Endocrine therapy resistance can be associated with high estrogen receptor α (ERα) expression and reduced ERα phosphorylation in breast cancer models

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Abstract

Hormone-dependent estrogen receptor (ER)-positive breast cancer cells may adapt to low estrogen environments such as produced by aromatase inhibitors. In many instances, cells become insensitive to the effects of estrogen but may still retain dependence on ER. We have investigated the expression, function, and activation of ERα in two endocrine-resistant MCF-7 models to identify mechanisms that could contribute to resistance. While MCF-7/LCC1 cells are partially estrogen dependent, MCF-7/LCC9 cells are fully estrogen insensitive and fulvestrant and tamoxifen resistant. In both MCF-7/LCC1 and MCF-7/LCC9 cell lines, high expression of ERα was associated with enhanced binding to the trefoil factor 1 (TFF1) promoter in the absence of estrogen and increased transcription of TFF1 and progesterone receptor. In contrast to the observations derived from hypersensitive and supersensitive models, these cells were truly estrogen independent; nevertheless, removal of ERα by siRNA, or fulvestrant, a specific ER downregulator, inhibited growth indicating dependence on ERα. In the absence of estrogen, neither ERα Ser118 nor Ser167 were phosphorylated as frequently found in other ligand-independent cell line models. Addition of estrogen activated ERα Ser118 in MCF-7 and LCC1 cells but not in LCC9 cells. We suggest that the estrogen-independent growth within these cell lines is accounted for by high levels of ERα expression driving transcription and full estrogen independence explained by lack of ERα activation through Ser118.

Endocrine-Related Cancer (2006) 13 1121–1133

Introduction

Estrogen receptor α (ERα) is a major growth regulator for many breast cancers and has provided an exploitable target for therapy (Ali & Coombes 2002). Estrogen binding to ERα promotes conformational changes in the receptor leading to dimerization and attachment to DNA, generally at the site of conserved estrogen response elements in the promoter regions of target genes (Ali & Coombes 2002). Functional regulation of ERα is additionally mediated via phosphorylation of key residues in the activation function 1 (AF-1) domain of ERα including Ser118 and Ser167 and these influence both DNA binding and recruitment of cofactor molecules (reviewed in Lannigan 2003). The activation of ER involves crosstalk with other growth factor-signaling pathways. There is extensive evidence that activation of the mitogen-activated protein kinase (MAPK)-signaling cascade and the phosphoinositol 3 kinase (PI3-K) pathway phosphorylate ERα at Ser118 and Ser167, via extracellular signal-regulated kinase (ERK)1/2 and Akt respectively (Bunone et al. 1996, Martin et al. 2000, Lannigan 2003). Transcriptional activation of ERα then involves a dynamic process where large transcription complexes incorporating co-activator proteins are assembled in an ordered and combinatorial manner...
Well-defined estrogen-regulated genes include trefoil factor 1 (TFF1)/pS2 (Masiakowski et al. 1982, Jakowlew et al. 1984) and progesterone receptor (PGR; Nardulli et al. 1988).

While tamoxifen has been the established form of treatment for ER-positive breast cancers for more than 20 years, other anti-estrogen strategies, notably aromatase inhibitors (Johnston & Dowsett 2003) and selective estrogen downregulators (SERDs), are increasingly being used (Robertson 2002). Despite initial responsiveness to these agents, most tumors eventually recur with acquired resistance (Clarke et al. 2001, 2003). Multiple mechanisms, dependent on the form of endocrine treatment, are involved in the development of resistance and, in many cases, these mechanisms remain unclear. During the acquisition of endocrine resistance, progressive changes are frequently observed, with ER-positive breast cancer cells progressing in a stepwise manner from a fully estrogen-sensitive phenotype to an estrogen-sensitive, but no longer dependent phenotype, to a fully resistant phenotype (Clarke et al. 2001, 2003).

