ERBB2 influences the subcellular localization of the estrogen receptor in tamoxifen-resistant MCF-7 cells leading to the activation of AKT and RPS6KA2

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Abstract

Acquired resistance to endocrine therapies remains a major clinical obstacle in hormone-sensitive breast tumors. We used an MCF-7 breast tumor cell line (Tam^R-1) resistant to tamoxifen to investigate this mechanism. We demonstrate that Tam^R-1 express elevated levels of phosphorylated AKT and MAPK3/1-activated RPS6KA2 compared with the parental MCF-7 cell line (MCF-7). There was no change in the level of total ESR between the two cell lines; however, the Tam^R-1 cells had increased phosphorylation of ESR1 ser^{167}. SiRNA blockade of AKT or MAPK3/1 had little effect on ESR1 ser^{167} phosphorylation, but a combination of the two siRNAs abrogated this. Co-localization studies revealed an association between ERBB2 and ESR1 in the Tam^R-1 but not MCF-7 cells. ESR1 was redistributed to extranuclear sites in Tam^R-1 and was less transcriptionally competent compared with MCF-7 suggesting that nuclear ESR1 activity was suppressed in Tam^R-1. Tamoxifen resistance in the Tam^R-1 cells could be partially overcome by the ERBB2 inhibitor AG825 in combination with tamoxifen, and this was associated with re-localization of ESR1 to the nucleus. These data demonstrate that tamoxifen-resistant cells have the ability to switch between ERBB2 or ESR1 pathways promoting cell growth and that pharmacological inhibition of ERBB2 may be a therapeutic strategy for overcoming tamoxifen resistance.

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Introduction

Estrogens classically exert their effects by binding to the estrogen receptor (ESR1) inducing a conformational change followed by hyperphosphorylation and dimerization of the receptor. Estradiol (E_2)-bound ESR1 interacts with estrogen response elements (ERE) regulating transcription on target genes that control cell proliferation and survival. This knowledge has been exploited clinically by the development of endocrine therapies that reduce E_2 activity either by blocking its biosynthesis using aromatase inhibitors or competing with E_2 for the ESR1 using anti-estrogens such as the selective ESR1 modulator tamoxifen. Tamoxifen has been the most commonly prescribed drug over the last 20 years both for treatment of advanced disease and in early breast cancer as adjuvant therapy impacting on disease free and overall survival (Cancer Trialists' Collaborative Group 1998). Unfortunately a large proportion of women (40%) will relapse with acquired endocrine-resistant disease.

Multiple causal events have been associated with endocrine resistance including loss of ESR1 (Gutierrez *et al.* 2005), selection of ESR1 mutants (Cui *et al.* 2004, Herynk *et al.* 2007), altered intracellular pharmacology (Johnston *et al.* 1993), crosstalk between the type I tyrosine kinase growth factor receptors resulting in ligand-independent activation of the ESR1 (Kato *et al.* 1995, Bunone *et al.* 1996) or hypersensitization to residual estrogens (Jeng *et al.* 1998, Shim *et al.* 2000,

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Chan *et al*. 2002, Martin *et al*. 2003). Increased growth factor signaling via EGFR has been associated with resistance to endocrine therapy (de Cremoux *et al*. 2003, Knowlden *et al*. 2003, Fan *et al*. 2007) while elevated ERBB2 has been implicated with perturbation of the interaction of ESR1 with transcriptional co-repressors (Kurokawa *et al*. 2000). Altered expression of coactivators such as NCOA3 and associated amplification of ERBB2 have been shown to predict a poor response to tamoxifen treatment (Osborne *et al*. 2003).

Recent studies have suggested that regulation of cell cycle by ESR1 does not solely rely on ESR1's genomic activity as a nuclear transcription factor. Rather that rapid effects resulting in the activation of MAPK3/1 in response to E_2 are attributed to non-genomic interactions of ESR1 at the plasma membrane (Migliaccio *et al*. 1996). For instance, studies suggest that ESR1 is capable of associating with an SHC/IGFR complex leading to elevated MAPK3/1 activity (Song *et al*. 2004). *In vitro* studies suggest that amplified ERBB2 or expression of EGFR may alter the physical location of ESR1 resulting in an accumulation in the cytoplasm as opposed to the nucleus (Yang *et al*. 2004, Fan *et al*. 2007). These observations suggest that interplay between these mechanisms provides a high degree of plasticity with the potential to generate these resistant phenotypes. Hence, further characterization of these mechanisms and their contribution to endocrine resistance is critical for a rational approach for the design of new therapeutic strategies. To address this, we have characterized an MCF-7 cell line, which has acquired resistance to the inhibitory effects of tamoxifen. We show that an ERBB2/ESR1 membrane-associated complex leads to non-genomic activation of both RPS6KA2 and AKT, which in turn provides these cells with a survival advantage. We demonstrate for the first time that the cell line shows a high degree of plasticity with the ability to drive proliferation independently, via ERBB2- or ESR1-driven signaling pathways. We also demonstrate that treatment with an inhibitor of ERBB2 phosphorylation only in the presence of tamoxifen leads to a reduction in proliferation, abrogation of cell signaling, and redistribution of ESR1 into the nucleus. These data provide further support for the combination of signal transduction inhibitors with endocrine agents as a therapeutic approach.

Materials and methods

Reagents

All cell culture media and serum were obtained from Invitrogen unless otherwise stated. The AKT inhibitor SH6 was obtained from Alexis (Nottingham, UK) and the MAP2K inhibitor, U0126 was purchased from Promega. SiRNA SMARTpool AKT1, siRNA SMARTpool MAPK3/1 (MAPK1), and non-specific control pool siRNA oligonucleotides were obtained from Dharmacon (Thermo Fisher Scientific, Loughborough, Leicestershire, UK).

Antibodies for western blotting

Primary antibodies were obtained from: New England Biolabs, Hitchin, Hertfordshire, UK (phospho-AKT (Ser^{473}) and (Thr^{308}), AKT, phospho-Raf (Ser^{259}), phospho-MAP2K1/2 ($Ser^{217/221}$), MAPK3/1, phospho-RPS6KA2 (Ser^{380}), phospho-ESR1 (Ser^{167}), phospho-ESR1 (Ser^{118}), phospho-Bad (Ser^{112}), and (Ser^{136}), Bad), Upstate Biotechnology, Lake Placid, NY, USA (ESR2, phospho-ERBB2, ERBB2, phospho-EGFR, and EGFR), Thermo Fisher Scientific, Neomarkers (ERBB3 and ERBB4), Leica Biosystem, Novocastra, Newcastle Upon Tyne, UK (ESR1, PGR), Sigma (phospho-MAPK3/1, actin), and Santa Cruz Biotechnology, Santa Cruz, CA, USA (IGF1Rβ, ESR1, ERBB2, PARP1). Secondary HRP-conjugated antibodies were from Amersham Pharmacia Biotech, UK.

Cell culture

MCF-7 cells were maintained in Phenol red-free DMEM/Ham's F12 (1:1 v/v) supplemented with 1% fetal bovine serum (FBS) and 6 ng/ml insulin referred to as 1% FBS medium. The level of estradiol in 1% FBS containing medium was quantified by RIA (Dowsett *et al*. 1987) and was routinely less than 3 pmol/l. The tamoxifen-resistant cell lines Tam^R-1 (Lykkesfeldt *et al*. 1994) and Tam^R-4 (Madsen *et al*. 1997) were maintained in the same medium plus 1 μM tamoxifen (Sigma). SKBR3 and BT474 cells were maintained in phenol red-containing RPMI 1640 supplemented with 10% FBS. Cells were passaged weekly with media changes every 2–3 days.

Cell proliferation

MCF-7 and Tam^R-1 cells were cultured in 1% FBS medium for 3 days then seeded into 12-well plates at a density of 1×10^4 cells per well. The cells were left for a further 2 days to acclimatize. Cell monolayers were subsequently treated with vehicle (0.1% v/v ethanol) E_2, Tam, or ICI182780 for 6 days with daily changes. The cell number was determined using a Z1 Coulter Counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK).

Real-time quantitative RT-PCR

mRNA from treated MCF-7 and Tam^R-1 cells was extracted using RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. All RNA quantification was performed using the Agilent 2100 Bioanalyzer (Expert Software version B.02.03) with RNA Nano LabChip Kits (Agilent Technologies, Wokingham, Berkshire, UK). The sequences of the primer/probe sets were as follows: ESR1: (forward) 5′-TTCTTCAAGAGAAGTATTCA-AGGACATAAC-3′, (reverse) 5′-TCGTATCCCACCT-TTCATCATTC-3′, (probe) 5′FAM-CCAGCCACCAA-CCAGTGCACCAT-TAMRA-p-3′; PGR: (forward) 5′-ACCTGAGGCCGGATTCAGAA-3′, (reverse) 5′-C-CACAGGTAAGGACACCATAATGAC-3′, (probe) 5′FAM-CCAGAGCCCACAATACAGCTTCGAGT-CATT-TAMRA-p-3′; PSEN2, (forward) 5′-GCCCA-GACAGAGACGTGTACAG-3′, (reverse) 5′-GTCGA-AACAGAGCCCTTATTT, (probe) 5′FAM-CCCCCG-TGAAAGACAGAATTGTGGTTT-TAMR-p-3′; and cathepsin D, (forward) 5′-ACATCGCTTGCTGGAT-CCA-3′, (reverse) 5′-GCTGCCCGAGCCATAGTG-3′, (probe) 5′-FAM-ACAAGTACAACAGCGACAAGT-CCAGCACCTA-TAMR-p-3′. GAPDH (Applied Biosystems, Warrington, Cheshire, UK) was used as a housekeeping gene to normalize the data. Analysis was performed in standard 96-well plates. Reactions were carried out in triplicate using 50 ng mRNA. The relative quantity was determined by ΔΔct according to the manufacturer's instructions (Applied Biosystems). In essence Δct was determined by normalizing against GAPDH. ΔΔct was then established by normalizing against the corrected control MCF-7 cells in 1% FBS.

