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Fulvestrant-induced expression of ErbB3 and ErbB4 receptors sensitizes ER-positive breast cancer cells to heregulin β1

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Abstract

Introduction: We have previously reported that induction of epidermal growth factor receptor (EGFR) and ErbB2 in response to antihormonal agents may provide an early mechanism to allow breast cancer cells to evade the growth inhibitory action of such therapies and ultimately drive resistant cell growth. More recently, the other two members of the ErbB receptor family, ErbB3 and ErbB4, have been implicated in antihormone resistance in breast cancer. In the present study we have investigated whether induction of ErbB3 and/or ErbB4, may provide an alternative resistance mechanism to antihormonal action in a panel of four oestrogen receptor (ER)-positive breast cancer cell lines.

Methods: MCF-7, T47D, BT474 and MDAMB361 cell lines were exposed to fulvestrant (100 nM) for 7 days and effects on ErbB3/4 expression and signalling and cell growth were assessed. Effects of heregulin β1 were also examined in the absence and presence of fulvestrant, to determine the impact of ER blockade on the capacity of this ErbB3/4 ligand to promote signalling and cell proliferation.

Results: Fulvestrant potently reduced ER expression and transcriptional activity and significantly inhibited growth in MCF-7, T47D, BT474 and MDAMB361 cells. However, alongside this inhibitory activity, fulvestrant also consistently induced protein expression and activity of ErbB3 in MCF-7 and T47D cells and ErbB4 in BT474 and MDAMB361 cell lines. Consequently, fulvestrant treatment sensitised all cell lines to the actions of the ErbB3/4 ligand heregulin β1 with enhanced ErbB3/4-driven signalling activity, re-expression of cyclin D1 and significant increases in cell proliferation being observed when compared to untreated cells. Indeed, in T47D and MDAMB361 heregulin β1 was converted from a ligand having negligible or

suppressive growth activity into one that potently promoted cell proliferation. Consequently, fulvestrant-mediated growth inhibition was completely overridden by heregulin β1 in all four cell lines.

Conclusions: These findings would suggest that although antihormones, such as fulvestrant, may have potent acute growth inhibitory activity in ER-positive breast cancer cells, their ability to induce and sensitize cells to growth factors may serve to reduce and ultimately limit their inhibitory activity.

Introduction

The ability of antihormones to inhibit growth of oestrogen receptor (ER)-positive breast cancer cells has principally been attributed to the ability of these agents to block the transcriptional activity of the ER and prevent activation of genes responsible for mediating cell cycle progression, such as cyclin D1 and c-Myc [1]. However, more recent findings have demonstrated that the majority of oestrogen (E2)-regulated genes in human breast cancer cells are repressed, rather than activated, and that many of these genes act as growth and/or transcriptional repressors [2]. Thus, antihormones may exert their anti-proliferative activity not only through suppression of growth promoting genes but also through an ability to induce negative regulators of cell proliferation. Although such an inductive mechanism will undoubtedly enhance the growth inhibitory activity of antihormones in ER-positive breast cancer there is now evidence that this inductive capacity may be a double-edged sword as it has also been demonstrated that estrogens can suppress, and antihormones induce, genes that promote cell proliferation and survival [3-5].

Two key pro-proliferative/survival genes that have been established as E2-suppressed/antihormone-induced genes in a range of ER-positive breast cancer cell lines are the ErbB receptors epidermal growth factor receptor (EGFR) and ErbB2 [2-4, 6-11]. As there is considerable preclinical and clinical evidence that both EGFR and ErbB2 play a central role in driving acquisition of antihormone resistance in breast cancer [12-19] it is possible that antihormones may actually play an active role in limiting their own activity through an ability to promote expression of these potent growth promoters. Indeed, there is now evidence, from *in vitro* and *in vivo* MCF-7 cell models of ER-positive breast cancer, that increased expression of EGFR and ErbB2 is an early response to tamoxifen treatment and that this induction of ErbB

signalling maintains residual activity of key downstream signalling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) cascades [10, 13]. Such signalling may allow cells to evade inhibition during the drug-responsive phase, as targeting these ErbB receptors in combination with tamoxifen suppresses the residual signalling activity and greatly improves and extends the growth inhibitory action of this antihormone in the two cell models [10, 13]. Similar preclinical findings have also been reported for the pure anti-oestrogen fulvestrant [10] and recent clinical studies examining co-targeting ER and EGFR and/or ErbB2 signalling pathways have reported improved response to a range of endocrine therapies in breast cancer patients [20, 21]. Despite these positive findings in clinical trials it should also be noted that a considerable number of patients did not benefit from such combination treatments suggesting that alternative, EGFR/ErbB2-independent, mechanisms of resistance to antihormones are active in the clinical setting [20].