With the increasing clinical use of aromatase inhibitors, such as letrozole, anastrazole, and exemestane which act by inhibiting estrogen synthesis (Johnston & Dowsett 2003), there has been great interest in how breast cancer cells can adapt to low estrogen environments and become resistant to the effects of these drugs. In most cases of acquired anti-estrogen resistance, expression of ERα is retained, suggesting that resistance involves either changed functionality or bypass of the receptor. Culturing breast cancer cells in estrogen-low conditions to produce long-term estrogen deprivation (LTED) has identified mechanisms of estrogen hypersensitivity and estrogen supersensitivity (Yue et al. 2002, Martin et al. 2003, 2005a,b, Santen et al. 2005). Estrogen hypersensitivity is characterized by the ability of cells to respond to levels of estrogen at concentrations 2–3 log lower than required to stimulate wild-type cells (Yue et al. 2002, Santen et al. 2005). This mechanism involves increased expression of ERα alongside enhanced phosphorylation of ERα Ser118 and is associated with activation of the ERK1/2 and PI3-K pathways. Estrogen supersensitivity, wherein cells are apparently estrogen independent, is a mechanism again associated with enhanced ERα expression, ERK activation, and activation of ERα Ser118 and involves ERα being supersensitized by growth factor activation (Martin et al. 2003, 2005a).

While higher levels of ERα expression are generally associated with enhanced estrogen response, in certain cases tumors expressing high levels of ERα can be insensitive to endocrine manipulation. High levels of ERα expression have been associated with increased proliferation rates (Black et al. 1983) and poor prognosis in breast cancer patients not receiving adjuvant therapy (Black et al. 1983, Thorpe et al. 1993). It has been suggested that a high level of ERα may lead to constitutive activation (Fowler et al. 2004). This mechanism has recently been demonstrated by Fowler et al. (2004, 2006) in a tetracycline-inducible ERα expression model of the MCF-7 cell line, wherein increased ERα expression resulted in aberrant promoter occupancy and gene activation in the absence of estrogen. The increased receptor activity required the amino-terminal domain and was not inhibited by tamoxifen, supporting the notion of AF-1 activation, yet was independent of Ser104/106 and Ser118 phosphorylation (Fowler et al. 2004).

In these models, the expression of ERα is still critical to the response and it has been suggested that use of a SERD such as fulvestrant (faslodex, ICI 182 780) would be a beneficial strategy once resistance to aromatase inhibitors has developed (Johnston et al. 2005, Martin et al. 2005b). A number of laboratories are developing models of resistance to this agent to identify strategies that might be tried at the onset of resistance (Dowsett et al. 2005, Howell 2005, Johnston et al. 2005, Martin et al. 2005b, Nicholson et al. 2005, Normanno et al. 2005).

We have investigated two MCF-7 cell lines (MCF-7/LCC1 and MCF-7/LCC9), which have acquired estrogen insensitivity and with variable sensitivity to tamoxifen and fulvestrant to identify novel mechanisms of endocrine resistance that might arise in clinical specimens. The wild-type ER-positive MCF-7 breast cancer cell line is both estrogen dependent and responsive to anti-estrogens, such as tamoxifen and fulvestrant. The MCF-7/LCC1 (LCC1) cell line was derived from an MCF-7 xenograft, which had grown in a low estrogen environment in an immuno-deprived mouse and which was known to be estrogen independent but with a degree of estrogen sensitivity (Brunner et al. 1993). Treatment of the cell line with fulvestrant produced the MCF-7/LCC9 (LCC9) cell line which is fully resistant to both estrogen and fulvestrant (Brunner et al. 1997). A number of novel features of these lines were identified within this study and are reported here.
Materials and methods