Transcriptional assays

MCF-7 and Tam^R-1 cells were cultured in 1% FBS medium for 3 days, then seeded in 24-well plates at a density of 1×10^5 cells per well in 1% FBS medium. The following day, the cells were transfected by Fugene 6 at a ratio of 6:1 (Invitrogen) with 0.25 μg EREIItkluc (ERE luciferase reporter construct) and 0.25 μg pCH110 (β-galactosidase for normalizing luciferase data) in 1% FBS medium. The next day, cells were treated with the appropriate concentration of E_2, Tam, ICI, or vehicle. In inhibitor studies, the monolayers were treated with SH6 (10 μM), U0126 (10 μM), or AG825 (10 μM) alone or in combination with tamoxifen (1 μM). After treatment for 24 h, the luciferase (Promega) and β-galactosidase (GalactoStar, Applied Biosystems) activity were measured using a luminometer.

For siRNA treatment prior to transcription assay, cells were grown in 1% FBS medium then seeded into 24-well plates, and left to attach overnight. Nonsense or siRNA against AKT was transfected at 100 nM using DharmaFECT 4 reagent (Dharmacon, Thermo Fisher Scientific). After 24 h, cells were transfected by Fugene 6 at a ratio of 6:1 with 0.25 μg EREIItkluc and 0.25 μg pCH110 in 1% FBS medium. The next day cells were treated with tamoxifen (1 μM) or vehicle. After 24 h, the luciferase and β-galactosidase activity were measured as stated above.

Chromatin immunoprecipitation

ChIP analysis was carried out as described by Shang *et al*. (2000) and Metivier *et al*. (2003). MCF-7 and Tam^R-1 cells were seeded at a density of 5×10^6 cells per 15 cm dish in the presence of 1% FBS medium. Once cells reached 90% confluence, they were synchronized in serum-free medium for 24 h. Monolayers were then treated with 2.5 μM α-amanitin for 2 h then treated with 1% FBS medium containing vehicle (0.01%v/v EtOH) or 1 μM tamoxifen for 45 mins. The chromatin was sheared to give a profile of 200–1000 bp with an average of 400–600 bp. Chromatin complexes were immunoprecipitated with antibodies raised against ESR1 (HC-20; Santa Cruz), NCOA3, NCOR, or CREBBP (Upstate) overnight at 4 °C. Eluted DNA was amplified with Ampli Taq Gold (Amersham) in the presence of primers downstream of the ERE within the promoter region of the *PSEN2* gene (forward) 5′-GGCCATCTCTCACT-ATGAATCACTTCTGCA-3′ and (reverse) 5′-GGCA-GGCTCTGTTTGCTTAAAGAGCGTTAGATA-3′. Amplification occurred between 22 and 30 cycles using an annealing temperature of 63 °C.

Preparation of whole-cell extracts for immunoblots

Cell monolayers were washed with ice-cold PBS, then lysed in extraction buffer (1%(v/v) Triton X100, 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 2 mM Na_3VO_4, and 1 tablet of Complete inhibitor mix (Roche) per 10 ml buffer) and homogenized by passage through a 26 gage needle. The lysate was clarified by centrifugation (14 000 ***g*** for 10 min at 4 °C) and the protein concentration was quantified using BioRad protein assay kit (BioRad). Equal amounts of protein (50 μg) were resolved by SDS-PAGE and transferred to nitrocellulose filters (Schleicher and Schuell, London, UK). Filters were probed with the specific antibodies described diluted in 2% BSA, 10mM Tris–HCl (pH 8.3), 150mM NaCl, 0.025% Tween-20, and 0.01% sodium azide. Immune complexes were detected using Ultra-Signal chemiluminescence kit from Pierce

(Chester, UK). For experiments involving MAP2K1 (U0126), AKT (SH6), or ERBB2 (AG825) inhibitors, cells were initially made quiescent by culture in serum-free medium prior to the addition of inhibitors. Cells were exposed to serum-free medium containing the inhibitors for 60 min before being re-stimulated using medium containing 1% FBS plus inhibitors in the presence of absence of tamoxifen (1 mM) for 60 min. Total protein was then extracted from the cells.

Immunoprecipitation

Five hundred μg of cell lysates were pre-cleared by incubation with protein G-conjugated agarose beads (ImmunoPure Immobilized Protein G, Pierce) for 1 h at 4 °C. Recovered supernatants were transferred to fresh tubes and incubated with appropriate primary antibody at 4 °C overnight with continuous agitation. Agarose beads were added to each tube and the samples were gently mixed for 4 h at 4 °C. Complexes were recovered by centrifugation, washed five to six times, and then boiled in SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE.

Inhibition of cell signaling by siRNA transfection

The MAPK3/1 and PI3K signaling pathways were blocked by transfecting specific siRNA oligonucleotides into cells. MCF-7 and Tam^R-1 cells growing in six-well tissue culture plates were washed in serum-free medium and left to incubate in 900 μl of this medium for 1 h. For each well, 3.75 μl Oligofectamine reagent (Invitrogen) was added to 46 μl serum-free medium in eppendorf tubes and left to incubate for 10 min at room temperature. Meanwhile, 200 pM siRNA was added to 40 μl serum-free medium to give a final volume of 50 μl. The lipid mixture and the diluted siRNA were combined, mixed gently, and left to incubate at room temperature for 30 min. The siRNA/Oligofectamine complex was then added dropwise to the medium in the wells, gently mixed, and left to incubate at 37 °C for 5 h. Monolayers were fed with fresh growth medium. Cells were cultured for 48 h prior to protein extraction.

Apoptosis assay

Apoptosis was measured using 'Cell Death Detection ELISA^{PLUS}' (Roche) according to the manufacturer's instructions. In essence, MCF-7 and Tam^R-1 cells cultured in 1% FBS medium were seeded into 6-well plates at a density of 2×10^5 cells per well. After 48 h, cells were transferred to serum-free medium for 24 h then treated for 24, 48, 72 or 96 h with 1% FBS medium containing vehicle or 1 μM tamoxifen.

Immunofluorescence and confocal studies

Cells were grown on glass coverslips in standard growth medium. Cells were fixed in 4% paraformaldehyde in PBS for 30 min, rinsed with PBS, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Cells were incubated in the presence of primary antibodies diluted in PBS containing 1% BSA and 2% FBS for 2 h at room temperature. Coverslips were washed with PBS and cells were incubated in the presence of appropriate Alexa Fluor 555 (red) or Alexa Fluor 488 (green)-labeled secondary antibodies (Molecular Probes, Invitrogen) diluted 1:1000 for 1 hr. Cells were washed in PBS and nuclei (DNA) were counterstained with Topro-3 (Molecular Probes, Invitrogen) diluted 1:10 000. This gives an emission in the far-red segment of the light spectrum and was pseudo-colored blue. Coverslips were mounted onto glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, Northamptonshire, UK). Images were collected sequentially in three channels on a Leica TCS SP2 confocal microscope (Milton Keynes, Buckinghamshire, UK). Each image represents Z-sections at the same cellular level and magnification (x63 oil immersion objective). Co-localization of two proteins (red and green) is indicated as yellow. Digital analysis for overlays was carried out using NIH ImageJ version 1.38m and the RG2B Co-localization plugin (Christopher Philip Mauer, Northwestern University).

Statistical analysis

Statistical analyses were carried out using unpaired Student's *t*-test or the Wilcoxon paired test. $P<0.05$ was taken as statistically significant.

Results

Tam^R-1 cells remain sensitive to the pure anti-estrogen ICI182780

The tamoxifen-resistant MCF-7 cell line (Tam^R-1) was derived from MCF-7 cells (which had been previously adapted to grow in phenol-red free medium supplemented with 1% FBS) by long-term exposure to tamoxifen (Lykkesfeldt *et al.* 1994). Both the Tam^R-1 and parental MCF-7 cells were refractory to estradiol in the range assessed (0.01–1000 nM; Supplementary Figure 1A, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/). The Tam^R-1 cells were entirely refractory to the inhibitory effects of tamoxifen on growth while, most importantly, the MCF-7 cells showed a dose-dependent decrease in cell growth (Supplementary Figure 1B, which can be viewed

online at http://erc.endocrinology-journals.org/supplemental/). Treatment with the pure anti-estrogen ICI 182780 resulted in a dose-dependent decrease in cell growth in both cell lines confirming their dependence on a functional ESR1. However, Tam^R-1 cells were significantly less sensitive to ICI182780 (IC_{50} 5 nM) compared with the MCF-7 cells (IC_{50} 0.25 nM; Supplementary Figure 1C, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/) suggesting that alternative pathways may also be implicated in controlling cell growth in this setting.

