

Inducible upregulation of oestrogen receptor- β 1 affects oestrogen and tamoxifen responsiveness in MCF7 human breast cancer cells

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Abstract

To investigate the effect of altered oestrogen receptor (ER) α and ER β expression on oestrogen and anti-oestrogen action in breast cancer, we have stably expressed an inducible ER β 1 in MCF7 breast cancer cells. Stably expressing clones were isolated and over-expression of ER β 1 correlated with increased levels of specific radiolabelled oestradiol (E2) binding. Increased ER β 1 did not affect endogenous levels of ER α but increased progesterone receptor (PR) levels. Over-expression of ER β 1 reduced growth responses to E2 in contrast to little if any effect of over-expression of ER α . In oestrogen-replete conditions, over-expression of ER β 1 but not ER α reduced proliferation. Over-expression of ER β 1 did not result in anti-oestrogen resistance but was associated with increased sensitivity to 4-hydroxytamoxifen. Our results suggested that over-expression of ER β 1 in the presence of an endogenously expressed ER α was associated with tamoxifen sensitivity but may negatively modulate ER α -mediated growth. However, not all ER α activities were inhibited since endogenous PR expression was increased by both ER α and ER β 1 over-expression. These data paralleled those seen in some *in vivo* studies showing a relationship between PR and ER β expression as well as ER β expression and tamoxifen sensitivity of ER-positive breast cancer patients. These models are relevant and will be useful for dissecting the role of ER β 1 expression in ER-positive breast cancer.

Journal of Molecular Endocrinology (2005) **34**, 553–566

Introduction

Oestrogens are considered to be major driving forces in breast tumorigenesis and breast cancer progression (Colditz 1998, Clemons & Goss 2001). Current evidence suggests that oestrogen action is primarily mediated through two receptors, oestrogen receptor (ER) α and ER β (Kuiper *et al.* 1996). These structurally related receptors are generally considered to be ligand-regulated transcription factors which classically modulate target gene transcription by binding to oestrogen responsive sequences (ERE) in target gene promoters (McDonnell & Norris 2002). There is evidence that ER α and ER β can be expressed together in some cell types and independently expressed in others (Dotzlaw *et al.* 1997, Enmark *et al.* 1997). Based on studies in which ER β expression was determined at the protein level by immunohistochemistry (IHC) (Jarvinen *et al.* 2000, Jensen *et al.* 2001, Mann *et al.* 2001, Omoto *et al.* 2002, Saunders *et al.* 2002) and ER α was determined by IHC or by enzyme-linked immunoassay, an estimated frequency of ER α and ER β status in breast tumors was

obtained (Murphy *et al.* 2003). The most frequently occurring status is ER α positive/ER β positive (59%) with similar frequencies of between 11 and 17% for the other three ER phenotypes. It is important to note that there are two groups of ER β -expressing breast tumors, those with coexpression of ER α , and those that express ER β alone. The former occurs most frequently and, under experimental conditions when ER α and ER β are coexpressed, they form heterodimers preferentially over homodimerization (Cowley *et al.* 1997). It is likely that homodimers of ER α , homodimers of ER β and ER α /ER β heterodimers will differentially affect gene expression. Further, transient coexpression of ER α and ER β in cell lines results in ER β -induced reduction of ER α activity at low ligand concentrations, as measured using ERE-regulated reporters (Hall & McDonnell 1999), suggesting that ER β can directly modulate ER α activity in an inhibitory fashion. This may be a mechanism for differential oestrogen sensitivity and some reports have documented the differential expression of these two receptors under conditions of altered oestrogen sensitivity (Leygue *et al.* 1998a,

Weihua *et al.* 2000). Interestingly, ER β expression is downregulated and ER α expression upregulated during human breast tumorigenesis, suggesting that the ability of ER β to modulate ER α may be altered during breast tumorigenesis (Leygue *et al.* 1998a, Roger *et al.* 2001). This correlates with altered oestrogen sensitivity and activity occurring during breast tumorigenesis (Murphy & Watson 2002). In addition, altered oestrogen action can occur during breast cancer progression and this is thought to underlie the development of tamoxifen resistance (Clarke *et al.* 2001). Increased expression and/or activity of ER β has been suggested as a mechanism of tamoxifen resistance, but the data are controversial (Paech *et al.* 1997, Speirs *et al.* 1999, Mann *et al.* 2001, Murphy *et al.* 2002).

To investigate further the effect of altered relative expression of ER α and ER β on oestrogen and anti-oestrogen action in human breast cancer cells, we have stably expressed a tetracycline-inducible (Tet-on) human ER β 1 in MCF7 human breast cancer cells, which endogenously express high ER α but low ER β levels (Dotzlaw *et al.* 1997, Fuqua *et al.* 1999). The effect of altering the relative expression of these two ERs on oestrogen- and anti-oestrogen-regulated proliferation, as well as on endogenous markers of ER activity such as progesterone receptor (PR) (Osborne 1998), has been determined in this model system.

Materials and methods

Plasmids

Epitope-tagged tetracycline-inducible human ER β 1 (full-length) expression plasmids were generated. Human ER β 1 long form of 530 amino acids (Leygue *et al.* 1998b, Ogawa *et al.* 1998b) was tagged at its N-terminus with a polyhistidine and an Xpress epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc., Burlington, Ontario, Canada) and characterized as previously described (Peng *et al.* 2003). This cDNA was then subcloned downstream of the tetracycline-inducible promoter in the pTRE2 hyg plasmid (Gossen *et al.* 1995) (Clontech Laboratories Inc., Palo Alto, CA, USA). This construct contains the selectable hygromycin resistance gene on the same plasmid as the inducible gene.

Hormones and reagents

Oestradiol-17 β (E2) and the tetracycline analogue doxycycline were obtained from Sigma Chemical Co. (Oakville, Ontario, Canada). Hygromycin B was obtained from Clontech and G418 was from Invitrogen Canada Inc.

Cell culture and transfection

The cells were routinely cultured in Dulbecco's modified Eagles' medium (DMEM) containing 5% (v/v) foetal calf serum (FBS), 0.4% (w/v) glucose, glutamine and penicillin-streptomycin and 10 nM E2 (5% CM) as previously described (Peng *et al.* 2003). To obtain oestrogen-depleted cells, the culture medium of stock cells (approximately 50% confluent) was changed to phenol red-free DMEM supplemented with 5% (v/v) FBS that has been treated twice with charcoal/dextran, glucose, glutamine and penicillin-streptomycin (5% CS) and replaced every 2 days. The cells were grown for at least a week and not more than 2 weeks in this oestrogen-depleted medium, and were passaged once during this time. When oestrogen-depleted cells were around 50% confluent, they were harvested and used for experiments. For transfection, MCF7-clone 89 cells (Venditti *et al.* 2002) were grown in 10% CM in 100 mm dishes until 90% confluent. The cells were then split into three 100 mm dishes and the next day transfected (2–4 μ g linearized plasmid per dish) using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, Ontario, Canada). Two days after transfection the medium was changed to 10% CM plus 500 μ g/ml G418 and 400 μ g/ml hygromycin, and the medium was changed every day for 4 days and then every 3 days. Approximately 2 weeks later, hygromycin resistance colonies began to appear and 120 large, healthy colonies were transferred to 96-well plates and screened after suitable numbers of cells were achieved as described below.

