For the following 18 articles, the authors note, in addition to the partial funding of this study from KaroBio AB, that J.-A.G. is co-founder, deputy board member, stockholder, and consultant of KaroBio AB.


(iv) **NEUROSCIENCES.** “Estrogen receptor (ER)β knockout mice reveal a role for ERβ in migration of cortical neurons in the developing brain,” by Ling Wang, Sandra Andersson, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 2, January 21, 2003, of Proc. Natl. Acad. Sci. USA (100, 703–708; first published January 6, 2003; 10.1073/pnas.242735799).


www.pnas.org/cgi/doi/10.1073/pnas.0602780103
Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D

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Contributed by Jan-Åke Gustafsson, December 15, 2003

Estrogen receptor (ER) β counteracts the activity of ERα in many systems. In agreement with this, we show in this study that induced expression of ERβ in the breast cancer cell line T47D reduces 17β-estradiol-stimulated proliferation when expression of ERβ mRNA equals that of ERα. Induction of ERβ reduces growth of exponentially proliferating cells with a concomitant decrease in components of the cell cycle associated with proliferation, namely cyclin E, Cdc25A (a key regulator of Cdk2), p45Skp2 (a key regulator of p27kip1 proteolysis), and an increase in the Cdk inhibitor p27kip1. We also observed a reduced Cdk2 activity. These findings suggest a possible role for ERβ in breast cancer and imply that ERβ-specific ligands may reduce proliferation of ER-positive breast cancer cells through actions on the G1 phase cell-cycle machinery.

In a recent report, Omoto et al. (15) expressed ERβ stably in MCF-7 cells under the control of a cytomegalovirus promoter and found that the receptor had a negative effect on proliferation of these cells and reduced the number of colonies in an anchorage–independence assay.

In the present study, we have investigated how ERβ affects cellular proliferation in response to E2 in T47D cells stably transfected with tetracycline-regulated ERβ expression plasmid. We have investigated the specific effects of ERβ expression on the components of the cell cycle machinery Cdk2, cyclin D1, Cdc25A, cyclin E, and p27kip1 in these cells.

Materials and Methods

Cell Culture. T47D cells were cultured in DMEM/Ham’s F-12 (1:1) supplemented with 5% FBS, penicillin, and streptomycin. For experiments using E2, DMEM without phenol red and FBS treated with Dextran-coated-charcoal (DCCFBS) were used.

Transfection and Plasmids. T47D cells stably transfected with tetracycline-regulated ERβ expression plasmid were generated in two steps. The cells were first transfected with pTet-TaK (GIBCO/BRL) modified to contain puromycin resistance by using Lipofectin according to the manufacturer’s instructions (GIBCO/BRL). Selection was performed with 0.5 μg/ml puromycin in the presence of 1μg/ml tetracycline. A clone showing high levels of induction upon tetracycline withdrawal and low basal activity was selected by using the pUC13-3 control plasmid (GIBCO/BRL). The short form of ERβ encoding 485 aa was fused to the flag tag (ERβ 485) and cloned into PBI-EGFP (Clontech). This construct was then transfected into the previously described inducible clone together with a neomycin resistance plasmid, and selection was performed with 500 μg/ml G418 (Calbiochem). For transient transfections of promoter constructs, normal T47D cells were used. Cells were plated in six-well plates at 50% confluency and synchronized as described under real-time PCR and primers (see below). The plasmids were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Real-Time PCR and Primers. Cells were added to six-well plates at a confluence of 40%; after 1 day, the normal medium was replaced by phenol red-free medium supplemented with 5% DCCFBS. After 24 h, 10 nM ICI 182,780 was added to the cultures, and incubation proceeded for an additional 48 h. For expression of ERβ, tetracycline was removed 12 h before initiation of treatment with E2. At time 0 h, the medium was changed to 0.5% DCCFBS, and E2 was added to a final concentration of 10 nM. RNA was prepared by adding 1 ml of TRIzol (Invitrogen) to each 35-mm plate at different time points after the start of treatment, and RNA was prepared according
to the manufacturer’s instructions. cDNA (100 ng) was amplified in a real-time PCR using TaqMan Universal Master Mix (PE Applied Biosystems) or, for cyclin E, OPCR Master Mix for Cybergreen (Medprobe). The real-time PCRs were performed in an ABI PRISM model 7700 sequence detector (Perkin–Elmer Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 1 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The optimum concentration of primers and probes was determined in preliminary experiments. All probes were labeled with 6-carboxyfluorescein as the 5′ reporter. The sequences of primers and probes are as follows. H cyclin D1 (16): F, 5′-CCGCTCAGGCGAGATGTC-3′; R, 5′-ATGGGC-CAGCGGAAAGAC-3′; probe, 5′-CTCTCTCACATACCGGCACAT; 200 nM primers, 200 nM.