Acquired tamoxifen-resistant MCF-7 cells down-regulate ESR1-genomic activity

Measurement of ESR1 mRNA levels by quantitative RT-PCR together with several other endogenous estrogen-regulated genes (PSEN2, cathepsin D, and PGR) revealed ~50% less expression in the Tam^R-1 versus the MCF-7 cells in the presence or absence of tamoxifen (Fig. 1A). Transfection of the Tam^R-1 and MCF-7 cells with a reporter construct consisting of two copies of an ERE upstream of a luciferase reporter gene showed that basal transactivation in the Tam^R-1 cells was fivefold less than MCF-7 cells in the absence of tamoxifen. Treatment of the MCF-7 cells with tamoxifen reduced ESR1/ERE transactivation by fivefold providing a profile similar to the Tam^R-1 (Fig. 1B). Treatment of both cell lines with escalating E_2 revealed a dose-dependent increase in transactivation (Fig. 1C). Similarly, treatment with ICI 182780 resulted in a dose-dependent decrease in ESR1/ERE transactivation in both cell lines (Fig. 1D). However, the MCF-7 cells appeared more sensitive to the inhibitory effects of ICI 182780. Taken together these data suggested that the ESR1 remained functional but down-regulated in the Tam^R-1 cell line. To analyze this further, we treated the Tam^R-1 cells with vehicle or tamoxifen for 45 min and assessed the recruitment of the basal transcription machinery to the PSEN2 promoter, using chromatin immunoprecipitation (Fig. 1E). In the absence of tamoxifen, ESR1 was recruited to the PSEN2 promoter together with the coactivator NCOA3 and the histone acetyl transferase CREBBP. This observation suggested that PSEN2 transcription was active and was supported by the expression of *PSEN2* mRNA (Fig. 1A). Treatment with tamoxifen significantly increased recruitment of the ESR1 to the PSEN2 promoter by ~50% together with a concomitant increase in NCOR, while a significant decrease in both NCOA3 and CREBBP recruitment was evident. Expression of *PSEN2* mRNA in the presence of tamoxifen was similarly decreased as noted in Fig. 1A. This suggested that although basal genomic ESR1 activity was suppressed in the Tam^R-1 cells, in the absence of tamoxifen (Fig. 1B), the ESR1 remained capable of recruiting coactivators and the basal transcription machinery and that this recruitment was suppressed by tamoxifen. Analyses of MCF-7 cells for the recruitment of ESR1 and CREBBP showed significantly reduced CREBBP recruitment in the presence of tamoxifen (Supplementary data Figure 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

Elevated ERBB2, MAPK3/1 and AKT are associated with the Tam^R-1 phenotype

Crosstalk between the ESR1 and the type I tyrosine kinase receptor family has been associated with the development of endocrine-resistant breast cancer (Ali & Coombes 2002, Hutcheson *et al*. 2003, Massarweh & Schiff 2007). Analysis of the Tam^R-1 versus MCF-7 cells revealed an elevation in both total and phosphorylated ERBB2 (Fig. 2A) that was not associated with gene amplification as assessed by FISH (data not shown). Tam^R-1 cells also showed a concomitant increase in total ERBB3 but no change in EGFR was noted (Fig. 2A). Most noteworthy, IGF1R appeared down-regulated. Increases in activated AKT, MAPK3/1 and its downstream partner RPS6KA2 were also detected (Fig. 2B and C). The basal protein expression level of ESR1 appeared similar in both the MCF-7 and Tam^R-1 cell lines, while ESR2 expression was significantly decreased (Fig. 2D). PGR expression was lost in the Tam^R-1 compared with the parental MCF-7 cell line. Analysis of the phosphorylation status of ESR1 showed that serine 118 (Ser^{118}) was phosphorylated in both cell lines while ESR1 serine 167 (Ser^{167}) was phosphorylated to a greater extent in the Tam^R-1 (Fig. 2D).

ESR1 Ser^{167} is phosphorylated in Tam^R-1 cells by both AKT and pRPS6KA2

In vitro studies have revealed that ESR1 can be activated in a ligand-independent manner by phosphorylation of Ser^{118} via MAPK3/1 or by Ser^{167} via AKT- or MAPK3/1-activated pRPS6KA2 (Kato *et al*. 1995, Bunone *et al*. 1996, Joel *et al*. 1998, Campbell *et al*. 2001). This phenomenon has been associated with resistance to endocrine treatment *in vitro*. To assess this in the Tam^R-1 cells, siRNAs were used to abrogate expression of AKT and MAPK1 alone or in combination. Inhibition of AKT resulted in a slight but noticeable decrease in the expression of ESR1 Ser^{167} but had no effect on ESR1 Ser^{118} (Fig. 3A). Inhibition of AKT also caused a slight but noticeable increase in MAPK1. Inhibition of MAPK1 had no effect on ESR1 Ser^{167} but resulted in a marked decrease in phosphorylated ESR1 Ser^{118}. Suppression of

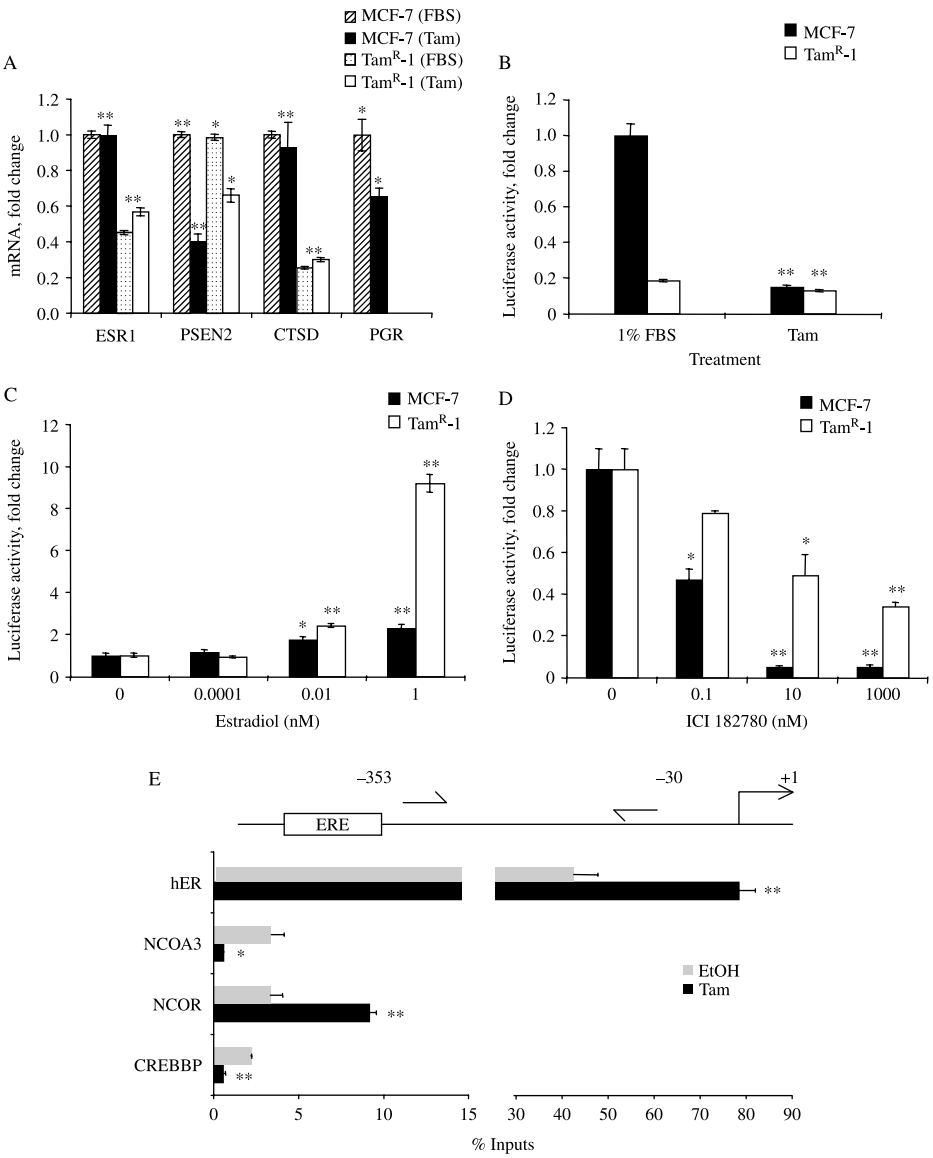

Figure 1 ESR1 transactivation is suppressed by tamoxifen in the Tam^R-1 cell line. (A) Both MCF-7 and Tam^R-1 cells were seeded into six-well plates then treated with 1% FBS medium containing vehicle or 1 μM tamoxifen for 24 h. mRNA was then isolated and qRT-PCR used to assess the expression of endogenous E2-regulated genes. Each data point represents the mean of triplicate wells and in each case was normalized to the vehicle control for each cell line; bars represent the SEM. *$P<0.05$, **$P<0.01$, by Student's unpaired *t*-test. The results are representative to three independent experiments. (B) To assess the ESR1/ERE basal transactivation, MCF-7 and Tam^R-1 monolayers were transiently transfected with an ERE-linked luciferase reporter construct followed by 24 h treatment with vehicle or tamoxifen (1 μM). Luciferase activity was normalized by β-galactosidase from co-transfected pCH110. Normalized luciferase activity from quadruplicate wells was expressed relative to the vehicle-treated MCF-7 cells. Bars represent SEM. **$P<0.01$, compared with 1% FBS vehicle-treated control by Student's unpaired *t*-test. Results were confirmed in four independent experiments. (C) Monolayers were transfected as described above and treated with escalating doses of E_2. Bars represent SEM. *$P<0.05$, **$P<0.01$, compared with vehicle-treated control by Student's unpaired *t*-test. Data were confirmed in three independent experiments. (D) Both MCF-7 and Tam^R-1 cells were treated with escalating doses of ICI 182780 in 1% FBS medium. In each case, normalized luciferase activity from quadruplicate wells was expressed relative to the vehicle-treated control. Bars represent SEM. *$P<0.05$, **$P<0.01$, compared with vehicle-treated control by Student's unpaired *t*-test. Results were confirmed in four independent experiments. (E) Chromatin immunoprecipitation was carried out as described in the 'Materials and Methods' to monitor recruitment of ESR1 together with CoA/CoR to the PSEN2 promoter in Tam^R-1 cells treated with vehicle or tamoxifen. qPCR was used to quantify DNA. The diagram indicates the position of the primers on the PSEN2 promoter. Bars represent SEM from for triplicate samples. *$P<0.05$, **$P<0.01$, compared with vehicle-treated control by Student's unpaired *t*-test. Data were confirmed in two independent experiments. ChIP analysis for ESR1 and CREBBP in MCF-7 cells was also assessed (Supplementary Data Figure 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