Cell proliferation

MCF-7 cells were routinely grown in phenol red containing Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 (g/ml). LCC1 and LCC9 cells (source: Dr Robert Clarke, V T Lombardi Cancer Research Center, Georgetown University Medical School, Washington, DC, USA) were routinely kept in phenol-free containing DMEM supplemented with 5% dextran-activated charcoal-stripped fetal calf serum (DCC), penicillin (100 units/ml), streptomycin (100 (g/ml), and 2 mM glutamine. All cells were grown at 37 °C in 5% CO2. To determine the effects of 17β-estradiol (E2) and tamoxifen on cell proliferation, MCF-7 cells were seeded in six-well plates in phenol red containing DMEM with 10% fetal bovine serum (FBS) for 24 h. The media were changed to phenol red-free DMEM with 5% DCC for 48 h. The cells were then supplemented with media containing either 1 nM E2, 1 μM tamoxifen or both. LCC1 and LCC9 cells were seeded in six-well plates in phenol red-free containing DMEM with 5% DCC and after 24 h supplemented with E2 and/or tamoxifen. Cell growth was evaluated using a Coulter counter. Fulvestrant was a kind gift from Dr Alan Wakeling (AstraZeneca, Macclesfield, Cheshire, UK). For studies exploring growth in DMEM without serum, the sulforhodamine-B (SRB) colorimetric assay was used.

Briefly, log phase cells were seeded into 96-well flatbottom tissue culture plates. The following day, cells were washed in PBS and media replaced with phenol red-free DMEM for 48 h. Cells were then treated with concentrations of E2 varying from 10 fM to 1 μM in the absence or presence of 100 nM fulvestrant. After 72 h, plates were removed from the incubator and ice-cold 25% trichloroacetic acid (TCA) solution (50 μl) added to each well. All plates were placed on ice for 60 min after which the TCA solution was removed. The plates were washed under running water and dried prior to staining with SRB dye solution (30 min at room temperature) and the trays were washed with 1% glacial acetic acid (×4) at room temperature, air-dried, and resuspended in 10 mM Tris buffer (pH 10.5; 150 μl) before reading at 540 nm.

RNA extraction and RT-PCR

Extraction of total RNA from whole cells was performed using Tri-Reagent (Sigma) as per the manufacturers’ instructions. RNA concentration was measured using a spectrophotometer. QuantiTect SYBR Green system (Qiagen, cat no. 204243) was used according to the manufacturers instructions for one step RT-PCR in a total of 15 μl reaction volumes, including 0.5 μM each primer and 40 ng RNA. Real-time cycler conditions were RT: 50 °C for 30 min; PCR: initial activation 95 °C for 15 min followed by 40 cycles of denaturation 94 °C for 15 s, annealing 57 °C for 30 s, extension 72 °C for 30 s, and a final extension of 72 °C for 60 s. The following primers were used:

TFF1: fwd TTGTGGTTTCTCTTGGTGTA 
rev CCGAGCTCCTGGACTAATCA 
ERα: fwd CCACACACAGTGCACATT 
rev GTCTTCTCGTATCCACCTTTC 
PGR: fwd GTCAGTGGGCGATGCTGTA 
rev ACGCCCTCACAGGAATT 
ACTIN: fwd CTACGTGGCGCTGGACTT 
rev GATGGAGGCCGCGATCCACGG

Western analysis

Cells were washed twice with PBS and lysed in ice-cold lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, aprotenin 10 μg/ml, and 1× protease cocktail inhibitor (Roche) for 10 min and the debris was cleared by centrifugation at 13 000 r.p.m. for 6 min at 4 °C). Protein lysates (100 μg) were resolved on 7.5–12% SDS-PAGE and electrophoretically transferred to Immobilon-P membranes. After transfer, membranes were blocked and probed with primary antibody overnight at 4 °C. Immunoreactive bands were detected using chemiluminescent reagents (ECL or SuperLuminol) and photographic paper (Hyperfilm, Amersham). The following antibodies were used: ERα (F-10; Santa Cruz Biotech, Santa Cruz, CA, USA sc-8002), PGR (ab-8; Neomarkers, Stratage Scientific Ltd, Newmarket, Suffolk, UK (MS-298)), P-ERK1/2 (1:1000, Cell Signaling, New England Biolabs, Hitchin, Herts, UK #9101), phospho-Ser118 ERα (1:500, Cell Signaling #2511), phospho-Ser167 ERα (1:500, Cell Signaling #2514), and actin (1:120 000, CP01, Calbiochem, La Jolla, CA, USA). Integrated optical density absorbance values were obtained by densitometric analysis using a gel scanner and analyzed by ‘Labworks’ gel analysis software (UVP Life Sciences, Cambridge, UK).

