Endocrine therapy resistance can be associated with high estrogen receptor α (ER α) expression and reduced ER α 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 ER α 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 ER α 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 ER α by siRNA, or fulvestrant, a specific ER downregulator, inhibited growth indicating dependence on ER α . In the absence of estrogen, neither ER α Ser¹¹⁸ nor Ser¹⁶⁷ were phosphorylated as frequently found in other ligand-independent cell line models. Addition of estrogen activated ER α 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 ER α expression driving transcription and full estrogen independence explained by lack of ER α activation through Ser¹¹⁸.

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Introduction

Estrogen receptor α (ER α) is a major growth regulator for many breast cancers and has provided an exploitable target for therapy (Ali & Coombes 2002). Estrogen binding to ER α 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 ER α is additionally mediated via phosphorylation of key residues in the activation function 1 (AF-1) domain of ER α 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 ER α 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 ER α then involves a dynamic process where large transcription complexes incorporating co-activator proteins are assembled in an ordered and combinatorial manner

(Glass & Rosenfeld 2000, Metivier *et al.* 2003). 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, anastrozole, 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 ER α 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 ER α alongside enhanced phosphorylation of ER α 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 ER α expression, ERK activation, and activation of ER α Ser¹¹⁸ and involves ER α being supersensitized by growth factor activation (Martin *et al.* 2003, 2005a).

While higher levels of ER α expression are generally associated with enhanced estrogen response, in certain cases tumors expressing high levels of ER α can be insensitive to endocrine manipulation. High levels of ER α 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 ER α 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 ER α expression model of the MCF-7 cell line, wherein increased ER α 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 Ser^{104/106} and Ser¹¹⁸ phosphorylation (Fowler *et al.* 2004).

In these models, the expression of ER α 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 17 β -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 μ M 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 μ M 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 μ l) 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 ($\times$ 4) at room temperature, air-dried, and resuspended in 10 mM Tris buffer (pH 10.5; 150 μ l) 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 μ l reaction volumes, including 0.5 μ M 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 TTGTGGTTTTCTGGTGTCA
rev CCGAGCTCTGGGACTAATCA
ER α : fwd CCACCAACCAGTGCACCATT
rev GTCTTTCCGTATCCACCTTTC
PGR: fwd GTCAGTGGGCAGATGCTGTA
rev AGCCCTTCCAAAGGAATT
ACTIN: fwd CTACGTCGCCCTGGACTTCGAGC
rev GATGGAGCCGCCGATCCACACGG

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, aprotinin 10 μ g/ml, and 1 $\times$ 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 μ g) 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: ER α (F-10; Santa Cruz Biotech, Santa Cruz, CA, USA sc-8002), PGR (ab-8; Neomarkers, Stratech Scientific Ltd, Newmarket, Suffolk, UK (MS-298)), P-ERK1/2 (1:1000, Cell Signaling, New England Biolabs, Hitchin, Herts, UK #9101), phospho-Ser¹¹⁸ ER α (1:500, Cell Signaling #2511), phospho-Ser¹⁶⁷ ER α (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, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, and 1× protease inhibitor cocktail). Chromatin were precleared with 1 µg anti-rabbit or anti-mouse IgG, 2 µg sheared salmon sperm DNA, and Protein-G-Agarose (50 µl of 50% slurry in dilution buffer) for 3 h at 4 °C. Immunoprecipitation using Protein-G-Agarose Beads (Roche) was performed overnight at 4 °C with anti-ERα HC-20 antibody (sc-543, Santa Cruz). Beads were washed sequentially for 5 min each at 4 °C with TSE I (20 mM Tris (pH 8.1), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), TSE II (20 mM Tris (pH 8.1), 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, and 0.1% SDS), and buffer III (10 mM Tris (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% NP40, and 1% deoxycholate). Precipitates were then washed twice with TE buffer and the protein/DNA complexes were eluted twice with 0.1 M NaHCO₃ and 1% SDS. Heat treatment at 65 °C overnight reversed formaldehyde cross-links. DNA fragments were purified using QIAquick Spin Kit columns (Qiagen) and amplified using the QuantiTect SYBR Green system (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 GAGTTTGGCCTCC-CACATTA and rev CTTGCCTCTGCATTCTCTCC.

Short interfering (siRNA) transfections

MCF-7 cells were seeded at 0.5×10^6 cells per T75 flask in DMEM as mentioned previously. After 24 h, the media were changed to phenol red-free containing DMEM with 5% DCC for 48 h. LCC1 and LCC9 cells were seeded directly into phenol red-free containing

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 growth assays, cells were seeded as for growth characterization as mentioned previously. 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'-AAACAGGAGGAA-GAGCTGCCA (Ambion; 40 nmol), ER RNAi 3; 5'-AACCTCGGGCTGTGCTCTTTT (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 17 β-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