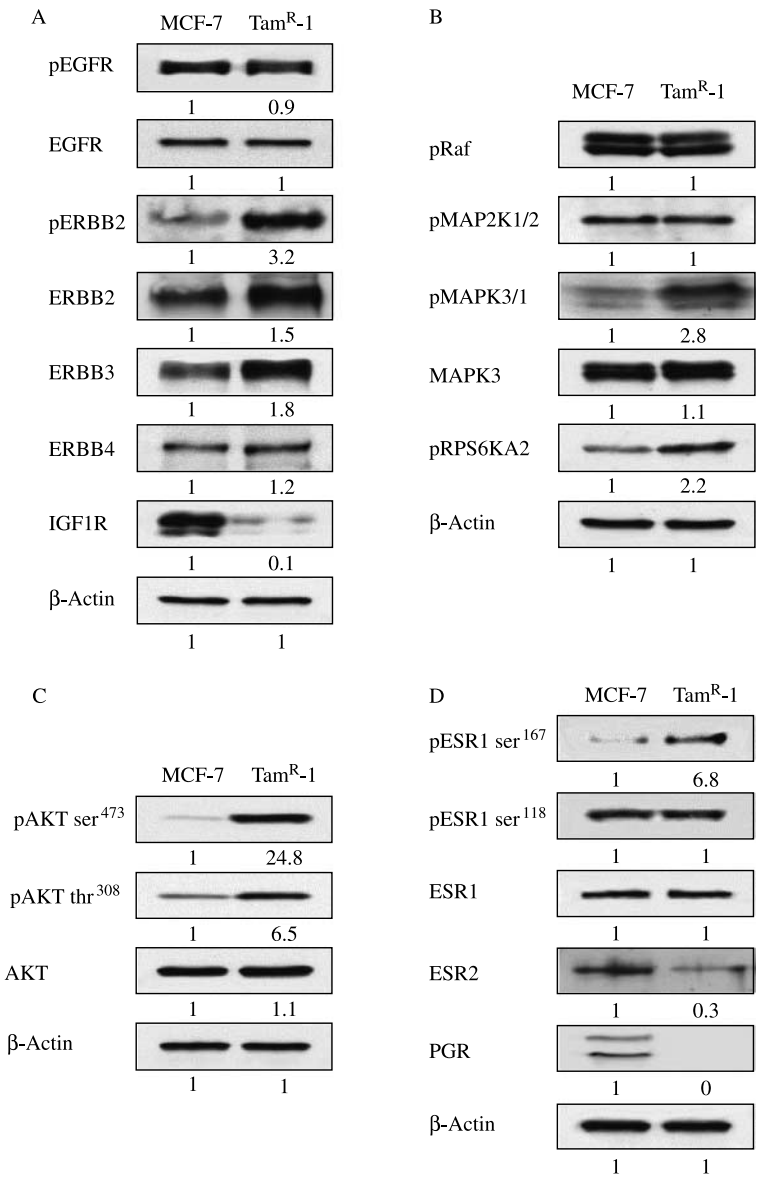

Figure 2 Tam^R-1 cells express ESR1 and elevated levels of phosphorylated ERBB2, AKT, and RPS6KA2. (A) Whole-cell extracts from MCF-7 and Tam^R-1 cells cultured in their basal medium were subjected to immunoblot analysis. Samples were tested for the presence of the growth factor receptors indicated. (B & C) Whole-cell extracts were screened for the presence of phosphorylated MAPK3/1, RPS6KA2, and AKT respectively, all of which have been associated with ligand-independent activation of ESR1. (D) Immunoblot analysis for ESR1 phosphorylated on Ser^{118} and Ser^{167} together with ESR2 and PGR. The data are representative of three independent experiments. Numbers indicate relative band intensities compared with control.

MAPK1 also led to a 2.5-fold increase in AKT. It is noteworthy that only when both pathways were blocked was phosphorylation of ESR1 Ser^{167} abrogated suggesting MAPK1, possibly via pRPS6KA2 and AKT were independently able to activate this epitope. Having established that alterations in the expression of both phosphorylated AKT and MAPK1 appeared to be associated with the tamoxifen-resistant phenotype, we assessed the effect of the MAP2K1/2 inhibitor UO126 and AKT inhibitor SH6, alone or in combination on cell proliferation and ESR1/ERE-mediated transactivation in the Tam^R-1 versus their parental cell line. Increasing doses of UO126 inhibited proliferation of both the Tam^R-1 and MCF-7 cells in a dose-dependent manner (Fig. 3B). However, SH6 caused a marked decrease in the proliferation of the Tam^R-1 cell line with an IC_{50} of 1 μM compared with 2.5 μM for the MCF-7 cells (Fig. 3C). Most notably, a combination of escalating doses of SH6

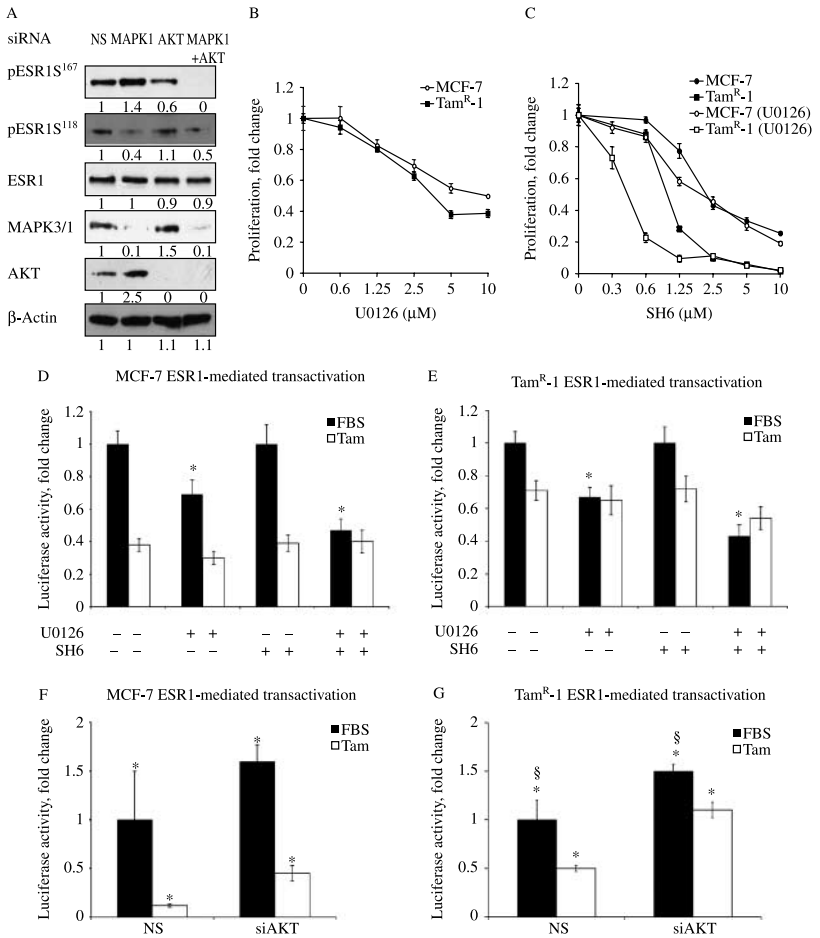

Figure 3 ESR1 can be phosphorylated by AKT or MAPK1, but while inhibition of AKT suppresses Tam^R-1 cell proliferation it has no effect on ESR1 transactivation. (A) Tam^R-1 and MCF-7 cells were seeded into six-well plates then transfected with siRNAs targeting MAPK1, AKT or a combination of the two. As a control nonsense (NS), siRNAs were used to check for off-target activity. After 48 h, whole-cell extracts were probed with antibodies against the markers indicated. Numbers indicate relative band intensities compared with control. (B) To test the effect of inhibiting MAPK3/1 on cell proliferation, both MCF-7 and Tam^R-1 cells were seeded into 12-well plates and treated with escalating doses of the MAP2K1/2 inhibitor U0126. Cell number was determined 6 days later using a Coulter counter. (C) To test the effect of inhibiting AKT alone or in addition to MAPK3/1 on cell proliferation, both MCF-7 and Tam^R-1 cells were treated with escalating doses of the AKT inhibitor SH6 $\pm$ U0126 (5 μM). Each data point represents the mean of triplicate wells and in each case was normalized to the vehicle control for each cell line. Bars represent SEM. Data were confirmed in three independent experiments. (D and E) To assess the effect of inhibiting AKT, MAPK3/1 or both pathways on ESR1/ERE transactivation, MCF-7 and Tam^R-1 monolayers were transiently transfected with an ERE-linked luciferase reporter construct, followed by 24 h treatment with U0126 (10 μM), SH6 (10 μM) or a combination of the two inhibitors $\pm$ tamoxifen (1 μM). Luciferase activity was normalized by β-galactosidase from co-transfected pCH110. In each case, normalized luciferase activity from quadruplicate wells was expressed relative to the vehicle-treated control. Bars represent SEM. *$P<0.05$, compared with vehicle-treated control by Student's unpaired *t*-test. All data shown were confirmed in four independent experiments. (F & G) MCF-7 cells and Tam^R-1 cells were seeded into six-well plates then transfected with siRNA targeting AKT. As a control nonsense (NS), siRNAs were used to check for off-target activity. Cells were then transiently transfected with an ERE-linked luciferase reporter construct. Cells were treated with either tamoxifen or vehicle for 24 h prior to harvesting. Luciferase activity was normalized by β-galactosidase from co-transfected pCH110. In each case, normalized luciferase activity from quadruplicate wells was expressed relative to the vehicle-treated control. Bars represent SEM. *$P<0.05$ compared with vehicle-treated control by Student's unpaired *t*-test; §, $P<0.05$ compared with nonsense siRNA transfected cells by Student's unpaired *t*-test.