Generation of MCF7 human breast cancer cells with doxycycline-inducible epitope tagged human ER β 1

The generation and characterization of MCF7 cells stably expressing reverse tetracycline transactivator (rTA), clone 89 rTA) have been previously described (Venditti *et al.* 2002). These cells (clone 89) were transfected and selected as described above. Resistant clones were screened by Western blotting for doxycycline-inducible His-Xpress-ER β 1 expression (tagged-ER β 1), using anti-Xpress antibody (no. R910–25; Invitrogen Canada Inc.; 1/5000 in 20 mM Tris base, 0.137 M NaCl, pH 7.5 and 0.1% (v/v) Tween-20 (TBST)) as previously described (Peng *et al.* 2003). Multiple positive clones were isolated and two independent clones were selected for further characterization.

RNA extraction and RT-PCR conditions

Total RNA was extracted from frozen cell pellets using Trizol reagent (Life Technologies, New York, NY, USA) according to the manufacturer's instructions. One

microgram of total RNA was reverse transcribed, as previously described (Miksicek *et al.* 2002).

The primers used consisted of PR-U primer (5'-CACAAAACCTGACACCTCCAGTTC-3'; sense; located in PR 2290–2313) and PR-L primer (5'-GCAAAATACAGCATCTGCCAC-3'; antisense; located in PR 2490–2511). Nucleotide positions given correspond to sequences of the human PR cDNA (accession number M15716). PCR amplifications were performed and PCR products analyzed as previously described (Miksicek *et al.* 2002). Each PCR consisted of 28 cycles (30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C). PCR products were then resolved by electrophoresis on 1.5% agarose gels. Following electrophoresis, the gels were stained with ethidium bromide, as previously described (Miksicek *et al.* 2002). Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was performed in parallel and PCR products were visualized on agarose gels stained with ethidium bromide as previously described (Miksicek *et al.* 2002). Three independent PCRs for PR and GAPDH were performed for each experiment. Signals visualized by u.v. irradiation on a GelDoc 2000/ChemiDoc System (Bio-Rad, Mississauga, Ontario, Canada) were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). PR was normalized to GAPDH in each experiment as previously described (Miksicek *et al.* 2002).

Whole cell ligand-binding assays

ER ligand-binding assays were done using the whole cell binding method as previously described (Coutts *et al.* 1996) following oestrogen depletion of the cells by two passages in 5% CS as previously described (Coutts *et al.* 1996).

Western blotting

Western blotting was carried out essentially as previously described (Adeyinka *et al.* 2002, Peng *et al.* 2003) with minor modifications. ER α was detected using 1/1000 dilution of an ER α mouse monoclonal antibody (NCL-ER-6F11; Novocastra Labs Ltd, Newcastle on Tyne, Tyne and Wear, UK) in TBST buffer, PR was detected using 1/1000 dilution of a PR mouse monoclonal antibody (NCL-PGR-AB; Novocastra Labs Ltd) in TBST and α -tubulin was detected using 1/12 000 dilution of tubulin- α antibody 2 (mouse monoclonal, clone DM1A; Neomarkers, Fremont, CA, USA). All membranes were incubated for 1 h at room temperature in 0.2% I-block (Tropix, Bedford, MA, USA) in Tris-buffered Saline (TBS). The blot was washed for 10 min at room temperature in TBST and then incubated with the appropriate dilution of primary antibody overnight at 4 °C. This incubation was

followed by five 5-min washes in TBST, and then incubation with secondary antibody for 1 h at room temperature (goat anti-mouse IgG-HRP; Jackson Immuno Research Labs Inc., West Grove, PA, USA; 1/5000 in TBST). The blot was washed five times for 5 min with TBST and the signal was visualized using Supersignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Signal detection and documentation used the ChemiDoc Gel Documentation System 2000 (Biorad Labs Canada, Mississauga, Ontario, Canada).

Immunofluorescence

Eleven days prior to slide preparation, stock cells were placed in medium containing 5% tetracycline-free FBS (BD Bioscience Clontech, Palo Alto, CA, USA), and the medium was changed every 3 days. These cells were then harvested and 1×10^4 cells in 1 ml medium was added to each well of 4-chambered Falcon culture slides (Becton Dickinson, Franklin Lakes, NJ, USA). For every cell line used, one well was treated with vehicle alone and the duplicate well was treated with 1 μ g/ml doxycycline for 48 h. Each chamber slide consisted of two wells of control MCF7-clone 89 cells and two wells of a tagged-ER β 1 subclone of cells. Following incubation, the medium was removed by aspiration and the slides were washed with $1 \times$ phosphate-buffered saline (PBS) for 2 min. The PBS was removed and the cells were fixed and permeabilized with paraformaldehyde solution (3.7% formaldehyde and 0.18% Triton-X-100 in PBS) for 30 min at 37 °C. The slides were then washed in $1 \times$ PBS for 5 min at room temperature, with agitation. Slides were then removed from the washing solution and, while still damp, they were coated with a hydrophobic barrier around the outside edge using an ImmEdge pen (Vector Laboratories Inc., Burlingame, CA, USA). Inside the barrier the cells were covered in 1 ml blocking solution (1% (w/v) BSA and 1% (v/v) normal goat serum in PBS) and incubated for 30 min at 37 °C in a humidified chamber to prevent evaporation. The blocking solution was removed and the cells were incubated with mouse anti-Xpress antibody (1/750 in blocking solution; Invitrogen Canada Inc.) for 1 h at room temperature. The slides were then washed three times with PBS for 5 min with agitation. Slides were then moved to a dark room and then incubated with CY3-labelled goat anti-mouse secondary antibody (1/10 000 in blocking solution) for 1 h at room temperature. This was followed by three 5-min washes at room temperature with PBS with agitation. The cells were stained with Hoechst dye (no. 33258; Sigma Chemical Co., catalogue number B2883; 1/10 000 in blocking solution) for 20 min at room temperature, followed by two washes in PBS for 6 min at room

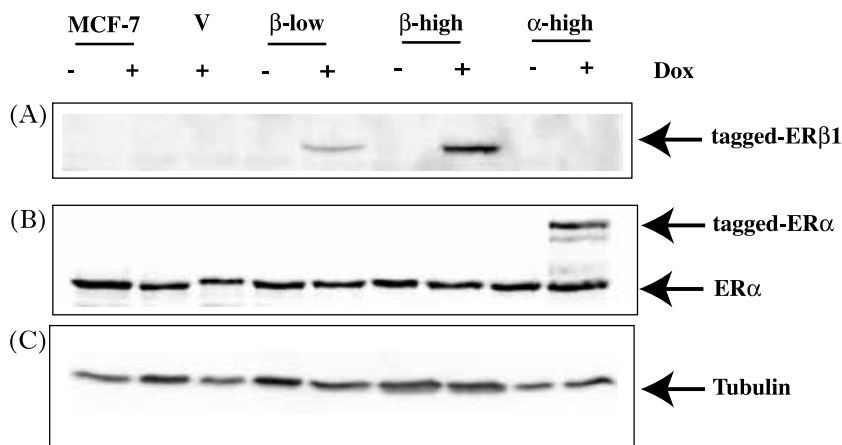


Figure 1 (A) Western blot of cell extracts from cell lines grown in 5% CS medium treated (+) or not treated (-) with doxycycline (Dox) for 48 h. Tagged-ER β 1 was visualized using anti-Xpress antibody as described in Materials and methods. MCF7 parent cells stably expressing rTA Tet-on transcription factor (Venditti *et al.* 2002); V=vector alone transfected MCF7 cells; β -low=Xpress-tagged ER β 1 low-expressing MCF7 clone; β -high=Xpress-tagged ER β 1 high-expressing MCF7 clone; α -high=GFP-tagged ER α high-expressing MCF7 clone. (B) Same blot as in (A), stripped and visualized using anti-ER α antibody as described in Materials and methods (note the larger GFP-ER α and the smaller approximately 66 kDa endogenously expressed ER α). (C) Same blot as in (A), stripped and visualized using anti-tubulin antibody as described in Materials and methods.

temperature. Slides were allowed to stand in PBS for a further 20 min at room temperature, then drained and one drop of Fluorsave reagent (Calbiochem, Hornby, Ontario, Canada) per well was applied. A coverslip was then added and sealed with clear nail polish. The slides were stored in the dark overnight at 4 °C and fluorescent images obtained using a Nikon Eclipse E1000 microscope (Nikon, Mississauga, Ontario, Canada) using ACT-1 software (Nikon). All exposure times were for 1 s.