**Perkin Real-time PCR was done in triplicate. The 18S rRNA (PDAR, Cybergreen (Medprobe). The real-time PCRs were performed in a real-time PCR using TaqMan Universal Master Mix (PE Applied Biosystems) under the following conditions: 50°C for 15 sec and 60°C for 1 min.**

**Results**

**ERβ Expression Inhibits Proliferation of T47D Cells.** T47D cells proliferate in response to treatment with E2 (5). In breast cancer cell lines, ERα has been shown to be the receptor responsible for the proliferative effect of E2, whereas, based on studies from knockout mice, ERβ has been proposed to reduce ERα-induced proliferation (18). With real-time PCR using oligos specific for ERα and ERβ, respectively, we found that the predominant ER mRNA in T47D cells is ERα, the ratio ERα/ERβ mRNA being 9:1. We could not detect any endogenous expression of ERβ protein in these cells (data not shown).

There is a lack of suitable breast cancer cell lines for studies on the function of endogenous ERβ. Our approach has been to stably transfect the breast cancer cell line T47D with a tetracycline-regulated vector for expression of ERβ. In these cells, we can regulate the expression level of the receptor by using different amounts of tetracycline (Fig. 1). We found that E2 inhibited proliferation of T47D cells completely when the mRNa levels of both receptors are equal (Figs. 1a and 2) in contrast to parental T47D cells where ERα predominates and E2 acts as a mitogen. Levels of ERβ protein detected with FLAG antibodies correlated well with ERβ mRNA expression (Fig. 1b).

**Cyclin D1 and Cdc25A mRNA Levels Are Affected by ERβ Expression.** Real-time PCR and Western blot analysis of cyclin D1 and Cdc25A expression were performed on extracts of mitogen-deprived T47D cells treated with E2 for different lengths of time. We could show that cyclin D1 mRNA and protein are induced by E2, 4OH-tamoxifen, raloxifene, and ICI 182,780. After an additional 48 h, the cells were harvested with a rubber policeman, and cell slurry was sonicated briefly before centrifugation at 30,000 × g for 5 min at 4°C. The supernatant was collected, and aliquots of 25 μg of protein were separated on SDS-PAGE. Antibodies used were as follows: mouse mAbs directed against Cdc25A (Ab-3) and E2F-1 (Ab-1) (NeoMarkers, Fremont, CA), cyclin D1 rabbit polyclonal antibody (sc-753), mouse monoclonal anticyclin E (HE12) and cyclin A (BF683), goat polyclonal anti-p27Kip1 (C19), and rabbit polyclonal anti-Skp2 (H435) (Santa Cruz Biotechnology). Histone kinase assays were performed as described (9, 17), using rabbit polyclonal antibodies to Cdk2 (M2) (Santa Cruz Biotechnology).