with a fixed dose of UO126 (5 μM) resulted in a marked shift in sensitivity in the Tam^R-1 (IC50 0.25 μM) compared with the MCF-7 cells whose sensitivity to SH6 was largely unaffected by U0126 (Fig. 3C). As both pAKT and pMAPK3/1 appeared associated with phosphorylation of the ESR1, we sought to assess the effect of inhibiting MAPK3/1 or AKT on ESR1/ERE-driven transactivation. The Tam^R-1 and MCF-7 cells were transfected with an ERE-luciferase-linked reporter construct. Cell lines were then treated with the inhibitors indicated, either alone or in combination (Fig. 3D & E). Unexpectedly, while inhibition of AKT had no effect on

ESR1 transactivation in the MCF-7 and Tam^R-1 cell lines, inhibition of MAPK3/1 significantly decreased activity by 30% in each cell line in the absence of tamoxifen. A combination of both inhibitors had a similar suppressive effect as inhibition of MAPK3/1 alone. As AKT has previously been implicated in ESR1-mediated transactivation (Campbell *et al.* 2001), we repeated this assay using siRNAs targeting AKT. Knockdown of AKT had no significant effect on ESR1-mediated transactivation in the parental cell line in the absence of tamoxifen compared with the nonsense control. As noted previously (Fig. 3D), addition of tamoxifen markedly decreased ESR1-mediated transcription compared with the FBS control in each case. Of note, knockdown of AKT in the Tam^R-1 cells in the absence of tamoxifen significantly enhanced ESR1-mediated transactivation compared with the nonsense control. Similar to the parental cell line, addition of tamoxifen reduced ESR1-mediated transactivation in both the nonsense and AKT treatment arms (Fig. 3F and G). This suggested MAPK3/1 as opposed to AKT was implicated in ESR1 transactivation in the absence of tamoxifen and that knockdown of AKT may enhance ESR1-mediated transactivation further as a result of increased MAPK3/1 activity as shown in Fig. 3. Of note, however, the combination of tamoxifen with the signal transduction inhibitors was no better than tamoxifen alone in either cell line. This suggested that the limited ESR1 genomic activity remaining in the Tam^R-1 cell line was still suppressed by tamoxifen in a similar manner to the MCF-7. This confirmed our previous ChIP analysis, which indicated a preferential recruitment of NCOR in the presence of tamoxifen in the Tam^R-1 cell line.

Elevated levels of AKT and RPS6KA2 provide tamoxifen-resistant MCF-7 cells with a survival advantage involving BAD

The data shown in Fig. 3A–G suggested that AKT and possibly pRPS6KA2 were playing another role in the Tam^R-1 cell line. It is well known that both AKT- and MAPK3/1-activated pRPS6KA2 are associated with cell survival by phosphorylating BAD and suppressing apoptosis (Zha *et al.* 1996). Further analysis showed that the Tam^R-1 cells had elevated levels of BAD phosphorylated on ser^{112} and ser^{136} (Fig. 4A), which was inhibited by U0126 and SH6 respectively, confirming the involvement of pRPS6KA2 and AKT respectively

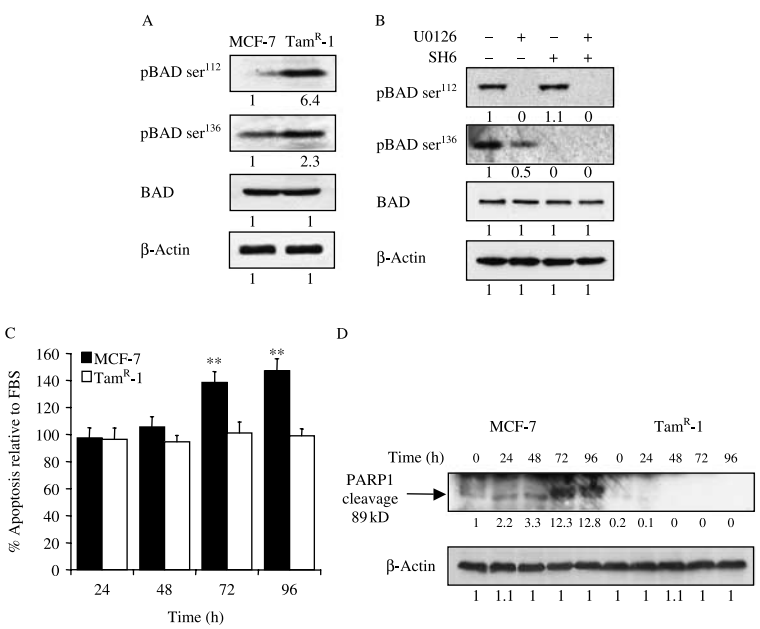

Figure 4 AKT potentiates Tam^R-1 cell survival in the presence of tamoxifen. (A) Whole-cell extracts from MCF-7 and Tam^R-1 cells cultured under basal conditions, were assessed by immunoblotting for expression of BAD. (B) As both AKT- and MAPK3/1-activated pRPS6KA2 have been associated with phosphorylation of BAD, MCF-7 and Tam^R-1 monolayers were treated with the U0126 (10 μM), SH6 (10 μM) or a combination of the two agents. Whole-cell extracts were then immunoprobed to assess the effect on pBAD ser^{112} and pBAD ser^{136}. (C) To assess the effect of tamoxifen on apoptosis, MCF-7 and Tam^R-1 cells were treated with tamoxifen (1 μM) over a 96 h time course. Cell apoptosis was monitored using a 'Live-Dead' assay as described in 'Materials and Methods'. Bars represent SEM of quadruplicate treatments at each time point. $^{**}P<0.01$, compared with vehicle-treated control at each time point by Student's unpaired *t*-test. Experiments were confirmed in two independent experiments. (D) MCF-7 and Tam^R-1 cell monolayers were treated with tamoxifen (1 μM) for the times indicated. Whole-cell extracts were then immunoprobed to show PARP1 cleavage. Numbers indicate relative band intensities compared with control.

(Fig. 4B). This provides the Tam^R-1 cells with a potential survival advantage. Further confirmation of this survival advantage was achieved by monitoring apoptosis in the Tam^R-1 versus MCF-7 cells over a 96-hour time course ± 1 μM tamoxifen using a 'Live/Dead assay' and by PARP1 cleavage. Apoptosis was elevated by 50% in the MCF-7 versus Tam^R-1 cells (Fig. 4C). Similarly, the onset of PARP1 cleavage was evident at 72 h in the MCF-7 while in the Tam^R-1 cells PARP1 cleavage was undetectable even after 96 h exposure (Fig. 4D).

Evidence suggests ESR1 interacts with ERBB2 and leads to elevation of phosphorylated AKT

As analysis of the growth factor receptors associated with the Tam^R-1 phenotype indicated elevated levels of both phosphorylated ERBB2 and total ERBB3, we postulated that these formed heterodimers activating the PI3 kinase pathway leading to elevated pAKT. However, immunoprecipitation studies revealed no evidence of these complexes. Previous studies have suggested that ERBB2 can associate with ESR1 (Chung *et al.* 2002) leading to rapid activation of both the AKT and MAPK3/1 signaling pathways. To investigate this, co-immunoprecipitation studies were undertaken in which ESR1 was shown to associate with ERBB2 in the Tam^R-1 but not MCF-7 cells (Fig. 5A). This association could be abrogated by treatment with ICI 182780 (Fig. 5B) and lead to a concomitant dose-dependent decrease in ESR1 and pAKT (Fig. 5C). We also investigated whether ESR1 associated with ERBB3 in the Tam^R-1 cell line but were unable to observe any association (Fig. 5A and Supplementary data Figure 3, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/). Confocal analysis showed that in the Tam^R-1 cells ESR1 appeared diffuse and mainly in the cytoplasm and at the plasma membrane compared with the MCF-7 cells where ESR1 remained nuclear (Fig. 5D). ESR1 within the Tam^R-1 cell line co-localized with ERBB2 at the membrane (see co-localized pixels in Fig. 5D) and this association was knocked out by treatment with ICI 182780 together with the loss of ESR1 staining as expected. To confirm that the staining for ESR1 was specific, SKBR3 human breast tumor cells that are ESR1-negative and ERBB2 amplified were used as a negative control. BT474 breast tumor cells, which are ESR1-positive and have amplified ERBB2, showed similar diffuse patterning of ESR1 seen in the Tam^R-1 cells. ERBB2 also co-localized with ESR1 in the BT474 cells in keeping with previous observations (Yang *et al.* 2004). The association between ERBB2 and ESR1 was reproduced using a second tamoxifen-resistant cell line, Tam^R-4 (Fig. 8A).

Inhibition of pERBB2 signaling in combination with tamoxifen suppresses growth of the Tam^R-1 cells

To test whether targeting ERBB2 could suppress Tam^R-1 cell proliferation, both the MCF-7 and Tam^R-1 cells were treated with increasing doses of AG825, a specific inhibitor of ERBB2 phosphorylation, alone or in combination with tamoxifen. Surprisingly, AG825 alone had no effect on the proliferation of the MCF-7 or Tam^R-1 cells (Fig. 6A and B). However, when in combination with tamoxifen, AG825 caused a 50% decrease in cell growth of the Tam^R-1 cells while providing no added effect in the MCF-7 cells (Fig. 6A and B). In keeping with the anti-proliferative effect of the combination, both AKT and MAPK3/1 were suppressed by AG825 in the Tam^R-1 cells (Fig. 6C). To assess the effect of inhibiting ERBB2 on ESR1/ERE transactivation, both MCF-7 and Tam^R-1 cells were transfected with an ERE-luciferase-linked reporter construct. At the highest dose of AG825 (10 μM), there was a slight but statistically insignificant 27% decrease in ESR1 transactivation in the MCF-7 cells compared with the vehicle-treated control. The combination of AG825 with tamoxifen provided no further decrease in ESR1 transactivation compared with tamoxifen alone in this setting (Fig. 6D). Assessment of the Tam^R-1 cells showed that suppression of ERBB2 phosphorylation alone or in combination with tamoxifen had no further suppressive effect on ESR1/ERE transactivation (Fig. 6E). To confirm this, we assessed the effect of combining AG825 with tamoxifen on PSEN2 transcription and showed no additive effect (Fig. 6F and G). There was, however, a significant increase in PSEN2 transcription in the MCF-7 cells treated with AG825 alone compared with the vehicle control.