Half-life determination of tagged-ER β 1

To determine the effect of various ligands on the half-life of tagged-ER β 1, cells were set-up in 5% CS and left overnight. The media were changed and then the cells were treated for 48 h with 2 μ g/ml doxycycline. The next day the cells were washed three times with 5% CS without doxycycline, and then treated with ethanol alone, E2 (10 nM), 4-hydroxy-tamoxifen (4-OH-tamoxifen; 500 nM) or ICI 182780 (500 nM; a gift kindly provided by AstraZeneca, Macclesfield, Cheshire, UK). Cells were harvested at 0, 2, 3, 4, 6, 8, 10, 12, 15 and 24 h after treatment. Cell extracts were analyzed by Western blot for tagged-ER β 1 and tubulin expression. Tagged-ER β 1 normalized to the tubulin signals from the same blot was quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). The half-life of tagged-ER β 1

when bound to various selective ER modulators (SERMs) was estimated using the line of best fit on a semi-log plot from two independent experiments.

Cell proliferation assay

Cells depleted of oestrogen for at least a week by passage in 5% CS were set-up at 5×10^4 cells per well in 6-well plates in 5% CS. The cells were left overnight and then treated with 2 μ g/ml doxycycline or vehicle alone for 48 h. Cells maintained in the presence or absence of doxycycline then were either treated with E2 (10 nM) or vehicle alone. Triplicate wells were counted electronically (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada) at the times indicated after the start of E2 treatment. Medium was changed every 2 days.

Cells grown in 5% CM were set-up at 10^4 cells per well in 6-well plates in 5% CM, left overnight and then treated with 2 μ g/ml doxycycline or vehicle alone for 48 h. Cells were maintained in the presence or absence of doxycycline and treated with varying concentrations of 4-OH-tamoxifen or vehicle alone. Triplicate wells were counted electronically (Beckman Coulter Canada Inc.) at the indicated times after the start of treatment. Medium was changed every 2 days. Doubling times were calculated from the initial (n_i) and final (n_f) cell numbers from the equation: doubling time (DT) = $\log 2 /$

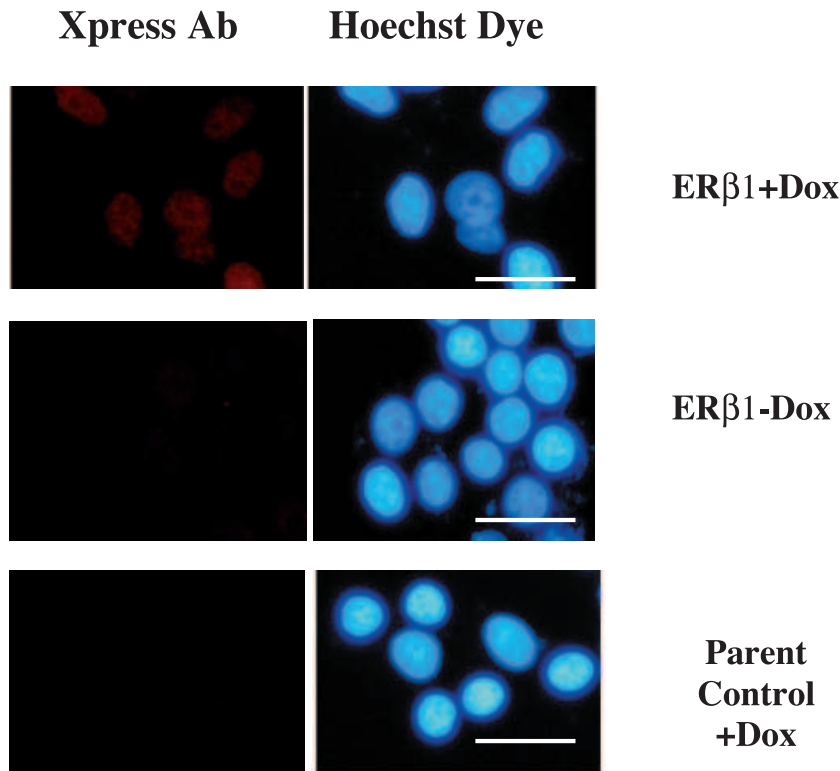


Figure 2 Immunofluorescent detection of tagged-ER β 1 in induced (+doxycycline (Dox)) and uninduced (-Dox) β -low and control parental MCF7 cells. The magnification was $\times 60$ and the white bar in each panel represents 25 μm . Ab, antibody.

$\log(n_t)/(n_0)$, where t is time in days between n_t and n_0 .
Proliferation rate = $\text{DT}(\text{control})/\text{DT}(\text{treated}) \times 100\%$.

Statistical analysis

ANOVA followed by t -test analyses was used as appropriate.

Results

Identification of doxycycline-inducible tagged-ER β 1-expressing MCF7 cell lines

Two clones, a low-expressor (β -low) and a high expressor (β -high) shown to express doxycycline-inducible tagged-ER β 1 by Western blot analysis were chosen for further characterization. Controls were the original previously described MCF7 cell line stably expressing rTA (Venditti *et al.* 2002) and these MCF7 cells transfected with the empty pTRE2 hyg plasmid alone (V). As an additional control we included an MCF7 cell line stably transfected with a doxycycline-

inducible GFP-tagged ER α expression vector (α -high) (Htun *et al.* 1999, Zhao *et al.* 2002). Preliminary experiments showed that induction of the tagged-ER β 1 was dependent on the dose of doxycycline used, and maximum induction in any one clone was achieved by treating the cells for 48 h with 1–2 $\mu\text{g}/\text{ml}$ doxycycline. A Western blot of the cell lines grown in 5% CS+doxycycline treatment for 48 h is shown in Fig. 1A. A single band of approximately 62 kDa was detected only in the doxycycline-treated tagged-ER β 1-transfected lines but not in control cell lines, MCF7, V and α -high. The level of tagged-ER β 1 expression in each of the clones was different with β -high > β -low. When these cell lines were grown on glass slides, fixed and the tagged-ER β 1 visualized using anti-Xpress antibody, significant fluorescence was only found over the nucleus (determined by Hoechst staining) of doxycycline-treated tagged-ER β 1 clones, with little, if any, signal detected in doxycycline-treated MCF7 cells. An example of the data obtained is shown in Fig. 2. These data were consistent with a general localization of the tagged-ER β 1 product in or associated with the nucleus, although we cannot exclude localization of minor levels

Table 1 ER levels measured by ligand-binding assay of doxycycline-induced and uninduced MCF7 cell lines. Values are means \pm s.d.

Clone	Sites per cell	
	Plus doxycycline (48 h)	No doxycycline
MCF7 (parent)	33910 \pm 5831 (<i>n</i> =3)	34150 \pm 11030 (<i>n</i> =3)
MCF7-V (vector alone)	42260 \pm 5445 (<i>n</i> =2)	45190 \pm 12110 (<i>n</i> =2)
MCF7- β -low (ER β 1)	70360 \pm 9004 (<i>n</i> =3)	38120 \pm 11210 (<i>n</i> =3)
MCF7- β -high (ER β 1)	98580 \pm 12350 (<i>n</i> =3)	37280 \pm 22850 (<i>n</i> =3)
MCF7- α -high (ER α)	108700 \pm 30630 (<i>n</i> =3)	49400 \pm 19180 (<i>n</i> =3)

of tagged-ER β to other subcellular compartments such as the mitochondria (Yang *et al.* 2004).