Figure 1 ER α expression in MCF-7, LCC1, and LCC9 cells. (A) ER α mRNA expression. Cells were grown in charcoal-stripped serum-containing medium for at least 48 h and RNA was collected. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate RT-PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars = s.d. Statistical significance noted for treatment groups versus matched control (one-way ANOVA and multiple comparison Tukey–Kramer test; $*P < 0.05$). (B) Western blot analysis of ER α (66 kDa) in breast cancer cell lines grown in charcoal-stripped serum-containing medium for 48 h prior to protein collection. One hundred micrograms of protein were loaded per lane and detected using either anti-ER α (Santa Cruz Biotech) or anti-actin (Calbiochem) antibodies as described in Materials and methods. (C) Western blot analysis of ER α (66 kDa) in breast cancer cell lines grown in charcoal-stripped serum-containing medium for at least 48 h prior to protein collection. One hundred micrograms of protein were loaded per lane and detected using either anti-ER α (Santa Cruz Biotech) or anti-actin (Calbiochem) antibodies as described in Materials and methods. (D) ER α binding to the TFF1 promoter. Basal recruitment of ER α to the TFF1 promoter was determined by ChIP analysis on untreated cells. The ChIP method used was as described in Materials and methods and immunoprecipitated TFF1 promoter was quantified by real-time PCR. The input-corrected expression values were determined by normalizing to the inputs. Data are presented as mean $\pm$ s.e. Groups were compared with the Kruskal–Wallis test with Dunn’s multiple comparison test ($*P < 0.05$). Binding to the promoter region is compared with binding to a region 3.5 kb distal to the promoter wherein only background binding was observed.

growing very rapidly in the absence of E₂ (Fig. 2C). Addition of 1 μ M 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 ER α Ser¹¹⁸ phosphorylation in LCC9 cells

Several frequently cited mechanisms of estrogen-independent activation of ER α involve phosphorylation of ER α at the Ser¹¹⁸ or Ser¹⁶⁷ residues mediated via ERK or Akt respectively (Bunone *et al.* 1996, Martin *et al.* 2000, Lannigan 2003). While the Ser¹¹⁸

residue is a major site of E₂-induced phosphorylation, Ser¹⁶⁷ is not (Lannigan 2003). The latter site is activated by growth factor signaling. In view of these previous observations, we first investigated whether ER α Ser¹¹⁸ or Ser¹⁶⁷ phosphorylation were increased in the absence of estrogen in the resistant cell lines. Neither was there evidence of increased Ser¹¹⁸ phosphorylation in the resistant lines relative to MCF-7 under basal conditions, nor was Ser¹⁶⁷ 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 Ser¹¹⁸ 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). Ser¹¹⁸ phosphorylation has been proposed to affect cofactor recruitment and this might explain the reduced

Figure 2 Growth characterization of MCF-7 and MCF-7 variant cells. (A) MCF-7 cells, (B) LCC1, and (C) LCC9 cells were plated for 24 h and maintained in reduced media for 48 h before treatment. Cells were then left untreated (control group), treated with 1 nM E₂, 1 μM tamoxifen or 1 nM E₂ and 1 μM tamoxifen. Cells were counted on day 0 (72 h after plating/day of treatment start) and days 2/4/6 using a Coulter counter. Mean cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars=s.d. A representative experiment is shown of at least four experiments carried out.

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

Figure 3 Effects of E₂ and tamoxifen on ERα phosphorylation in the resistant cell lines. (A) Western analysis of ERα phospho-Ser¹¹⁸ after 30-min treatment with control (no treatment), 1 nM E₂, 1 μM tamoxifen, or 1 nM E₂ and 1 μM tamoxifen. Lysates were run on a 10% SDS gel and membranes probed with anti-phospho ERα Ser¹¹⁸ antibody (1:1000). Lysates were also probed for actin expression to compare protein loading. (B) Histogram representing optical densities from triplicate western blots of ERα phospho-Ser¹¹⁸ after 30-min treatment with control (no treatment), 1 nM E₂, 1 μM tamoxifen, or 1 nM E₂ and 1 μM tamoxifen. Values were actin corrected and then standardized to a control sample. The control sample was a 30 min E₂-treated MCF-7 sample and was used on all gels as a standard to allow comparisons between runs. Statistical comparisons were made with each cell line's control level; **P* < 0.01; [†]*P* < 0.001 (ANOVA). (C) Western analysis of ERα phospho-Ser¹⁶⁷ and phospho-ERK1/2 in cell lines. Untreated lysates were probed with antibodies specific for ERα phospho-Ser¹⁶⁷ and phospho-ERK1/2. A positive control lane of MCF-7 cells treated with 1 nM HRGβ was used. Lysates were also probed for actin expression.

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'-AAACAGGAG-GAAGAGCTGCCA) 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.

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 μ M tamoxifen, 1 nM E₂ + 1 μ M 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).

This was investigated further using fulvestrant. Fulvestrant abrogates E₂-induced gene transcription by binding, blocking, and causing the degradation of ER α (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 ER α 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 ER α . In contrast, LCC9 cells were completely insensitive to both E₂ and

fulvestrant. Fulvestrant degraded ER α protein in all three lines which is shown in Fig. 7A.