**Flow Cytometric Analysis.** For flow cytometric analysis, T47D cells were harvested in saline-EDTA, fixed in cold 70% ethanol, and stored at −20°C. Fixed cells were subsequently washed, treated with 100 μg/ml RNase A, and stained with 50 μg/ml propidium iodide. Analysis of DNA content was performed in a Becton Dickinson FacsCan with a minimum of 15,000 events collected for analysis with Becton Dickinson cellQuest software. The proliferative fraction in the culture was determined as cells in the S and G2/M phases of the cell cycle based on DNA content.
Furthermore, in the presence of tetracycline (i.e., with ER\textsubscript{β} expression suppressed), Cdc25A mRNA and protein are induced by E2 within 12 h of treatment of ICI 182,780 pretreated cells. When tetracycline was absent (ER\textsubscript{β} expressed), induction of Cdc25A mRNA and protein was weak (Fig. 4). To investigate whether regulation occurred at the transcriptional level, we transiently transfected a luciferase reporter regulated by a minimal Cdc25A promoter into T47D cells. The cells had been treated with ICI 182,780 in low serum for 48 h. The basal activity of the Cdc25A promoter was unchanged by cotransfection with ER\textsubscript{β}, but upon treatment with 10 nM E2, there was a substantial decrease in promoter activity (Fig. 5). In agreement with a study by Hodges et al. (11), 4OH-tamoxifen induced the promoter to the same extent as E2 in cells maintained in tetracycline, whereas expression of ER\textsubscript{β} eliminated this induction (Fig. 5). Activity of Cdk2 complexes precipitated from cells expressing ER\textsubscript{β} was markedly decreased relative to those from cells treated with E2 in the absence of ER\textsubscript{β}, causing a decrease of cells in the proliferative fraction S\textsubscript{G2}/M (Fig. 6). These complexes could not be activated by adding purified GST-Cdc25A (data not shown). A similar inability of Cdc25A to activate Cdk2 was observed by Musgrove et al. (19) with progesterin inactivated Cdk2. Levels of the Cdk inhibitor p27\textsuperscript{kip1} increased in ER\textsubscript{β}-expressing cells and did not decline in re-
As cyclin E by ER expression/H9252 cells with or without cotransfection with 100 ng of pcDNA3 or Flag 485 ER in presence of 2-fold, stimulate proliferation in the presence of antiestrogens. In the presence of antagonists (20), it could be argued that the receptor would activate AP-1 in the presence of estrogen (10, 22). Moreover, studies referred to (13, 22) used HeLa cells, which lack endogenous ER, and, in this system, the activity of ER may be different from that in T47D cells where ER is naturally expressed.

**Antiestrogens Do Not Induce Proliferation in the Presence of ERβ in T47D Cells.** Because ERβ can activate AP-1 in the presence of antiestrogens (20), it could be argued that the receptor would stimulate proliferation in the presence of antiestrogens. In the presence of 2 μg/ml tetracycline (no ERβ expression), E2 induced proliferation 2-fold, whereas 1 μM 4OH-tamoxifen and raloxifene stimulated proliferation only weakly, and ICI 182,780 did not. The weak stimulation by 4OH-tamoxifen is likely caused by the weak agonistic of this agent on ERα (21). Expression of ERβ completely prevented proliferation in response to E2, 4OH-tamoxifen, raloxifene, and ICI 182,780 (Fig. 8).

**Discussion**

We have shown here that expression of ERβ in the breast cancer cell line T47D inhibits proliferation in response to E2 treatment. By monitoring cell-cycle components, we found that, surprisingly, cyclin D1 is induced earlier and to a higher level if ERβ is expressed. This finding is in apparent contradiction with the current hypothesis (based on analysis with the ~936 cyclin D1 promoter) that ERβ inhibits proliferation of breast cancer cells by reducing E2-mediated induction of cyclin D1 (1, 22). The reason for the discrepancy might be that additional regulatory elements situated further upstream or downstream of the cyclin D1 gene are targets of regulation by ERβ. Furthermore, the studies referred to (13, 22) used HeLa cells, which lack endogenous ER, and, in this system, the activity of ER may be different from that in T47D cells where ER is naturally expressed. Although it is well established that E2 activates the cyclin D1 expression is illustrated by studies indicating that the major antiproliferative effect of tamoxifen is related to its inhibition of cyclin D1 expression (11) and that overexpression of cyclin D1 or c-Myc is sufficient to increase the proliferation of MCF-7 cells (23). However, this is not the case in all situations: when ERα was expressed from an adenovirus vector in the breast cancer cell line MDA-MB-231, there was increased expression of cyclin D1 but a reduction in proliferation (24).

In MCF-7 cells, ERα is known to induce expression of c-Myc and E2F-1, which are, in turn, important for E2 induction of Cdc25A mRNA and protein levels (9, 25). In contrast, we found that ERβ expression in T47D cells results in repression of Cdc25A mRNA and protein expression. The inhibited Cdk2 complex precipitated from ERβ-expressing cells could not be activated with purified GST-Cdc25A (data not shown), indicating that, as shown in other studies (11, 19), Cdc25A is not solely responsible for the lack of Cdk2 activation. A further examination of the regulation by ERβ of other cell-cycle factors revealed that...
expression of both cyclin E and cyclin A mRNA was decreased by ERβ. Cyclins E and A are critical components of the active Cdk2 complex driving passage from G1 into S phase. Overexpression of cyclins E or A is associated with poor prognosis in breast cancers (26–29). We cannot at this stage discriminate between the alternative possibilities that the reduction in cyclin E and A levels is caused (i) by ERβ’s inhibition of ERα’s transcriptional activity or (ii) by ERβ directly repressing the activity of the cyclin E and A promoters. There is no evidence at this time that ERα or ERβ binds directly or indirectly to enhancer elements in the cyclin E or A promoters.