Effect of tagged-ER β 1 expression on ER α expression and E2 ligand-binding assays

Under conditions where the medium was depleted of oestrogen (5% CS as described in Materials and methods), little if any effect of tagged-ER β 1 expression on endogenous ER α expression was seen (Fig. 1B), and similar amounts of endogenous ER α were expressed in control and tagged-ER β 1 over-expressing cells. Protein loading was assessed by alpha-tubulin levels on the same blot (Fig. 1C). In order to determine the functional level of tagged-ER β 1 over-expression achieved relative to the endogenous level of ER (primarily due to ER α since only very low levels of ER β have been identified in these cells (Dotzlaw *et al.* 1997, Fuqua *et al.* 2003)) under these conditions, whole cell ligand-binding assays were carried out on cells grown in 5% CS with and without treatment for 48 h with 2 μ g/ml doxycycline. The results of several independent experiments are presented in Table 1. A significant increase in specific [3 H]E2 binding compared with the non-expressing controls (MCF7 and V) as well as the no doxycycline treatment controls was measured in all doxycycline-treated tagged-ER β 1 (β -high, β -low) and the α -high over-expressing cell lines. In addition, the levels of binding induced by doxycycline correlated with the level of tagged-ER β 1 induced by doxycycline as determined by Western blot analysis, β -high $>$ β -low (Fig. 1A). Doxycycline induction of tagged-ER α in the α -high cell line under these same conditions led to an increase in ligand binding similar to that obtained in β -high tagged-ER β 1 cells. These data were consistent with the increased E2 binding being due to the induced expression of tagged-ER β 1 or tagged-ER α after 48 h doxycycline treatment in 5% CS.

Treatment of cells containing doxycycline-induced ER β 1 with E2 (10 nM) for 24 h resulted in downregulation of the tagged-ER β 1 (Fig. 3A) in parallel to the expected downregulation of the endogenous ER α (Fig. 3B), as did treatment with ICI 182780 (500 nM). In

contrast, the tagged-ER β 1 was stabilized by 24 h of 4-OH-tamoxifen (500 nM) treatment (Fig. 3A). These data are similar to previously published data demonstrating differential effects of various SERMs on steady-state levels of ER α (Fig. 3B) (Wijayaratne & McDonnell 2001). The functional consequences of the various ligands were assessed by PR expression and are consistent with previous reports (Read *et al.* 1988, Aronica & Katzenellenbogen 1991). Signals obtained from Western blot analyses of time-course experiments of tagged-ER β normalized to the tubulin signals from the same blot were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). The half life of tagged-ER β using the line of best fit on a semi-log plot was estimated when bound to various SERMs. The estimated half-life of tagged-ER β 1 in the absence of E2 was 7.75 h, while in the presence of E2 it was 2.25 h. Treatment with 4-OH-tamoxifen stabilized the receptor, increasing its half-life to approximately 28.5 h, while treatment with ICI 182780 resulted in a decreased tagged-ER β 1 half-life compared with vehicle-treated controls. The estimated half-life of tagged-ER β 1 in the presence of ICI 182780 was 4.5 h.

Effect of tagged-ER β 1 expression on endogenous PR expression in MCF7 human breast cancer cells

To determine if tagged-ER β 1 expression affected known oestrogen-responsive endogenous targets, we investigated the level of expression of a known oestrogen-responsive gene product, PR. Under oestrogen-depleted conditions in MCF7 cells, the level of PR expression is markedly reduced and usually not detectable using our Western blot conditions. However, after doxycycline treatment, PR levels were significantly increased in all tagged-ER β 1 over-expressing clones (Figs 3B and 4). Furthermore, the increase in level of PR expression was correlated to the level of transgene expression in the different clones, β -high $>$ β -low (Fig. 4). When the cells were treated with E2 for 24 h following 48 h of doxycycline, there was a further induction of PR expression in all tagged-ER β 1-expressing clones (Fig. 3B

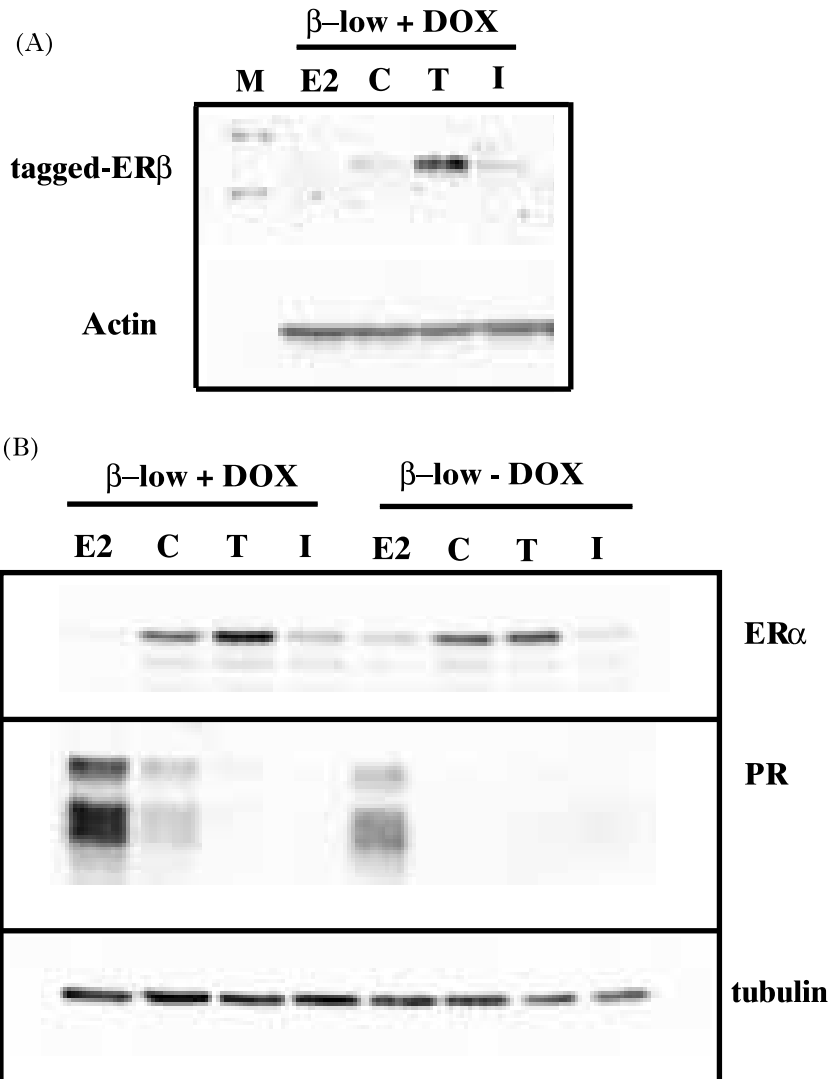


Figure 3 (A) Effect of oestrogen and anti-oestrogens on detection of tagged-ER β 1 in MCF7 cells in oestrogen-depleted medium. Upper panel visualized with anti-Xpress antibody; lower panel visualized with anti-actin antibody. E2=treated with 10 nM E2; C=vehicle (ethanol)-treated control; T=treated with 500 nM 4-OH-tamoxifen; I=treated with 500 nM ICI 182780. β -low=low ER β 1 expressor+doxycycline (DOX) for 48 h followed by +ligands for 24 h. (B) Effect of E2 and anti-oestrogens on endogenous ER α (top panel) and PR (middle panel) expression in the presence of over-expression of ER β 1 (+DOX) or no over-expression of ER β 1 (-DOX). Bottom panel visualized with anti-tubulin antibody. The same blot used for ER α was stripped and visualized with anti-PR antibody and then anti-tubulin antibody as described in Materials and methods. The results shown in this figure are from β -low treated (+DOX) or not treated (-DOX) for 48 h, followed by treatment+ligand for 24 h, as described above.

for β -low, data not shown for β -high) as well as the expected E2-induced PR expression in the non-tagged-ER β 1-expressing MCF7 control cells (data not shown). Since PR is regarded as a gold standard downstream marker of ER α activity we determined what effects upregulation of tagged-ER α (α -high) would have on PR expression. As shown in Fig. 4, increased tagged-ER α

(α -high) also resulted in an induction of PR expression found under these conditions. A further increase in PR expression was seen in α -high cells with E2 treatment as well (data not shown).