Chromatin immunoprecipitation assays (ChIP)

Cells were grown to 85–90% confluence in phenol red-free DMEM with 5% DCC for at least 48 h. Cells were
cross-linked with 1% formaldehyde (37 °C for 10 min) at 10-min interval over a 90-min time course. Unreacted formaldehyde was quenched by gentle agitation at room temperature for 10 min with 0.125 M glycine. Cells were then washed twice with ice-cold PBS, collected into PBS containing protease inhibitors (Roche), and centrifuged for 4 min at 2000 r.p.m. at 4 °C. The pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), and 1× protease inhibitor cocktail), incubated on ice for 10 min, and sonicated (12×20 s at two amplitude microns, Soniprep 150, MSE) to fragment DNA to ∼500 bp. Following centrifugation for 15 min at 13 000 r.p.m. and 4 °C, supernatants were collected and resuspended in dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate). Precipitates were then washed twice with 0.1 M NaHCO3 and 1% SDS. Heat with TE buffer and the protein/DNA complexes were washed sequentially for 5 min each at 4 °C overnight reversed formaldehyde cross-links. DNA fragments were purified using QIAquick Spin Kit columns (Qiagen, cat no. 204242). TFF1 PCR conditions were: initial activation of 95 °C for 15 min followed by 45 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min. TFF1 primer sequences: fwd GACGGAATGGGCTTCATGAGC and rev CTGAGACAATAATCTCCACTG. For the distal region, primers were: fwd GAGTTTGGCCTCCACATTA and rev CTTGCCCTGCTTCTTCTCC.

Short interfering (siRNA) transfections

MCF-7 cells were seeded at 0.5×10⁶ cells per T75 flask in DMEM with 5% DCC for 24 h prior to transfection. Cells were transfected with siRNA for 4 h using Oligofectamine reagent (Invitrogen) after which time 1 nM E2 was added for a further 48 h prior to RNA and protein extraction. For the 7-day time course, the media were left unchanged after the initial changes. For siRNA transfections were carried out as described earlier but scaled down for 24-well plates. Following siRNA treatment for 4 h, cells were treated with 1 nM E2 or 100 nM fulvestrant or a combination and cell counts on days 0, 3, and 6 were estimated using a Coulter counter. The following siRNA sequences were used: ER RNAi 1; ESR1 SMARTpool (four pooled sequences; Upstate Biotechnology, Lake Placed, NY, USA; M-003401; 100 nmol), ER RNAi 2; 5'-AACAGAGGAAGAGCTGCCA (Ambion; 40 nmol), ER RNAi 3; 5'-AACCTGGGCTGTGCTCTTT (Ambion, Huntingdon, Cambridgeshire, UK; 40 nmol), and negative RNAi: Upstate (D-001206; 100 nmol).

Results

Increased ERα expression in resistant cell lines

To explore the possibility that high ERα expression leads to estrogen-independent growth in endocrine-resistant cells, the expression levels of ERα in resistant lines (LCC1 and LCC9) were compared with levels in wild-type MCF-7 cells. Both resistant lines expressed between four- and elevenfold more ERα mRNA than wild-type cells (Fig. 1A). ERα protein levels were clearly elevated in LCC1 cells relative to MCF-7 cells (sevenfold) and less markedly in LCC9 cells (Fig. 1B). E2 decreased ERα protein in MCF-7 cells at 48 h and this has been explained by proteosomal degradation, a process speculated to limit the action of estrogen signaling (Nawaz et al. 1999; Fig. 1C). Similarly, both resistant lines demonstrated ERα turnover, suggesting that ERα is binding to E2 in all cases. In contrast, tamoxifen treatment results in maintenance of the receptor expression levels in all three cell lines (Fig. 1C).

