harvested. Cell extracts were analyzed for both luciferase and β-galactosidase activity to correct for transfection efficiency as described previously [34, 47]. All transfection studies were repeated at least three times in triplicate. The results shown are the mean $\pm$ standard error of means (S.E.M.). The data were analyzed by ANOVA and post-hoc comparison was made using Bonferroni's multiple range test.

GST pull-down assay

The GST fusion proteins were produced in *Escherichia coli* BL21 (DE3) and purified by glutathione-Sepharose resin (GE Healthcare, Piscataway, NJ). In vitro binding assays were performed by incubating GST resin (20 µl, 2 µg) and [^{35}S]-methionine-labeled, in vitro-translated proteins (5 µl) that were produced by rabbit reticulocyte lysate (Promega, Madison, WI), according to the manufacturer's instructions. Similar amounts of loading fusion proteins bound to the beads were used, as determined by Coomassie Blue staining/SDS-polyacrylamide gel electrophoresis analysis. Proteins were incubated at 4°C for 16 h in the binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Triton X-100, 10% glycerol, 1 mM dithiothreitol) in the presence or absence of E_2 or TAM. Bound proteins were washed three times with binding buffer in the presence or absence of ligand and subjected to SDS-PAGE and autoradiography. Kodak 1D Image Analysis Software was used to analyze quantitative data. The data were analyzed by ANOVA and post-hoc comparison was made using Bonferroni's multiple range test.

Gel mobility shift assay

Methods for electrophoretic mobility shift assay (EMSA) were described previously [55]. In brief, double-stranded oligonucleotides containing ERE (sense, 5′-gtccaaagtcaggtcacagtcacctgatca-3′; antisense, 5′-aactttgatcaggtcactgtgacctgactt-3′) or SXRE (sense, 5′-agctatatgaactcaaaggaggtcagtg-3′, antisense, 5′-agctcactgacctcctttgagttcatat-3′) was

labeled using Klenow fragment with [α-^{32}P]-dCTP. In vitro-translated ERα, SXR, RXR, and/or GST-fused-SMRT or -N-CoR, and 1×10^4 cpm of labeled templates were incubated in binding buffer (25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 4 mM EDTA, 110 mM NaCl, 5 μg/μl bovine serum albumin, 1 μg/μl poly (dI-dC), 20% glycerol, and 2 mM dithiothreitol) and in the presence or absence of E_2 or rifampicin for 30 min on ice. Different amounts of control reticulocyte lysate were added to some samples so that the total volume of the reticulocyte lysate was consistent. After incubation, samples were subjected to electrophoresis and analyzed with the use of autoradiography.

Liquid chemiluminescent DNA pull-down assay

This novel assay is to detect DNA–protein–protein complexes in solution. A DIG Gel Shift Kit from Roche Diagnostics (Basel, Switzerland) was used for this assay with several modifications. Double-stranded oligonucleotides containing an ERE was labeled by DIG using the DIG Gel Shift Kit (Roche Diagnostics). GST-fused protein such as GST-SMRT-RID on the glutathione sepharose beads was incubated with in vitro-translated ERα at 4°C for 16 h in the binding buffer (same as GST pull-down assay) in the presence or absence of E_2 or TAM. Bound proteins were washed three times with binding buffer in the presence or absence of ligand. Then, the beads–protein complex was incubated with DIG-labeled ERE with or without in vitro translated SXR in the binding buffer from the DIG Gel Shift Kit in the presence or absence of E_2 or TAM. Then beads were washed with washing buffer from the Kit. DNA binding was visualized by an enzyme immunoassay using anti-Digoxigenin, Fab-fragments conjugated with alkaline phosphatase and the chemiluminescent substrate CSPD from the Kit. Light activities were measured with a luminometer. Data represent means ± S.E.M. of experiments performed in triplicate. The data were analyzed by ANOVA and post-hoc comparison was made using Bonferroni's multiple range test.

Preparation of total cell lysate, immunoprecipitation, and western blotting

Expression vectors containing ERα and HA-tagged-SMRT and FLAG-tagged-SXR were cotransfected into HEK-293 cells. Approximately 1×10^5 cells/cm^2 were cultured in the absence or presence of E_2 (10^{-8} M). Cells were lysed on ice in lysis buffer containing 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% (w/v) Nonidet P-40, 1% sodium deoxycholate, protease inhibitors (1 mM PMSF, 2.5 μg/ml leupeptin, 10 μg/ml aprotinin), and 1 mM sodium orthovanadate (a phosphatase inhibitor). Total cell lysate was cleared by centrifugation at 16,400*g* for 15 min at 4°C. For immunoprecipitation, lysates with adjusted protein concentration (Bradford assay, Bio-Rad Laboratories, Hercules, CA) were mixed with anti-HA antibody (12CA5, Roche Diagnostics) and incubated end-over-end at 4°C for 1.5 h. Protein G Sepharose beads (GE Healthcare) were added for 1 h and subsequently washed three times in ice-cold lysis buffer. Proteins were eluted and separated by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) and probed with anti-ERα antibody (D-12, Santa Cruz Biotechnology, Santa Cruz, CA). The antibody–antigen complexes were detected using the ECL-Advance system (GE Healthcare) and visualized with a Lumi-Imager imaging analyzer (Roche Diagnostics). The intensity of bands was quantified by image analysis software (LumiAnalyst, Roche). The data were analyzed by ANOVA and post-hoc comparison was made using Bonferroni's multiple range test.

Realtime quantitative RT-PCR study

Total RNA was isolated from MCF-7 cells, which were cultured in the absence or presence of E_2 (10^{-8} M). The RNA was treated with DNase to remove any contaminating DNA, and was reverse transcribed using random primers and Superscript III RNase H reverse transcriptase (Invitrogen). The enzyme was heat inactivated for 20 min at 65°C, and the mixture was ethanol precipitated. Samples were then assayed by quantitative PCR with a LightCycler (Roche Diagnostics) using SYBR Premix Ex Taq (TaKaRa, Otsu, Japan) according to the manufacturer's recommendations. Specific primer for PgR was purchased from Search LC (Heidelberg, Germany), primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were sense: 5′-gtgacaaagtggacattgttgcc-3′, antisense: 5′-agatgatgacccttttggcccc-3′. A standard dilution series of control DNA was included in every experiment to allow relative quantification of each sample. Each of the experiments was repeated at least three times, with independent RNA preparations. LightCycler Software Ver.3.5 was used to analyze data. The results for each sample were normalized by GAPDH mRNA level as an internal control.

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