To determine if this apparently ligand-independent increase in PR following doxycycline-induced tagged-ER β 1 or tagged-ER α was due to effects at the

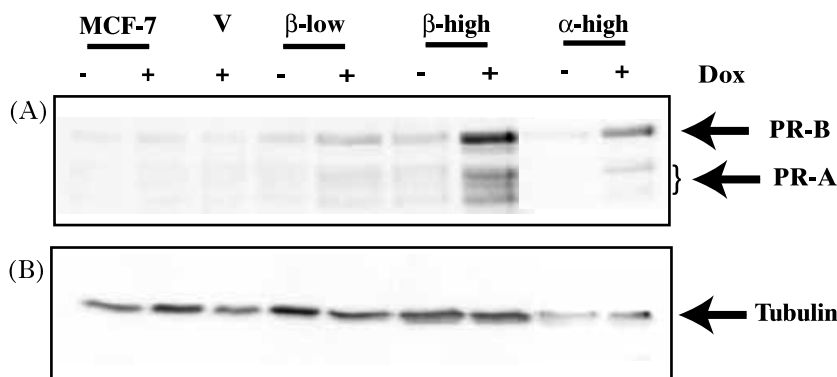


Figure 4 (A) Effect of tagged-ER expression (+ doxycycline (Dox) for 48 h) on endogenous PR expression in cells grown under oestrogen-depleted conditions. Western blot visualized with anti-PR antibody. MCF7 parent cells stably expressing rTA Tet-on transcription factor (Venditti *et al.* 2002); V=vector alone transfected MCF7 cells; β -low=Xpress-tagged ER β 1 low-expressing MCF7 clone; β -high=tagged-ER β 1 high-expressing MCF7 clone; α -high=GFP-tagged ER α high-expressing MCF7 clone. (B) Same blot as in (A), stripped and visualized with anti-tubulin antibody.

transcriptional level, PR mRNA was measured under the conditions described above. PR mRNA levels (determined by RT-PCR) were increased by $126 \pm 9\%$ in β -low, $148 \pm 7\%$ in β -high compared with $93 \pm 5\%$ in MCF7 control cells (means \pm S.E.M. or range, $n=2-7$) in a ligand-independent manner due to doxycycline induction of the tagged-ER β 1 expression. The increased PR mRNA level in the tagged-ER β 1-expressing cells was significant (ANOVA, $P=0.0014$), suggesting that the effect of ER β over-expression on PR protein level was, at least in part, due to altered steady-state levels of RNA, suggesting a transcriptional effect.

These data suggest that over-expression of ER β 1 does not universally inhibit the activity of the endogenous ER α , at least at the level of a known marker gene transcription in breast cancer cell lines.

Effect of tagged-ER β 1 expression on proliferation in MCF7 human breast cancer cells

When the different cell lines were grown in complete medium containing 5% FBS, induction of tagged-ER β 1 but not tagged-ER α inhibited the growth of the cells, compared with MCF7, the control parent cell line. Both β -high (DT+doxycycline, 1.84 ± 0.24 days, means \pm S.E.M., $n=6$; DT - doxycycline, 1.23 ± 0.09 days, $n=6$) and β -low (DT+doxycycline, 1.84 ± 0.30 days, $n=6$; DT - doxycycline, 1.34 ± 0.10 days, $n=6$) tagged-ER β 1 over-expressors showed a significant increase in DT (change in DT shown as the ratio of DT+doxycycline/DT - doxycycline, Fig. 5) in the presence of doxycycline than without (ANOVA, $P=0.002$, $n=6$) compared with no significant alterations in the parent (MCF7: (DT+doxycycline, 1.11 ± 0.13 days, $n=6$; DT - doxycycline, 1.11 ± 0.13 days) and α -high cells (DT+doxycycline,

1.32 ± 0.10 days, $n=6$; DT - doxycycline, 1.29 ± 0.10 days). Therefore over-expression of ER β 1 but not ER α can decrease the growth of MCF7 breast cancer cells in culture.

To determine the effect of over-expression of tagged-ER β 1 on the proliferation of MCF7 cells in the

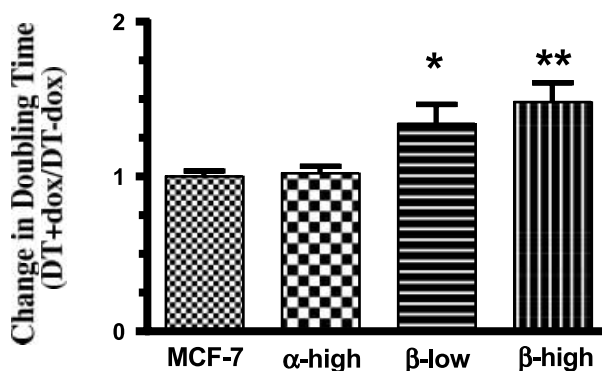


Figure 5 Effect of tagged-ER induction on proliferation of cells in 5% CM medium. Uninduced cells were set-up at 10^4 cells per well in 5% CM, followed by +doxycycline (dox) (2 μ g/ml). Media were changed every 2 days. Cells were harvested and counted electronically at various times after treatment (usually day 4 and day 8). DT were calculated as described in Materials and methods. The change in DT between no tagged-ER induction (-dox) and induction of tagged-ER (+dox) was calculated as a ratio. The means \pm S.E.M. of six independent experiments are shown. ANOVA ($P=0.002$) followed by *t*-testing indicates that the increase in DT seen in the β -low over-expressor and the β -high over-expressor is significantly different from that of the control parent cell line, MCF7 ($P=0.022$ and $P=0.004$ respectively); the over-expressing α -high cell line is not significantly different from the control parent cell line, MCF7; the β -low and the β -high over-expressors are significantly different from α -high (* $P=0.03$ and ** $P=0.005$).