Inhibition of phosphorylated ERBB2 leads to nuclear localization of ESR1 in the presence of tamoxifen

Recent studies (Yang *et al.* 2004) have suggested that amplification of *ERBB2* or overexpression of EGFR (Fan *et al.* 2007) can influence the cellular localization of ESR1. Although the Tam^R-1 cells do not possess an amplification of *ERBB2*, we postulated that overexpression of ERBB2 (possibly via a transcriptional mechanism) may provide an explanation for the diffuse cytoplasmic ESR1 staining (Fig. 5D). To investigate this further, we treated both the MCF-7 and Tam^R-1 cells with AG825 alone or in combination with tamoxifen. In the MCF-7 cells, ESR1 remained nuclear irrespective of treatment (Fig. 7A). However, in the Tam^R-1 cell line, treatment of the cells with tamoxifen in combination with

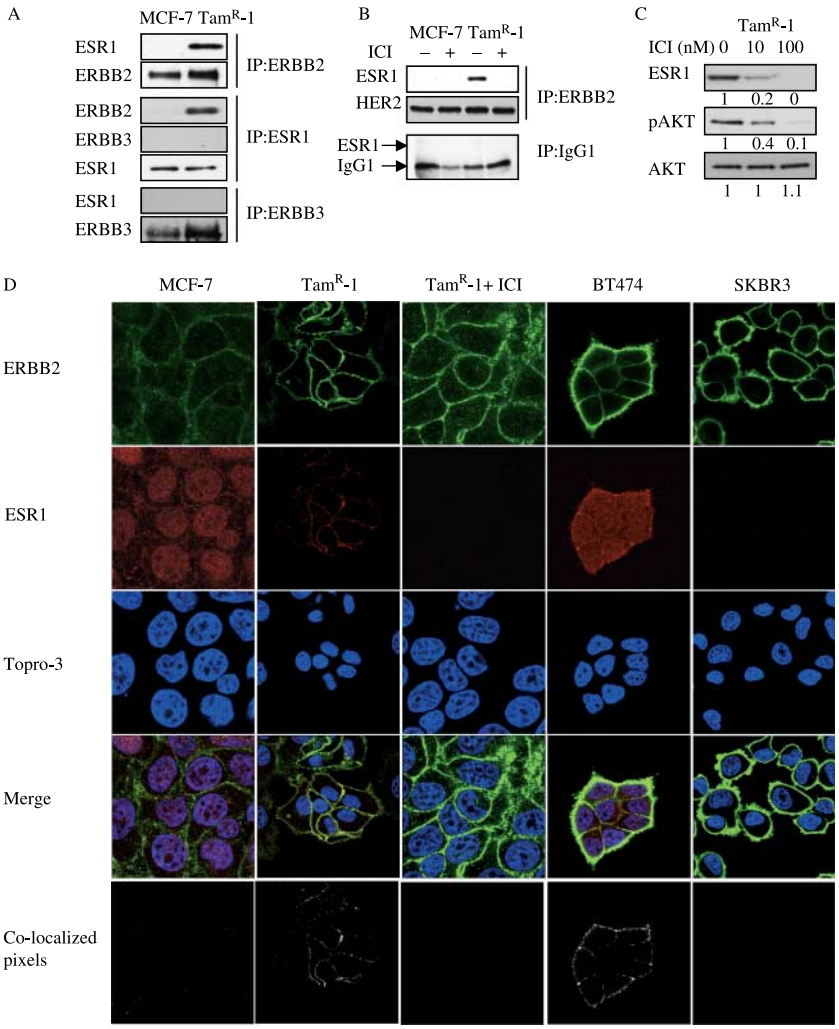

Figure 5 ESR1 associates with ERBB2 at the plasma membrane in Tam^R-1 but not MCF-7 cells. (A) ERBB2 was immunoprecipitated from MCF-7 and Tam^R-1 whole-cell extracts and immune complexes were subjected to ESR1 or ERBB2 immunoblot procedures. Alternatively, ESR1 was immunoprecipitated followed by immunoblot analysis with ERBB2, ERBB3, or ESR1. Finally, ERBB3 was immunoprecipitated and immunoblotted with ESR1 or ERBB3. (B) To establish the specificity of the ERBB2/ESR1 interaction, both MCF-7 and Tam^R-1 monolayers were treated ± ICI 182780 (100 nM). ERBB2 was immunoprecipitated and immune complexes were probed for ESR1 content. Immunoprecipitation with IgG was used as a negative control. (C) To assess the effect of destroying the ESR1 on expression of pAKT, Tam^R-1 monolayers were treated with increasing doses of ICI182780. Whole-cell extracts were immunoprobed for the presence of ESR1 and pAKT. Numbers indicate relative band intensities compared with control. (D) To determine the subcellular localization of the ERBB2/ESR1 interaction, confocal analysis was carried out as described in the 'Methods and Materials'. MCF-7 and Tam^R-1 cells were plated onto coverslips and stained with antibodies against ERBB2 and ESR1. Nuclei were visualized using Topro-3. To show that the interaction between ESR1 and ERBB2 were specific, Tam^R-1 cells were treated with ICI 182780, which destroyed the ESR1. Similarly, SKBR3 cells, which are ESR1-negative, ERBB2 amplified, were used as a further negative control showing the specificity of the ESR1 antibody. BT474 cells which are ESR1 and ERBB2 positive were used as a comparison to show diffuse ESR1 staining in the presence of ERBB2.

AG825 restored localization of ESR1 back to the nucleus (Fig. 7B). These observations were reproduced in a second tamoxifen-resistant cell line, Tam^R-4 (Fig. 8B).

Discussion

It is widely accepted that overexpression of ERBB2 is associated with acquisition of resistance to tamoxifen in human breast cell lines (Benz *et al*. 1992, Kumar *et al*. 1996, Kurokawa *et al*. 2000, Shou *et al*. 2004) and in patients with ESR1-positive, hormone-dependent tumors (Borg *et al*. 1994, Leitzel *et al*. 1995, Dowsett 2001, Dowsett *et al*. 2001, Gutierrez *et al*. 2005). However, the molecular mechanisms associated with the generation of resistance are poorly understood. To address this, we have characterized an

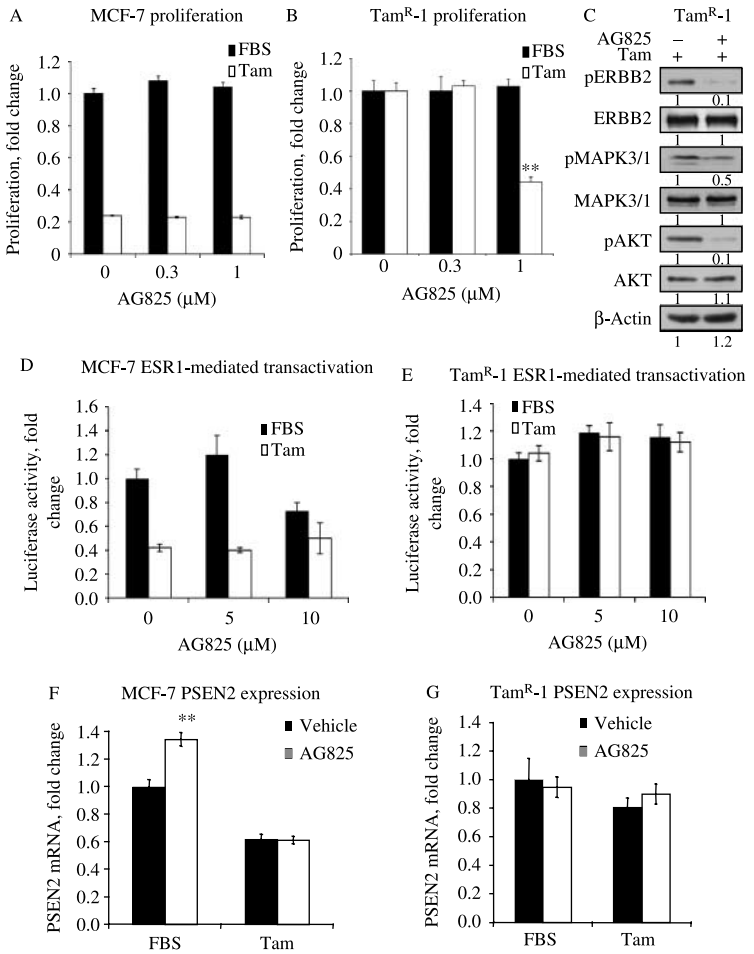

Figure 6 AG825 in combination with tamoxifen suppresses Tam^R-1 cell proliferation but has no effect on ESR1/ERE transactivation. (A & B) MCF-7 and Tam^R-1 cells were plated into 12-well plates and treated with the concentrations of AG825 shown ± tamoxifen (1 μM). Cell number was established after 6 days. Bars represent SEM of triplicate wells. $^{**}P<0.01$, compared with control by Student's unpaired *t*-test. Data shown were confirmed in three independent experiments. (C) To establish that AG825 suppressed ERBB2 phosphorylation, Tam^R-1 cells were treated with tamoxifen (1 μM) ± AG825 (10 μM). Whole-cell lysates were screened for the signal transduction molecules shown. Numbers indicate relative band intensities compared with control. (D & E) To assess the effect of AG825 on ESR1-transactivation, MCF-7 and Tam^R-1 monolayers were transiently transfected with an ERE-linked luciferase reporter construct followed by 24 h treatment with AG825 (10 μM) ± tamoxifen (1 μM). Luciferase activity was normalized by β-galactosidase from co-transfected pCH110. Normalized luciferase activity was expressed relative to the vehicle-treated cells. Bars represent SEM of quadruplicate wells. Experiments were confirmed in three independent experiments. (F & G) To assess the effect of AG825 on ESR1 regulation of an endogenous ESR1 regulated gene, qRT-PCR was used to measure the expression of PSEN2 after the treatments indicated. Bars represent SEM of triplicate wells. $^{**}P<0.01$, compared with vehicle-treated control by Student's unpaired *t*-test. Data were confirmed in two independent experiments.