Addition of 1 nM 17β-estradiol (E2) to MCF-7 cells produced a marked stimulation of growth to cells cultured in estrogen-depleted (double charcoal-stripped FCS) medium (Fig. 2A). In the absence of E2, MCF-7 cells are essentially static (Fig. 2A). In contrast, LCC1 cells grow rapidly in estrogen-depleted conditions and show an approximately twofold stimulation of growth on addition of E2 (Fig. 2B). LCC9 cells showed a lack of response to E2, again

![Image](https://via.placeholder.com/150.png?text=Endocrine+therapy+resistance+in+breast+cancer)
growing very rapidly in the absence of E2 (Fig. 2C). Addition of 1 mM tamoxifen to MCF-7 cells antagonized the E2-stimulated growth in this cell line. Tamoxifen also inhibited the E2-stimulated growth of LCC1 cells but had no effect on LCC9 cells (Fig. 2B and C). These results are consistent with wild-type cells being estrogen dependent, LCC1 cells demonstrating partial estrogen dependence and LCC9 cells being fully estrogen independent.

**Reduced ERα Ser^{118} phosphorylation in LCC9 cells**

Several frequently cited mechanisms of estrogen-independent activation of ERα involve phosphorylation of ERα at the Ser^{118} or Ser^{167} residues mediated via ERK or Akt respectively (Bunone et al. 1996, Martin et al. 2000, Lannigan 2003). While the Ser^{118} residue is a major site of E2-induced phosphorylation, Ser^{167} is not (Lannigan 2003). The latter site is activated by growth factor signaling. In view of these previous observations, we first investigated whether ERα Ser^{118} or Ser^{167} phosphorylation were increased in the absence of estrogen in the resistant cell lines. Neither was there evidence of increased Ser^{118} phosphorylation in the resistant lines relative to MCF-7 under basal conditions, nor was Ser^{167} phosphorylation increased (Fig. 3A–C). Furthermore, phospho-ERK1/2 expression was unchanged in the lines (Fig. 3C). On E2 addition, there was a marked increase in Ser^{118} phosphorylation in MCF-7 cells and this was also observed in the LCC1 cell line (Fig. 3A and B). However, minimal change was observed on E2 addition to LCC9 cells (Fig. 3A and B). Ser^{118} phosphorylation has been proposed to affect cofactor recruitment and this might explain the reduced
transcriptional (as mentioned below) and growth responses observed on E2 addition to this cell line. Tamoxifen alone produced a small increase in Ser118 phosphorylation in MCF-7 and LCC1 cells but not in LCC9 cells (Fig. 3A and B). Tamoxifen also produced a reduction of estrogen’s Ser118 phosphorylation in the MCF-7 and LCC1 cell lines (Fig. 3A and B).

**Modified DNA binding of ERα in resistant cell lines**

To explore whether high ERα expression was reflected in enhanced DNA binding in the absence of E2, ChIP methodology was used to examine ERα binding to the promoter of the E2-responsive gene TFF1 in the MCF-7, LCC1, and LCC9 cell lines. LCC9 cells had 2.5-fold greater ERα binding to the TFF1 promoter than MCF-7 cells (Fig. 1D). However, this binding was significantly higher in LCC1 cells with levels greater than eightfold above MCF-7 cells. This enhanced ERα binding in LCC1 cells was equivalent to the increased expression of ERα protein and is consistent with the suggestion by Fowler et al. (2004) that enhanced ERα protein expression can lead to increased DNA binding. As a control, binding to a region 3.5 kb distal to this region indicated only background levels as expected (Fig. 1D).