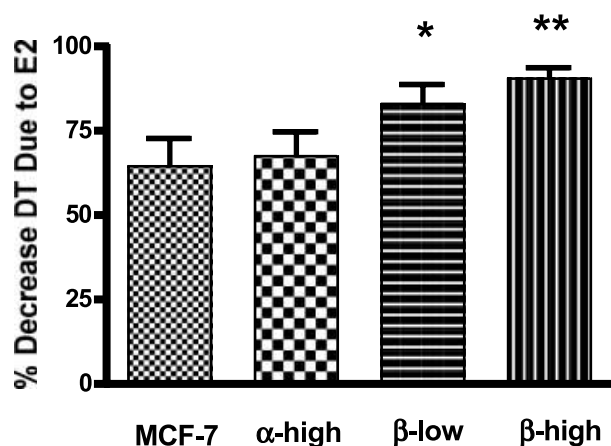


Figure 6 Effect of tagged-ER over-expression on growth of cells in response to oestrogen. Cells were depleted of oestrogen and set-up on day zero at 5×10^4 cells per well in 5% CS-containing medium, treated for 48 h with doxycycline and then treated with E2 (10 nM) or vehicle alone (ethanol). Cells were harvested and counted at the various times after E2 and medium was changed every 2 days. DT were calculated as described in Materials and methods. The % change in DT due to E2 treatment was calculated as $\text{DT-E2}/\text{DT-vehicle alone} \times 100$. The means \pm S.E.M. of $n=12$ (parent MCF7 cells, and ER α over-expressing cells= α -high) and $n=13$ (ER β 1 low over-expressor= β -low and ER β 1 high over-expressor= β -high) independent experiments is shown. Significant differences were found for all cell lines in the % decrease DT due to E2 treatment (ANOVA, $P < 0.0001$, followed by t -testing). In addition, differences between cell lines in % decreased DT due to E2 were found (ANOVA, $P = 0.012$) where β -low is significantly less than both MCF7 and α -high ($*P = 0.033$, $P = 0.049$ respectively); similar comparisons for β -high were $**P = 0.002$, $P = 0.003$. No significant differences were seen between MCF7 and α -high. DT (means \pm S.E.M. days, $n = 13-12$) in the presence of E2: MCF7, 1.75 ± 0.1 days; α -high, 1.62 ± 0.1 days; β -low, 1.83 ± 0.12 days; β -high, 2.04 ± 0.12 days. DT (means \pm S.E.M. days, $n = 13-12$) in the absence of E2: MCF7, 3.73 ± 0.81 days; α -high, 2.82 ± 0.36 days; β -low, 2.33 ± 0.19 days; β -high, 2.24 ± 0.13 days.

presence and absence of 10 nM E2, the cells were depleted of oestrogen as described in the Materials and methods section, treated with doxycycline for 2 days, followed by treatment with or without 10 nM E2 in the continued presence of doxycycline. All cell lines, including those expressing either tagged-ER β 1 or tagged-ER α , showed a statistically significant increase in their growth, i.e. a decrease in DT (ANOVA, $P < 0.0001$, $n = 12-13$, followed by t -tests) in response to 10 nM E2 (Fig. 6). However, those cell lines that over-express tagged-ER β 1 (either at a low or a high level) had a reduced response to E2 as determined by a reduced % decrease in DT in the presence of E2 (ANOVA, $P = 0.012$, $n = 12-13$, followed by t -tests). Both the low and high tagged-ER β 1 over-expressors were statistically significantly different from both the parent MCF7 and the high ER α over-expressor (α -high) controls (Fig. 6).

Effect of tagged-ER β 1 expression on the proliferative response of MCF7 human breast cancer cells to 4-OH-tamoxifen

An important hypothesis developed from basic studies on the function of ER β 1 using transient transfection and various reporter constructs is that upregulated expression of ER β 1 may result in tamoxifen resistance. Therefore in the inducible tagged-ER β 1 over-expressing cells the responsiveness to 4-OH-tamoxifen-induced growth inhibition was determined. We determined the effect of various concentrations of 4-OH-tamoxifen on the proliferation rate of β -high, β -low, α -high and MCF7 parent cell lines. Interestingly, with induction of tagged-ERs (+doxycycline) a biphasic dose-response was observed in the β -high and β -low cells, but not in α -high or MCF7 control cells (Fig. 7A). At low 4-OH-tamoxifen concentrations (< 50 nM), a significant stimulation of proliferation rate was observed (ANOVA, $P < 0.0001$, $n = 5$). This increase in proliferation rate was higher in β -high compared with β -low cells (ANOVA, $P < 0.0001$, $n = 5$). At higher concentrations (> 50 nM), growth was inhibited in all cell lines (Fig. 7A). Comparing the growth inhibition phase, both β -high and β -low were significantly more sensitive to 4-OH-tamoxifen than either α -high (ANOVA, $P < 0.0001$) or MCF7 parental cells (ANOVA, $P < 0.0001$). Both β -high and β -low dose-response curves were significantly to the left of both α -high and MCF7 curves. However, β -low was more sensitive than the β -high so a dose-dependent effect of tagged-ER β 1 expression was not observed for growth inhibition. The biphasic growth response to 4-OH-tamoxifen was also not found in MCF7 cells inducibly over-expressing the tagged-C-terminally truncated ER β variant, ER β 2/cx (Ogawa *et al.* 1998a). In contrast to the tagged-ER β 1, the tagged-ER β 2/cx over-expressing MCF7 cells only showed a growth inhibition in response to 4-OH-tamoxifen (data not shown). Together, these data support the specificity of the biphasic growth response to tagged-ER β 1 over-expression in multiple independent clones.

In the absence of tagged-ERs ($-$ doxycycline, Fig. 7B), there was a complete loss of the 4-OH-tamoxifen-induced growth stimulation in β -low and β -high. In addition, lack of tagged-ER expression (no doxycycline) eliminated any significant differences in sensitivity to 4-OH-tamoxifen growth inhibition between the β -high, β -low and α -high, but not the parental MCF7 cell line (Fig. 7B).

Discussion

During human breast tumourigenesis and breast cancer progression a marked alteration of oestrogen action occurs. First, the responsiveness of ER+ breast tumour cells to the proliferative effect of oestrogen is direct