MCF-7 breast cancer cell line cultured long-term in the presence of tamoxifen, to model acquired resistance. The level of E_2 within medium containing 1% FBS, the routine medium of culture for both MCF-7 parental cells and Tam^R-1 cells, was routinely quantified as less than 3 pmol/l. This is closely similar to the mean level seen in postmenopausal women receiving an aromatase inhibitor and could account for the lack of a growth response of both the parental MCF-7 and Tam^R-1 cell lines to added E_2 (Masamura *et al*. 1995, Long *et al*. 2002, Martin *et al*. 2003). Most importantly, the parental MCF-7 cells in this study are clearly sensitive to tamoxifen despite this lack of response to exogenous E_2 in contrast to the Tam^R-1 cells (Supplementary Figure 1B, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

The ESR1 mRNA content of the Tam^R-1 cell line was twofold less compared with the parental MCF-7. However, western blot analysis showed that the level of ESR1 protein was similar between the two lines, probably as tamoxifen is able to stabilize ESR1 protein (Horner- Glister *et al*. 2005). Both MCF-7 and Tam^R-1

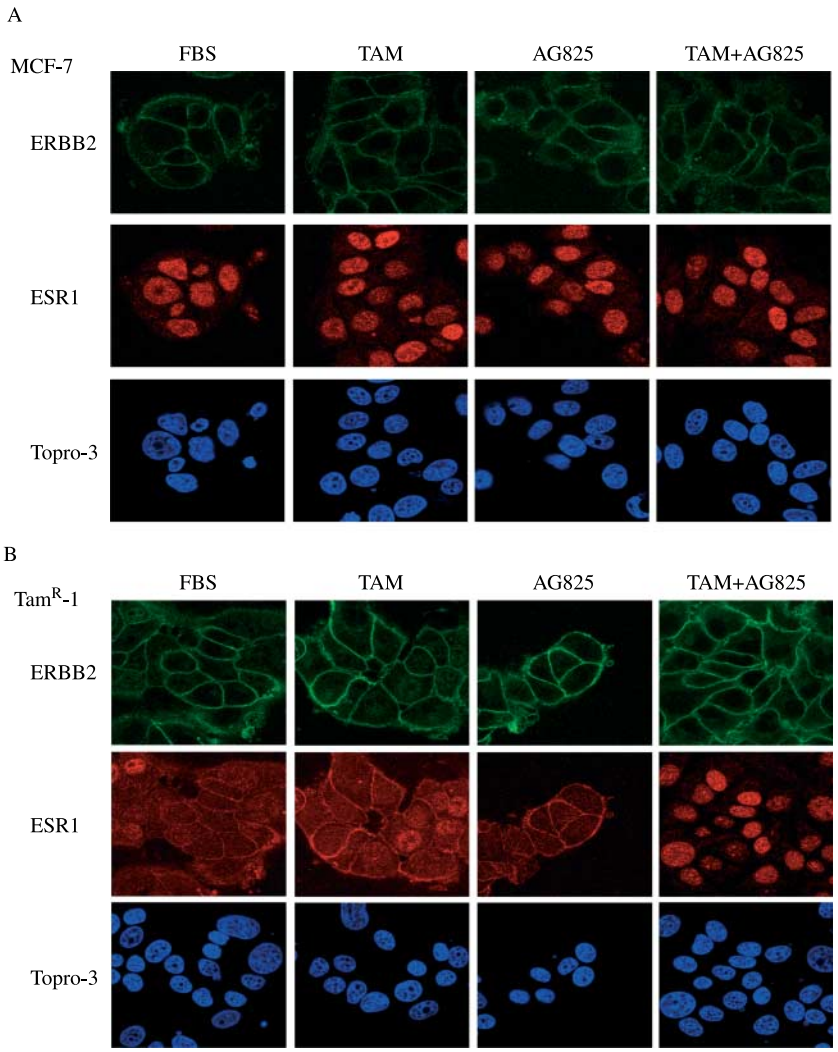

Figure 7 Inhibition of ERBB2 with AG825 in combination with tamoxifen promotes nuclear localization in the Tam^R-1 cells. (A) MCF-7 and (B) Tam^R-1 cells were plated onto coverslips and treated with AG825 (10 μM), tamoxifen (1 μM) or a combination of the two agents for 24 h. Coverslips were stained with antibodies against ERBB2 and ESR1. Nuclei were stained with Topro-3. Confocal analysis was used to establish the cellular localization of ERBB2 and ESR1.

cells remained sensitive to the growth-suppressive effects of the pure anti-estrogen ICI 182780 suggesting both cell lines utilized the ESR1 for growth although less so with the Tam^R-1, as indicated by a higher IC50 value. As with previous studies of tamoxifen-resistant cell lines, the Tam^R-1 expressed increased levels of ERBB2 and pMAPK3/1 (Kurokawa *et al.* 2000, Shou *et al.* 2004) compared with MCF-7. In these previous studies in which MCF-7 cells were engineered to overexpress ERBB2, tamoxifen-bound ESR1 in the presence of elevated pMAPK3/1 was shown to recruit coactivators such as NCOA3 in preference to the co-repressor NCOR (Kurokawa *et al.* 2000, Shou *et al.* 2004), providing a strong rationale for crosstalk between the ESR1 and growth-factor signaling pathways. However, by contrast, studies in BT474 cells, which are ESR1+ and naturally ERBB2 amplified showed reduced ESR1 genomic signaling (Chung *et al.* 2002). Of note, ESR1 transactivation was also down-regulated in an MCF-7 cell line modeling acquired resistance to tamoxifen in which EGFR overexpression was associated with the phenotype (Hutcheson *et al.* 2003). Our data are in keeping with these later studies as ESR1-mediated transcription was fivefold lower in the Tam^R-1 compared with MCF-7 cells irrespective of the presence or absence of tamoxifen. It has been suggested that hyperactivation of MAPK3/1 induces a reduction in ESR1 signaling (Oh *et al.* 2001, Creighton *et al.* 2006). This may explain why the level of ESR1 mRNA is reduced in the Tam^R-1 compared with the parental MCF-7 cells, and would support the loss of

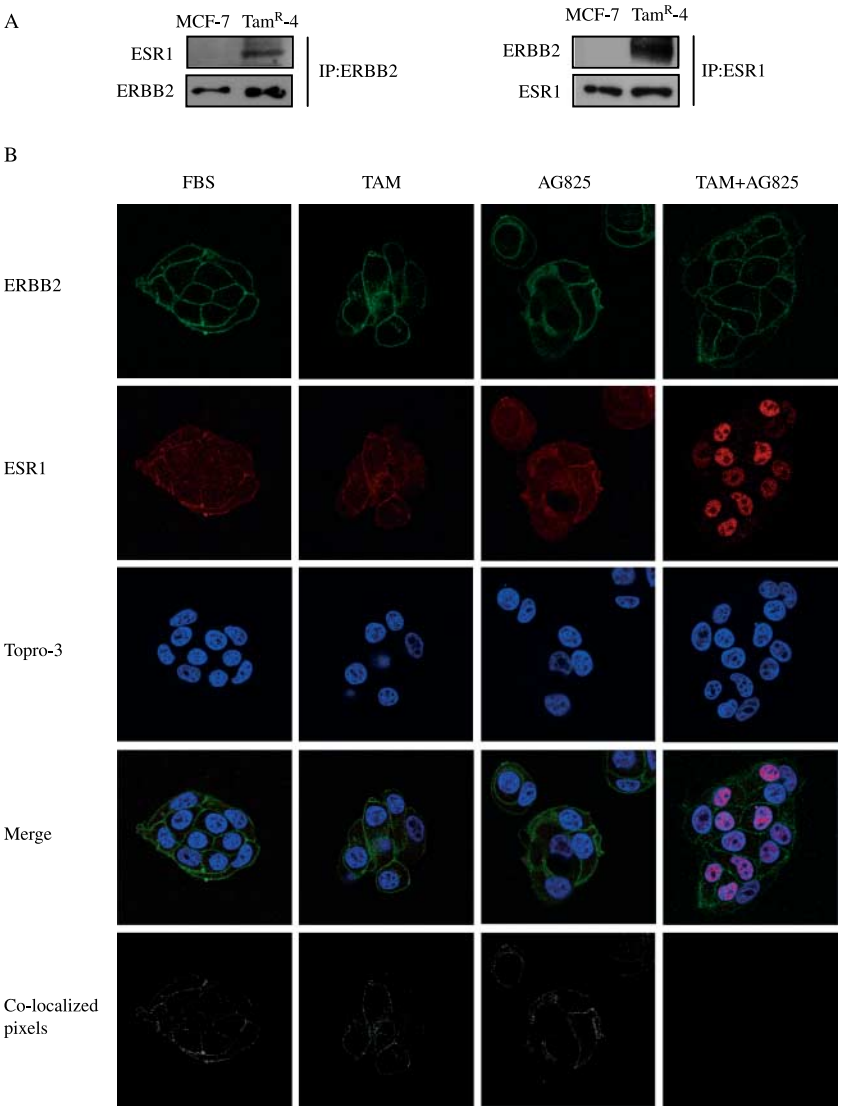

Figure 8 ESR1 associates with ERBB2 at the plasma membrane in Tam^R-4. (A) ERBB2 was immunoprecipitated from Tam^R-4 whole-cell extracts and immune complexes were subjected to ESR1 or ERBB2 immunoblot procedures. Alternatively, ESR1 was immunoprecipitated followed by immunoblot analysis with ERBB2 or ESR1. Immunoprecipitation with IgG was used as a negative control. (B) Tam^R-4 cells were plated onto coverslips and treated with AG825 (10mM), tamoxifen (1 mM) or a combination of the two agents for 24 h. Coverslips were stained with antibodies against ERBB2 (green) and ESR1 (red). Nuclei were stained with Topro-3 (blue). Confocal analysis was used to establish the cellular localization of ERBB2 and ESR1.