**Growth responses to estrogen and tamoxifen in the wild-type and variant cell lines are reflected in transcriptional changes**

To investigate the differences in estrogen and anti-estrogen activation processes, indicator genes that reflected the different growth responses were next investigated. Transcriptional changes in the estrogen-regulated genes TFF1 and PGR were measured and modulated expression was compared with the growth changes.

Expression of TFF1 mRNA in the absence of E2 was higher in both resistant lines compared with MCF-7 cells (Fig. 4A). After 48-h E2 (1 nM) treatment, TFF1 mRNA was increased by >20-fold in MCF-7 cells, but only one-to twofold in the resistant lines although this increase was significant. Tamoxifen (1 μM) produced a small increase in TFF1 expression in MCF-7 and LCC1 cells but not in the LCC9 cell line (Fig. 4A). These levels broadly reflect the growth differences observed.

The expression of PGR mRNA in the absence of E2 was greater in LCC1 and LCC9 lines compared with MCF-7 cells (Fig. 4B). As for TFF1, after 48-h E2 treatment, PGR mRNA was increased by >20-fold in MCF-7 cells and 2–5-fold in LCC1 and LCC9 cell lines (Fig. 4B). Tamoxifen also increased the PGR mRNA
expression level not only in MCF-7 cells, but also in LCC1 cells producing effects equivalent to that of E2 in the latter cell line. No change was observed in the LCC9 cell line.

These results are consistent with transcription of TFF1 and PGR being increased by ligand-independent mechanisms in LCC1 and LCC9 cell lines with estrogen and tamoxifen producing an additional ligand-dependent increase.

**Effect of removal of ERα on the growth of the cell lines**

To determine the relative importance of ERα on downstream gene expression and growth of MCF-7, LCC1, and LCC9 cells, we investigated the effects of removing ERα, either by specific siRNA inhibition of receptor synthesis or through inhibition and degradation of the receptor by fulvestrant.

A panel of interfering RNAs (siRNAs) were initially compared for their ability to transiently reduce ERα expression and were transfected into the MCF-7 cell line. RNAi 1 is a pooled set of four targeted sequences (Imai et al. 2005) while RNAi 2 (5'-AACAGGGAG-GAAAGAGCTGCCA) and RNAi 3 (5'-AACCT-CGGGCTGTGCTCTTTT) are individually targeted sequences (Leu et al. 2004). Of the three, RNAi 2 produced the best reduction of ERα mRNA and protein and was selected for further experiments (Fig. 5A and B). Quantitative RT-PCR analysis showed that, 48 h after transfection, ERα RNAi 2 treatment resulted in an 85% decrease in ERα mRNA expression and an 87% decrease in the presence of E2 (Fig. 5C). LCC1 and LCC9 cells have significantly higher basal expression of ERα mRNA and siRNA removal caused an 82 and 73% decrease respectively with similar reductions in the presence of E2 (Fig. 5C). Western analysis of the MCF-7 and LCC1 cell lines demonstrated that RNAi 2 produced ERα protein knockdown over a 7-day period (Fig. 5D) and it was effective in all three cell lines (Fig. 5E). This reduction in ERα protein was accompanied by a decrease in PGR protein (Fig. 5E). Thus, it appeared that gene expression in all three cell lines was ERα dependent.
This was investigated further using fulvestrant. Fulvestrant abrogates E2-induced gene transcription by binding, blocking, and causing the degradation of ERα (Parker 1993). Fulvestrant treatment in MCF-7 cells blocked E2-induced expression of TFF1 and PGR (Fig. 4A and B). In addition, ligand-independent and E2-induced TFF1 and PGR expression in LCC1 cells were reduced on fulvestrant treatment. These data confirm that for LCC1 cells TFF1 and PGR induction are dependent on ERα expression. However, LCC9 cells are resistant to fulvestrant treatment and as such no change in TFF1 expression and only a minor change in PGR expression was observed. The effect of fulvestrant on the growth of all three cell lines was also investigated in the complete absence of serum (Fig. 6). Under these conditions, MCF-7 cells did not grow over a 72-h period. LCC1 cells, however, still proliferated and the addition of E2 had little effect on growth confirming their independence of E2. Under these conditions, fulvestrant was able to oppose the effect of low concentrations of E2 again indicating dependence on ERα. In contrast, LCC9 cells were completely insensitive to both E2 and fulvestrant. Fulvestrant degraded ERα protein in all three lines which is shown in Fig. 7A.