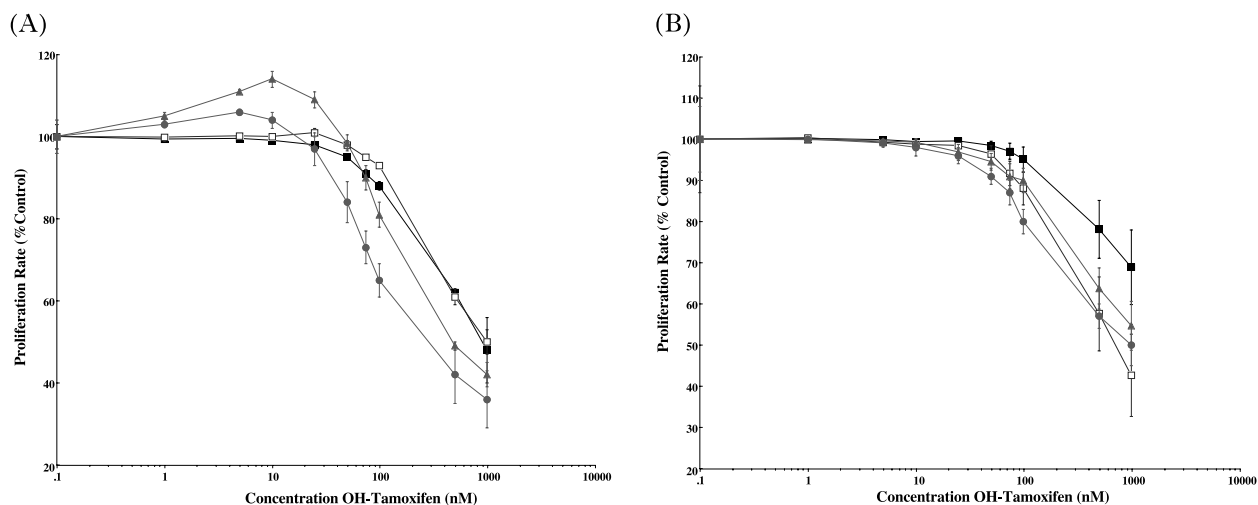


Figure 7 (A) Effect of tagged-ER over-expression (+doxycycline) on 4-OH-tamoxifen sensitivity in MCF7 breast cancer cells. Cells were grown in 5% CM and set-up on day zero at 10^4 cells per well in 5% CM-containing medium, treated for 48 h with doxycycline and then various concentrations of 4-OH-tamoxifen or vehicle alone. Cells were harvested and counted electronically at various times after anti-oestrogen treatment. DT and proliferation rates were calculated as described under Materials and methods, and the means \pm S.E.M. of $n=5$ (for 4-OH-tamoxifen concentrations 1–75 nM) and $n=3$ (for 4-OH-tamoxifen concentrations 100–1000 nM) independent experiments with results expressed as proliferation rate % of control (ethanol alone-treated cells). Medium was changed every 2 days. Solid squares are parental control cells, MCF7 clone 89 (rTA, Tet-on transcription factor expressor only). Open squares are α -high over-expressing MCF7 cells. Solid circles are β -low over-expressing MCF7 cells. Solid triangles are β -high over-expressing MCF7 cells. (B) 4-OH-tamoxifen sensitivity in MCF 7 breast cancer cell lines in the absence of tagged ER over-expression (–doxycycline). Cells were grown as described above except that they were not treated with doxycycline. Cells were harvested and counted electronically at various times after anti-oestrogen treatment. DT and proliferation rates were calculated as described under Materials and methods, and the means \pm S.E.M. of $n=3$ independent experiments with results expressed as proliferation rate % of control (ethanol alone-treated cells). Medium was changed every 2 days. Solid squares are parental control cells, MCF7 (rTA, Tet-on transcription factor expressor only). Open squares are α -high over-expressing MCF7 cells. Solid circles are β -low over-expressing MCF7 cells. Solid triangles are β -high over-expressing MCF7 cells.

(Lippman & Bolan 1975, Clarke *et al.* 1997), in contrast to normal breast epithelial cells where the growth effects of oestrogen appear to be indirect (Anderson *et al.* 1998). Secondly, the expression of ERs is markedly altered between normal breast tissue and breast tumours (Leygue *et al.* 1998a, Roger *et al.* 2001, Murphy & Watson 2002). ER α is up-regulated in ER+ tumours relative to normal breast epithelium whereas current data suggest that ER β is downregulated in breast tumours compared with normal breast epithelium (Leygue *et al.* 1998a, Roger *et al.* 2001). Since there are both *in vitro* and *in vivo* studies (Hall & McDonnell 1999, Weihua *et al.* 2000, Forster *et al.* 2002, Peng *et al.* 2003) suggesting that ER β may be a negative modulator of ER α action, it has been speculated that the downregulation of ER β during breast tumourigenesis removes a modulatory factor of ER α that is important for maintaining normal sensitivity of breast epithelial cells to oestrogen action. However, despite the general decreased expression of ER β in ER+ breast tumours compared with normal tissue, many breast tumours continue to express ER β and the levels of expression can vary widely amongst tumours (Dotzlaw *et al.* 1997,

Jarvinen *et al.* 2000). And although still controversial, often when ER β protein is determined, its expression can be correlated with good prognostic markers such as ER α and PR expression (Jarvinen *et al.* 2000, Murphy *et al.* 2002, Saunders *et al.* 2002, Skliris *et al.* 2003). This is also consistent with data suggesting that higher ER β expression is associated with sensitivity to tamoxifen therapy rather than resistance to tamoxifen therapy. However, other data have suggested ER β association with anti-oestrogen resistance and poor prognostic markers (Speirs *et al.* 1999, Speirs 2002). The development of an inducible tagged-ER β 1-expressing MCF7 breast cancer cell line has allowed us to address the functional outcome of altering the relative expression of ER β 1 to ER α in the ‘gold standard’ model of oestrogen/anti-oestrogen-responsive human breast cancer. The development of such breast cancer cell line models is highly relevant to human breast cancer *in vivo*, since the majority of human breast tumours (~59%) have been shown to co-express both ER α and ER β 1 (Murphy *et al.* 2003).

Consistent with studies in which stable over-expression of ER β 1 alone in cell lines was investigated, a

predominantly nuclear location of the transfected derivative ER β 1 (in our case epitope tagged) was observed (Lazennec *et al.* 2001). We have isolated various clones of the tetracycline-inducible tagged-ER β 1 that under maximal induction conditions express low and high levels of the tagged-ER β 1 protein. Using the ligand-binding assay, we have determined that in low ER β clones the tetracycline analogue doxycycline increased specific binding of radiolabelled E2 around 200% that of the non-induced (no doxycycline treatment) and/or non-expressing control cells, while doxycycline treatment of the high ER β 1-expressing clones resulted in around a 300% increase in specific E2 binding over controls. Since parental MCF 7 cells contain predominantly ER α , the control and uninduced levels of ligand binding reflect predominantly endogenous ER α levels. After doxycycline treatment, ER β was induced to less than or equimolar levels (estimating from ligand-binding assays) to the endogenous ER α in the β -low clones, suggesting that the majority of the ER β would be in the form of heterodimers with ER α (Cowley *et al.* 1997) in these clones. However, this depends on the kinetics of dimer formation *in vivo* and hence some homodimers of ER α may also exist. In β -high, the tagged-ER β 1 was induced to higher levels than β -low clones, suggesting that all the ER α molecules may be heterodimerized with ER β 1, with the possibility that a small population of ER β 1 homodimers may also exist. Although *in vitro* ER α -ER β 1 heterodimer is apparently favoured (Cowley *et al.* 1997) it is unclear if this is the case *in vivo*. However, recently it was demonstrated using Fluorescence resonance energy transfer (FRET) analysis in living cells that ER homodimers of each receptor and ER α -ER β 1 heterodimers can occur *in vivo* independently of the presence of ligand (Bai & Giguere 2003), supporting the presence in our cell line models of ER β 1 predominantly in the form of ER α -ER β 1 heterodimers. Little, if anything, however is known about the differential function of the various homo- and heterodimers of the ER isoforms, although the models described in this study may be useful in addressing such questions.

ER β 1 over-expression alone in the absence of ER α -negative MDAMB231 human breast cancer cells has resulted in variable results with both growth inhibition (Lazennec *et al.* 2001) and growth stimulation being reported (Tonetti *et al.* 2003). However, when over-expression of ER β 1 in an ER α -positive background has been achieved, more consistent results have been obtained, and in particular over-expressed ER β 1 results in growth inhibition (Paruthiyil *et al.* 2004, Strom *et al.* 2004). Our results are consistent with these latter data in that ER β 1 but not ER α over-expression results in reduced growth and increased DT when cells are grown in complete medium containing FBS and oestrogen. In addition, over-expression of ER β 1 but not ER α reduced the magnitude of oestrogen-induced growth when cells are grown in oestrogen-depleted medium. These effects

of ER β 1 on the proliferation of ER α -expressing breast cancer cells are dose-dependent since smaller effects were seen in the low ER β 1-expressing cells compared with the high ER β 1-expressing cells. These data are consistent with the hypothesis that ER β 1 can negatively modulate the activity of ER α when the two ERs are co-expressed. They also suggest that the downregulation of ER β 1 in breast tumours compared with normal breast tissue which altered the relative expression of the two ERs is functionally involved in breast tumourigenesis at least for ER+ breast cancer.