To determine how critical levels of ER α 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 ER α protein (Fig. 7B–D). E₂-induced MCF-7 cell growth was significantly decreased (33%) by ER α 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 ER α was removed through RNAi, but, unlike MCF-7 cells, fulvestrant alone was not enough to abolish growth – this, however, could be accomplished through combination with RNAi. LCC9 cell growth in the absence of E₂ was reduced by ER α 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 ER α for growth in the three cell lines.

Figure 5 Effects of ER α RNAi on ER α and PGR expression in the cell lines. (A) Expression of ER α mRNA after treatment with a range of ER α mRNA-targeted RNAis. ER α mRNA expression was measured by quantitative RT-PCR of mRNA from MCF-7 cells 48 h after RNAi treatment in the presence of 1 nM E₂. Data are presented as mean $\pm$ s.d. of actin-corrected values from triplicate samples. The RNAi transfection method and RNAi sequences used are described in Materials and methods. Statistical significance noted for treatment groups compared with matched control where $*P < 0.05$, untreated control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test). (B) Western analysis of ER α protein expression in MCF-7 cells 48 h after siRNA treatment. ER α was probed with the F-10 antibody and actin is shown as a loading control. (C) Expression of ER α mRNA after treatment with RNAi 2. ER α mRNA expression was measured by quantitative RT-PCR of mRNA from cell lines 48 h after RNAi 2 treatment in the presence or absence of 1 nM E₂. Data are presented as mean $\pm$ s.d. of actin-corrected values from triplicate samples. The RNAi transfection method and RNAi sequences used are described in Materials and Methods. Statistically significant differences are noted for treatment groups compared with matched control where $*P < 0.05$, untreated control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test). (D) Western analysis time course of the effect of RNAi 2 treatment on ER α protein expression in the MCF-7 and LCC1 cell lines. ER α was probed with the F-10 antibody and actin is shown as a loading control. (E) Western analysis of ER α and PGR protein expression in the cell lines 48 h after siRNA treatment. ER α was probed with the F-10 antibody, PGR with Ab 8 and actin is shown as a loading control.

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 ER α , 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 ER α 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 ER α via Ser¹¹⁸ phosphorylation, LCC1 and

Figure 6 Effect of the growth of the cell lines in serum-free media and treated with varying concentrations of E_2 in the absence or presence of fulvestrant (1 nM). Cells were plated and after establishment placed in serum-free medium for 48 h. E_2 with or without fulvestrant was added and plates left for 72 h. Relative cell numbers were then assessed by SRB assay as described in Materials and methods. Inset in MCF-7 figure: effect of E_2 on MCF-7 cells grown in 5% double charcoal-stripped fetal serum.

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. ER α is also downregulated by the addition of estrogen and markedly phosphorylated at Ser¹¹⁸. Additionally, the ER α downregulator fulvestrant reduces expression of TFF1 and inhibits growth. These effects are more marked when cells are exposed to fulvestrant with siRNA removal of ER α .

While constitutive activation of ER α may be achieved in some instances by phosphorylation of

Ser¹¹⁸ mediated by growth factor-driven activation of ERK, an increased expression of ER α alone might account for increased DNA binding. In support of this, there was enhanced binding of ER α 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 ER α -binding driving transcription. Direct support for such a mechanism has recently been demonstrated in an MCF-7 cell line using a tetracycline-inducible ER α overexpression model (Fowler *et al.* 2004, 2006). As with the data mentioned earlier, the results suggested that elevated levels of ER α 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 ER α 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 ER α . 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 ER α 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

Figure 7 Effect of fulvestrant on ER α expression and combined with ER α siRNA on the growth of the cell lines. (A) Western blot analysis of ER α (66 kDa) in breast cancer cell lines in control, 1 nM E₂, 100 nM fulvestrant, or 1 nM E₂ and 100 nM fulvestrant-treated groups at 48 h. One hundred micrograms of protein were loaded per lane and detected using anti-ER α (Santa Cruz Biotech) antibody as described in Materials and Methods. Actin expression is also shown. (B–D) Effects of fulvestrant, ER α siRNA, or combinations on the growth of the cell lines. (B) MCF-7, (C) LCC1, and (D) LCC9 cells were treated with 1 nM E₂, 100 nM fulvestrant, 40 nM ER α siRNA, or combinations of these. siRNA treatment was for 4 h only, while E₂ and fulvestrant were present throughout the time course. Comparisons are made with the negative siRNA control which gave an equivalent growth effect to no treatment. Data are presented as mean $\pm$ s.e. from quadruplicate samples. Statistical significance noted for treatment groups compared with matched control where * P < 0.05, negative RNAi control versus treatment group; † P < 0.05, negative RNAi + E₂ control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test).

ER α 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 μ M) were unable to influence growth (data not shown). Despite this, siRNA removal of ER α produced some growth inhibition suggesting a reduced but still measurable dependency on ER α .

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 ER α 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.

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