To determine how critical levels of ERα expression were for the growth of MCF-7, LCC1, and LCC9 cell lines, we used RNAi removal with or without fulvestrant to inhibit the synthesis of ERα protein (Fig. 7B–D). E2-induced MCF-7 cell growth was significantly decreased (33%) by ERα removal and abolished by all combinations of fulvestrant alone or with RNAi. LCC1 cells grew in the absence of E2 and RNAi removal had only a minor effect on growth. E2-induced LCC1 cell growth was reduced by approximately 40% when ERα was removed through RNAi, but, unlike MCF-7 cells, fulvestrant alone was not enough to abolish growth – this, however, could be accomplished though through combination with RNAi. LCC9 cell growth in the absence of E2 was reduced by ERα RNAi. A similar decrease was observed in the presence of E2. LCC9 cells are fulvestrant resistant and no effect on growth was observed with this agent. No combinations of fulvestrant or RNAi were able to totally abolish growth. These results indicate a varying degree of dependence on ERα for growth in the three cell lines.

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**Figure 4** Effect of estrogen, tamoxifen, and fulvestrant on (A) TFF1 expression and (B) PGR mRNA expression in the cell lines. Relative mRNA expression values of TFF1 and PGR in the cell lines were measured by real-time RT-PCR using specific primer pairs. RNA was collected at 48 h and was extracted from either untreated (control) cells or cells treated with 1 nM E2, 1 μM tamoxifen, 1 nM E2+1 μM tamoxifen, 100 nM fulvestrant, or 1 nM E2+100 nM fulvestrant. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars = s.d. Statistical significance noted for treatment groups compared to matched control where *P<0.05, untreated control versus treatment group; †P<0.05, E2 control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test).
Discussion

Aromatase inhibitors are now used for the adjuvant treatment of most hormone receptor-positive early breast cancer. Despite the improvement they offer over tamoxifen alone, recurrences still occur, and thus models of resistance to both tamoxifen and estrogen deprivation are required. The series of MCF-7-derived cell lines provides an excellent model system for the exploration of mechanisms of stepwise acquisition of resistance to tamoxifen and estrogen deprivation. Most models to date have been derived in vitro, which makes LCC1 cells interesting as the initial estrogen deprivation was achieved in vivo and therefore might reflect features that could arise in a primary breast cancer (Brunner et al. 1993). In many of the in vitro-derived LTED models, acquired resistance is due to enhanced sensitization to low concentrations of estrogen, which often involves crosstalk with growth factor-signaling pathways (Martin et al. 2003, 2005a,b). LCC1 cells have certain of the characteristics of the LTED phenotype (Yue et al. 2002, Martin et al. 2003, 2005a,b, Santen et al. 2005) such as a higher expression level of ERα, an ability to grow in low-estrogen conditions and elevated TFF1 expression. The continuous culturing of LCC1 cells in low estrogen conditions may well contribute to the increased expression of ERα in this cell line.

However, unlike most LTED-derived cells, which show little response to physiological levels of estrogen yet are sensitive to very low levels of estrogen, LCC1 cells appear truly insensitive to the addition of low levels of exogenous estrogen. Similarly, while most LTED cells show basal activation of ERK1/2 activation and ERα via Ser118 phosphorylation, LCC1 and
LCC9 cells do not. The ER, however, is still clearly functional in LCC1 cells and linked to growth regulation as estrogen addition can produce an increase in growth which could be reversed by tamoxifen. ERα is also downregulated by the addition of estrogen and markedly phosphorylated at Ser118. Additionally, the ERα downregulator fulvestrant reduces expression of TFF1 and inhibits growth. These effects are more marked when cells are exposed to fulvestrant with siRNA removal of ERα.