PGR. Taken together these data indicated that while ESR1 remained functional in the Tam^R-1 cell line, its genomic activity was down-regulated. Assessment of ESR1 function in the Tam^R-1 cell line by ChIP analysis indicated that in the absence of tamoxifen, ESR1 was recruited to the PSEN2 promoter but to a greater degree than NCOA3 and the histone acetyltransferase CREBBP. Although these data suggest that ESR1 remained capable of recruiting coactivators in the Tam^R-1 cell line, an alternative interpretation would be that NCOA3 and CREBBP are recruited by transcription factors other than ESR1. For instance, regulation of PSEN2 transcription has been shown to involve a complex interplay in which ESR1 together with AP1 and coactivators of the p160 family have been implicated (DeNardo *et al.* 2005). In the presence of tamoxifen, ESR1 within the Tam^R-1 cells recruited NCOR and there was an associated decrease in CREBBP binding (Fig. 1). This together with the observation that long-term treatment with tamoxifen is associated with increased ERBB2 suggested that ERBB2 might be the dominant control driving cell proliferation rather than genomic ESR1.

Previous *in vitro* and clinical studies have implicated elevated AKT activity in tamoxifen resistance (Jordan *et al.* 2004, Frogne *et al.* 2005, Kirkegaard *et al.* 2005). However, the mechanisms and functional consequences remain somewhat elusive. Analysis showed that the Tam^R-1 cells also expressed elevated levels of pAKT and most notably, MAPK3/1-activated pRPS6KA2. MAPK3/1, AKT, and pRPS6KA2 have been associated with the ligand-independent phosphorylation of ESR1 on ser^{118} and ser^{167} respectively. Analysis of the ESR1 in the Tam^R-1 cells indicated that it was phosphorylated on both sites. Using siRNA knockout, we were able to show that while MAPK3/1 was predominantly responsible for phosphorylation of ser^{118}, either AKT or pRPS6KA2 could phosphorylate ser^{167} showing a high degree of plasticity. Inhibition of MAPK3/1 reduced ESR1 transactivation in the absence of tamoxifen by ~30% while inhibition of AKT by siRNA knockdown appeared to enhance ESR1-mediated transactivation. This suggested that MAPK3/1 was involved in phosphorylation of the ESR1 and/or coactivators, as has been shown for NCOA3 (Font de Mora & Brown 2000). Increased MAPK3/1-activated pRPS6KA2 has also been shown to enhance the association of CREBBP with the basal transcription machinery potentially increasing the sensitivity of the ESR1 for E_2 (Nakajima *et al.* 1996). In the presence of tamoxifen, inhibition of MAPK3/1 or AKT provided no added benefit. This is in keeping with our ChIP data, which suggested tamoxifen suppressed ESR1-mediated transactivation in both cell lines almost to baseline. By contrast, inhibition of AKT via siRNA knockdown in the absence of tamoxifen appeared to enhance ESR1 transcription.

The significant decrease in proliferation of the Tam^R-1 cells upon inhibition of AKT suggested that this kinase plays a pivotal role in these cells despite the lack of effect on ESR1 transactivation. AKT is known to promote cell survival by phosphorylating the pro-apoptotic protein BAD at ser^{136} preventing its association with Bcl2 and Bcl_{XL} and promoting binding to the protein 14-3-3 (Zha *et al.* 1996). Of note, BAD can also be phosphorylated by pRPS6KA2 at ser^{112} (Bonni *et al.* 1999, Tan *et al.* 1999) and phosphorylation of either or both residues prevents apoptosis. Analysis of the Tam^R-1 cells revealed elevated levels of BAD phosphorylated on both ser^{136} and ser^{112} compared with the MCF-7 cells. Inhibition of both AKT- and MAPK3/1-activated pRPS6KA2 suppressed BAD phosphorylation, providing evidence for the role of these pathways in cell survival. In support, we demonstrated that in the presence of tamoxifen, MCF-7 cells showed a time-dependent increase in apoptosis and concomitant increase in PARP1 cleavage while the Tam^R-1 showed no change. These data support previous observations in which AKT overexpression in MCF-7 cells led to up-regulation of Bcl2 and macrophage inhibitory cytokine 1 providing a link between activation of PI3 kinase and survival pathways leading to inhibition of tamoxifen-induced apoptotic regression (Campbell *et al.* 2001). Similarly in BT474 cells, tamoxifen-induced apoptosis as indicated by assessment of caspase 3 and PARP1 cleavage was inhibited as a result of an ERBB2–ESR1 association (Chung *et al.* 2002).

The question that remained was what mechanism increased the level of both pAKT and pRPS6KA2 In a tamoxifen-resistant variant of MCF-7 cells, Knowlden *et al.* (2003) demonstrated increased basal expression of activated EGFR and ERBB2. They detected heterodimers formed by these growth factor receptors and showed downstream activation on MAPK3/1. Interestingly, phosphorylated ERBB3 levels were lower in their tamoxifen-resistant line compared with the wild-type control cells. Based upon our data, which showed increased expression of ERBB2 and ERBB3 (Fig. 2), we postulated that heterodimers formed from these receptors could be responsible for the downstream activation of signaling pathways in Tam^R-1 cells (Hynes & Lane 2005). However, using immunoprecipitation, we found no evidence for heterodimers between ERBB2 and ERBB3, EGFR and ERBB2, EGFR and ERBB3, EGFR and ERBB4, or ERBB2 and ERBB4. Studies have suggested that ESR1 can be associated with ERBB2 at the plasma membrane leading to the activation of downstream signal transduction pathways (Chung *et al.* 2002, Yang *et al.* 2004). Immunoprecipitation and co-localization studies showed that ESR1 associated with ERBB2 but not with ERBB3 in the Tam^R-1 while no interactions between ESR1 and growth factor receptors were noted in the MCF-7 cells. Treatment with ICI 182780 destroyed the interaction and led to a dose-dependent decrease in pAKT (Fig. 5). Of note, ESR1 staining within the Tam^R-1 cells appeared diffuse and mainly at the membrane compared with the MCF-7 cells in which ESR1 remained nuclear. These observations were also noted in a second tamoxifen-resistant cell line (Fig. 8). Recent studies have shown that the subcellular localization of the ESR1 can be altered. For instance, Kumar *et al.* (2002) demonstrated that a truncated MTA1 protein sequestered ESR1 to the cytoplasm promoting MAPK3/1 activity. Similarly, amplified ERBB2 has been shown to modulate the subcellular localization of the ESR1 and its ability to interact with ERBB2 (Yang *et al.* 2004). In addition, in the presence of tamoxifen, ESR1 may be associated with EGFR in the absence of either protein being up-regulated (Fan *et al.* 2007). These studies further showed that by targeting ERBB2 or EGFR, localization of ESR1 to the nucleus was restored

and proliferation was suppressed (Yang *et al*. 2004, Fan *et al*. 2007). In our studies, neither treatment of Tam^R-1 cells with Herceptin (data not shown) nor AG825 alone showed any growth-suppressive effects. ESR1 remained predominantly at the membrane (with some cytoplasmic or nuclear staining observable) upon treatment with FBS, tamoxifen, or AG825 alone. However, when the Tam^R-1 cells were treated with AG825 in the presence of tamoxifen, a growth-suppressive effect was observed (Fig. 6) accompanied with redistribution of the ESR1 to the nucleus (Fig. 7).

Studies have shown that E_2-bound ESR1 represses ERBB2 transcription by competing for transcription factors such as NCOA1 (Bates & Hurst 1997, Perissi *et al*. 2000). However, tamoxifen-bound ESR1 recruits NCOR allowing ERBB2 transcription to be activated (Newman *et al*. 2000). Hence, long-term treatment with tamoxifen could potentially lead to increased ERBB2 expression influencing the cellular localization of the ESR1. In this setting, inhibition of ERBB2 alone may be ineffective since non-genomic ESR1 may retain the capacity to activate both the MAPK3/1 and AKT signal transduction pathways enabling the promotion of cell survival and ligand-independent phosphorylation of genomic ESR1 resulting in the recruitment of coactivators. This hypothesis is partially supported by recent studies demonstrating that BT474 cells that had acquired resistance to the receptor tyrosine kinase inhibitor lapatinib, used ESR1 signaling to override the growth-suppressive effects of the drug and that by blocking both ESR1 signaling (by E_2 withdrawal) and ERBB2 with lapatinib growth-suppressive effects were maintained (Xia *et al*. 2006). Overall, the data suggest that the Tam^R-1 cells have a high degree of plasticity with the ability to switch between ESR1 and ERBB2 signal transduction pathways being determined by the presence or absence of tamoxifen and further supports the rationale for the combined use of signal transduction inhibitors together with endocrine agents as oppose to monotherapies.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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