Inhibition of expression of cyclin E, cyclin A, Cdc25A, and E2F-1 does not necessarily indicate a direct regulation by ERβ. It may simply be an indication that the cells are arrested in G1 phase (9). Because expression of all of these cell cycle-associated factors is regulated by E2F-dependent mechanisms, the effect of ERβ activation on G1-phase regulators may be the consequence of cell-cycle inhibition and a lack of release from E2F/pocket protein transcriptional repression. Such cell-cycle inhibition may result from a failure to down-regulate Cdk inhibitors such as p27kip1 (Fig. 6), whose regulation in MCF-7 breast cancer cells is cell cycle independent (8, 9). This failure in down-regulation of p27kip1 may, in turn, reflect the observed decrease in p45Skp2 levels (Fig. 6). We have previously shown that E2 up-regulates p45Skp2 expression and elicits nuclear export of p27kip1 in MCF-7 cells, leading to degradation of this Cdk inhibitor in both nucleus and cytoplasm (8).

It is clear that active cyclin E-Cdk2 is essential for the formation of the origins of replication complexes when cells reenter the cell cycle from a quiescent state, but several recent studies have raised questions about the primacy of cyclin E-Cdk2 in regulating G1/S transition in continuously proliferating mouse embryo fibroblasts and colon cancer cells (30–34). In breast cancer cells, Cdk2 activity does not necessarily correlate with cyclin E levels (35) and may instead reflect the relative amounts of cyclin E and p27kip1 (36). Cdk2 activity is turned on in quiescent MCF-7 cells by E2 without significant changes in cyclin E expression and instead relates to changes in association of cyclin E-Cdk2 with CIP/KIP Cdk inhibitors (37).

Our observation that the cyclin E content of Cdk2 immuno-precipitates was unchanged by ERβ expression (Fig. 6) leaves it unclear as to whether cyclin E suppression is the primary cause of the low Cdk2 activity in ERβ-expressing cells. The basis of Cdk2 and cell-cycle inhibition in ERβ-expressing T47D cells thus remains to be fully characterized.

Under our experimental conditions, antiestrogens like 4OH-tamoxifen, raloxifene, and ICI 182,780 did not increase proliferation of cells expressing ERβ at high levels. This finding was surprising because the antiestrogen–ERβ complex is known to activate AP-1 sites. Clearly, the interaction of ERβ with antiestrogens is not sufficient to increase proliferation in T47D cells. Palmieri et al. (38) have studied the expression of both ERα and ERβ in ductal cancers of various grades and shown that although ERβ is the dominant receptor in normal breasts, it is not expressed in grade 1 ductal cancer. Grade 1 ductal cancer shows high expression of ERα, and these are the cancers that respond well to tamoxifen. In grade 2 ductal cancer, both ERα and ERβ are highly expressed, whereas in grade 3 ductal cancer, which has the poorest prognosis, there is commonly neither ERα nor ERβ (38). Fuqua et al. (39), analyzing 242 breast tumors with a monoclonal antibody against the N terminus of ERβ, showed that this receptor isoform is present in 62% of the cases expressing ERα. No correlation of ERβ expression with tumor grade or S-phase fraction was observed; the study, however, did not take the relative levels of the receptors into consideration. Another study by Iwao et al. (40) using 112 breast tumors showed
that, whereas ERα mRNA is up-regulated during the progression of ER-positive breast cancers, ERβ mRNA is down-regulated. It is now clear that it is insufficient to measure only one form of ERβ in breast cancer. Another ERβ splice variant, ERβ cx (41), is well expressed in breast cancer (42), which is important because if ERβ cx is expressed in the same cells as ERα, it quenches ERα action (41). Any meaningful study on the relationship between ER expression and prognosis with antiestrogen treatment must measure ERα, ERβ, and ERβ cx and, in addition, must examine the cellular localization of these receptors.

Our studies were done in cells coexpressing ERα and ERβ and show that when these two receptors are together in a cell ERβ can inhibit the proliferative response of ERα to E2.

This work was supported by the Swedish Cancer Fund, KaroBio, and the National Institutes of Health.