While constitutive activation of ERα may be achieved in some instances by phosphorylation of Ser118 mediated by growth factor-driven activation of ERK, an increased expression of ERα alone might account for increased DNA binding. In support of this, there was enhanced binding of ERα to the TFF1 promoter in the absence of added estrogen in both the LCC1 and LCC9 cell lines. In addition, TFF1 transcription was markedly increased in the resistant cell lines consistent with this enhanced ERα-binding driving transcription. Direct support for such a mechanism has recently been demonstrated in an MCF-7 cell line using a tetracycline-inducible ERα overexpression model (Fowler et al. 2004, 2006). As with the data mentioned earlier, the results suggested that elevated levels of ERα resulted in activation of receptor transcriptional function in a manner distinct from mechanisms that involve ligand binding or growth factor-induced phosphorylation of the Ser104, Ser106 or Ser118 sites. The mechanism required the amino-terminal A/B domain and was not inhibited by tamoxifen. It was also uncoupled from ERK activation. The hypothesis proposed was that overexpression of unliganded ERα stabilized interactions with the basal transcriptional machinery, which at normal receptor levels may be too weak to support effective transcription (Fowler et al. 2004).

These results together support a model wherein growth (and TFF1 transcriptional activation) in LCC1 cells is dependent on ERα. This dependency has some ligand (i.e., estrogen) responsiveness but is largely ligand independent. The ligand-dependent component may be reversed by tamoxifen. The ligand independence appears to involve neither growth factor activation via the Ser118 or Ser167 phosphorylation routes nor hypersensitization (where low levels of estrogen produce apparent independence). Instead the ligand independence appears to be explained by the high level of ER expression leading to constitutive activation and promoting DNA binding and transcriptional activation.

We have shown that ERα is functionally active in the LCC1 model and since this has also been shown in models demonstrating LTED, a logical clinical strategy to attempt after development of resistance in a low estrogen environment (such as produced by aromatase inhibitor treatment) is to downregulate the receptor using fulvestrant (Johnston et al. 2005, Martin et al. 2005a,b). This strategy clearly is effective at inhibiting growth in LCC1 cells. However, the LCC9 variant was derived after exposure and development of resistance to fulvestrant (Brunner et al. 1997) and showed no growth response to either estrogen or tamoxifen. In this cell line, the negligible changes of
ERα Ser118 phosphorylation obtained on estrogen or tamoxifen addition contrasted with observations in the other cell lines. Markedly reduced phosphorylation is likely to affect cofactor binding and our initial findings suggest that p160 binding (specifically AIB1) is reduced in this cell line, again consistent with endocrine insensitivity (Kuske et al. 2004). However, it is quite clear that fulvestrant can downregulate the receptor and even extremely high levels of fulvestrant (10 μM) were unable to influence growth (data not shown). Despite this, siRNA removal of ERα produced some growth inhibition suggesting a reduced but still measurable dependency on ERα.

In conclusion, these results suggest that multiple changes contribute to endocrine resistance. While ER still demonstrates functionality in LCC1 cells, there is a major shift to ligand independence. This independence can be explained by the high level of ER expression found in these cells and could lead to constitutive activation of the receptor. These cells still show a degree of dependency on estrogen and this can be blocked by tamoxifen. Further changes were produced by exposure and development of resistance to fulvestrant including a loss of ERα Ser118 activation, which could account for its loss of sensitivity to estrogen. These data support the view that in the early stages of resistance, SERDs may provide a useful therapeutic option, but other approaches will be required when resistance has developed to these agents.

Acknowledgements

The authors gratefully acknowledge support from Cancer Research UK for this study. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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