However, negative modulation of ER α activity is not a universal effect of over-expression of ER β 1 since, in contrast to the effect on E2-induced proliferation, ER β 1 over-expression significantly increased endogenous PR expression, previously considered a specific downstream marker of ER α activity in breast cancer cells. Again, in contrast to the growth effects, the increase in PR expression is not ER β 1 specific since over-expression of ER α as seen in α -high cells similarly increased PR expression. These results support a mechanistic connection associated with the observation *in vivo* where increased ER β expression determined at the protein level in human breast tumours is often correlated with PR status and expression (Jarvinen *et al.* 2000, Mann *et al.* 2001, Murphy *et al.* 2002). Therefore ER β 1 regulation of PR expression has *in vivo* physiological and/or pathophysiological relevance. Interestingly, the effect of ER over-expression on PR expression occurs apparently without added ligand (E2) suggesting ligand independence, although further increases in PR expression were seen when the over-expressed ER was bound to E2 (ligand dependent). Indeed, since the majority of human breast tumors arise in older, and therefore likely postmenopausal women, one could speculate that PR expression in many breast tumors may reflect the ligand-independent activity of the combination of ER α and ER β 1 expressed in the tumour. Alternatively, oestrogen generated due to aromatase expression by tumor cells as well as the surrounding stroma would also contribute to the steady-state PR expression in breast tumours. However, we cannot exclude the possibility that trace amounts of oestrogens are still present in the medium and contribute to the increased PR expression. The molecular mechanism by which ER β 1 may regulate PR remains to be determined, although it is due, at least in part, to increased transcription since steady-state PR RNA levels were also increased. However, since ERE half-sites adjacent to both AP-1 and Sp1 sites have been previously implicated in the mechanism by which ER α regulates PR expression, it is speculated that these may also be involved in the mechanism by which ER β 1 can regulate PR expression. However, if the AP-1 site is important in this regard, this would be an example of E2 activation of ER β 1 being involved in increased rather

than decreased transcription of an AP-1-regulated oestrogen responsive promoter. We cannot however exclude effects on PR mRNA stability at this stage.

The involvement of ER β 1 in tamoxifen sensitivity is controversial, and in a previous study when inducible over-expression of ER β 1 was investigated after stable expression in T-47D breast cancer cells, which also express endogenous ER α , only growth inhibition due to tamoxifen was observed (Strom *et al.* 2004). No stimulation of growth was reported. However, full dose-response analyses covering low and high concentrations of 4-OH-tamoxifen were not undertaken (Strom *et al.* 2004). To the best of our knowledge, our data are the first to show any effect of altered ER β 1 expression on tamoxifen-regulated proliferation in breast cancer cells. In the presence of oestrogen-replete (phenol-red and FBS-containing) medium, a biphasic growth response to 4-OH-tamoxifen was observed only in tagged-ER β 1 over-expressing cells. Neither the parental control nor the tagged-ER α over-expressing cells showed a biphasic response. The biphasic growth response was lost when tagged-ER β 1 over-expression was not induced (no doxycycline), and was not present when the tagged-ER β variant, ER β 2-cx, was over-expressed under the same conditions. The growth stimulatory response seen at low doses of 4-OH-tamoxifen was only seen when tagged-ER β 1 was over-expressed, its magnitude was correlated with the level of ER β 1 and was completely eliminated in the absence of ER β 1 induction (i.e. no doxycycline). These data suggest that tagged-ER β 1 expression was functionally involved in the 4-OH-tamoxifen stimulation of proliferation seen at low doses of the anti-oestrogen. When considering the growth inhibition phase, doxycycline treatment of all cell lines except α -high significantly moved the dose-response curves to the left, compared with the no doxycycline treatment, i.e. no induction of expression of the tagged-ER. This suggested that a higher ER β 1 but not ER α increased sensitivity to the growth-inhibitory effects of 4-OH-tamoxifen. However, removal of doxycycline also reduced the sensitivity of the MCF7 control cells to 4-OH-tamoxifen, suggesting that clonal variation may also in part contribute to, but not entirely explain, the differences seen in sensitivity to the growth-inhibitory, but not the growth-stimulatory effects of tamoxifen in these cell lines. In particular, there was no evidence that ER β 1 over-expression resulted in a shift of the dose-response curve to the right of any of the control cells, supporting the conclusion that acute over-expression of ER β 1 is not associated with resistance or decreased sensitivity to tamoxifen. Overall, these data support the hypothesis that increased expression of ER β 1 but not over-expression of ER α in the presence of endogenous expression of ER α increases the sensitivity of human breast cancer cells to the growth-modulatory effects of tamoxifen.

Our results showing that ER β 1 over-expressing cells display an increased sensitivity to tamoxifen are consistent with increased expression being associated with tamoxifen sensitivity and not resistance *in vivo* in breast cancer patients (Mann *et al.* 2001, Murphy *et al.* 2002, Esslimani-Sahla *et al.* 2004, Myers *et al.* 2004). The stimulation of proliferation due to 4-OH-tamoxifen seen at low doses is interesting and we would argue that rather than be interpreted as representing a potential form of tamoxifen resistance (Clarke *et al.* 2003), since it only occurs at very low concentrations (tamoxifen resistance occurs *in vivo* at varying periods of time following or during high doses of tamoxifen), it is likely to resemble and/or be a model of 'tamoxifen flare' that has been observed clinically (Plotkin *et al.* 1978, Mortimer *et al.* 2001, Biersack *et al.* 2004). In this condition, an apparent worsening of the disease is seen in some patients in the first few weeks of starting tamoxifen treatment; likely before sufficiently high steady-state concentrations of tamoxifen and its active metabolites have accumulated. This is often a favourable sign for responsiveness to tamoxifen therapy since, in the cases where tamoxifen therapy was continued, subsequent tumour regression often occurred (Plotkin *et al.* 1978, Vogel *et al.* 1995). Interestingly, only one other breast cancer cell culture model of 'tamoxifen flare' has been documented previously. In this case the cell line was T-47D and several laboratories have often described a relatively higher natural level of ER β expression in T47-D cells compared with other ER+ breast cancer cell lines. The mechanism by which ER β 1 may increase the sensitivity of breast cancer cells to tamoxifen is unknown, but the higher relative binding affinity of ER β 1 compared with ER α for 4-OH-tamoxifen may contribute in part (Kuiper *et al.* 1997).

In conclusion, our data using inducible, ER β 1 over-expressing MCF7 cells do not support the hypothesis that up-regulation of ER β 1 in the presence of ER α is a mechanism of anti-oestrogen resistance *per se*. However, we cannot exclude a role of over-expression of ER β in resistance in combination with other changes, such as altered cofactor expression and/or activity. In addition, our data support the hypothesis that ER β 1 can be a negative modulator of ER α -mediated growth response since over-expression of ER β in a dose-dependent fashion inhibits the magnitude of the E2-induced growth response in ER+ human breast cancer cells. However, over-expression of ER β 1 does not inhibit or reduce all ER α -regulated activities since endogenous PR expression is not decreased in these cells. Indeed, ER β 1 upregulates the endogenous expression of PR in possibly both a ligand-independent and -dependent fashion. It is acknowledged that due to space limitations our interpretation of the data shown here is likely too narrow since it underestimates the complexity of the variables (only one of which is relative

expression of ER isoforms) involved in the response to E2 as previously demonstrated (Hewitt *et al.* 2003, Stossi *et al.* 2004). The parallels seen with our over-expressing ER β 1 MCF7 cell lines and some *in vivo* studies showing a relationship between PR and ER β expression as well as ER β expression and tamoxifen sensitivity of breast cancer patients suggest that these models are relevant and useful for dissecting the role of ER β 1 expression in ER+ breast cancer.

Acknowledgements

We thank Christina Walker for her technical assistance. This work was supported by the Canadian Breast Cancer Research Alliance and the USA Medical Research and Materiel Command. AL holds a CancerCare Manitoba Foundation/Guardian Angel Studentship Award. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 25 November 2004

Accepted 17 December 2004