Micro-array studies have now revealed a vast range of E2-suppressed/antihormone-induced genes, in addition to EGFR and ErbB2, which may also play a significant role in modulating response and resistance to endocrine therapies [2-5]. These genes include ErbB3 and ErbB4, the remaining members of the ErbB receptor family which are receptors for the neuregulin (NRG) family of growth factors, NRG1 or heregulin (HRG), NRG2, NRG3 and NRG4 [2, 22-24]. Like EGFR and ErbB2, ErbB4 can form active homodimers, however, ErbB3 is catalytically inactive and thus requires heterodimerization with another ErbB family member to promote signalling [25]. Both ErbB3 and ErbB4 have been shown to be E2-suppressed and tamoxifen induced in ER-positive breast cancer cells, suggesting a potential role for both receptors in antihormone response and resistance [2, 23]. This is supported by the findings of Tang

and colleagues who demonstrated that inoculation of HRG-transfected MCF-7 cells into the mammary fat pads of ovariectomised athymic nude mice can generate oestrogen-independent and anti-oestrogen-resistant tumours [26]. More recently, the weight of evidence has supported a role for ErbB3, rather than ErbB4, with increased activation of ErbB3 being reported in both acquired tamoxifen- and fulvestrant-resistant MCF-7 cells [27, 28] and its down-regulation enhancing responsiveness of ErbB2-overexpressing, de novo antihormone-resistant, breast cancer cells to tamoxifen [29]. Moreover, ErbB1-3 overexpression has been reported to predict for early relapse on tamoxifen therapy in ER-positive breast cancer patients [30, 31]. The involvement of ErbB4 remains unclear as in acquired tamoxifen-resistant MCF-7 cells expression of this receptor is enhanced [28], whereas, in a panel of fulvestrant-resistant MCF-7 cells it is decreased [27]. Furthermore, in agreement with the fulvestrant-resistance studies loss of ErbB4 expression has been reported to be an independent marker of tamoxifen resistance in patients with primary breast cancer [32].

In the present study we have examined the acute inductive capacity of the pure anti-oestrogen fulvestrant on ErbB3 and ErbB4 receptor expression in a panel of four ER-positive breast cancer cell lines, two ErbB2-negative MCF-7 and T47D and two ErbB2-positive BT474 and MDAMB361, and assessed the effect of ligand-activation of these receptors on antihormone-response. We demonstrate that a 7 day fulvestrant treatment induces expression of both ErbB3 and ErbB4 receptors resulting in enhanced sensitivity to the action of HRGβ1 and enabling this ligand to readily promote fulvestrant-resistant cell growth in all four cell lines.

Materials and methods

Cell culture

All tissue culture medium and constituents were purchased from Gibco Europe Ltd. (Paisley, Scotland) and tissue culture plastics were was obtained from Nunc (Roskilde, Denmark). A panel of four ER-positive breast cancer cell lines were used in this study MCF-7, T47D, BT474 and MDAMB361. These cell lines were all maintained in phenol-red-free RPMI (wRPMI) medium containing 5% foetal calf serum (FCS), penicillin-streptomycin (10 iU/ml-10 μg/ml), fungizone (2.5 μg/ml), glutamine (4 mM) at 37^{o}C in a humidified 5% CO_2 atmosphere.

Western Blotting and RT-PCR

Experimental Cell Culture

The 4 cell lines were grown in wRPMI supplemented with 5% FCS for 7 days in the presence of either fulvestrant (100 nM) alone, HRGβ1 (10 ng/ml) alone, a combination of the two agents or the appropriate vehicle control. Further studies were also performed where cell lines were grown in wRPMI supplemented with 5% FCS for 7 days in either the absence or the presence of fulvestrant (100 nM) and subsequently exposed to either HRGβ1 (0.1-10 ng/ml in ethanol; Sigma, Poole, UK) or vehicle control for 5 min. All experiments were performed at least three times.

Protein Cell Lysis

Cells were washed three times with phosphate buffered saline (PBS) and lysed using ice-cold lysis buffer (for composition see [12]. The cellular contents were transferred to microfuge tubes, clarified by centrifugation at 13,000 rpm for 15 minutes at 4^{o}C and supernatant aliquots were stored at -20^{o}C until required. Total protein

concentrations were determined using the DC BioRad protein assay kit (BioRad Labs Ltd, Hemel Hempstead, UK).

Western Blotting

Protein samples from total cell lysates (50 μg) were subjected to electrophoresis separation on a 7.5% polyacrylamide gel and trans-blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Blots were blocked at room temperature for 1 h in 5% skimmed milk powder made up in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated for a minimum of 1 hour in primary antibody diluted 1/40,000 for β-actin (reference control) or 1/1000 for EGFR, ErbB2, ErbB3, ErbB4, AKT , ERK1/2 MAPK, PKC and cyclin D1 in 1% marvel/TBS-Tween. The membranes were washed three times in TBS-Tween and then incubated for 1 hour with the required secondary IgG horseradish peroxidase-labelled donkey anti-rabbit or sheep anti-mouse (Amersham Biosciences UK Ltd., Buckinghamshire, UK), diluted 1/20,000 in 1% marvel/TBS-Tween. Detection was performed using West Dura chemiluminescent detection reagents (Pierce and Warriner Ltd, Chester, UK). Antibodies used were total EGFR (SC-03) ErbB2 (SC-284), ErbB3 (SC-285), ErbB4 (SC-283), phospho-ErbB4 (pY1056, SC-33040) and cyclin D1 (SC20044; Insight Biotechnology Ltd, Wembley, UK). Phospho-ErbB3 (pY1289, 4791), phospho-ErbB4 (pY1248, 4757), phospho-ErbB2 (pY1248, 2247), phospho-EGFR (pY1068, 2234), total AKT (9272), phospho-AKT (pS473, 9271), total ERK1/2 (9102) and phospho-ERK1/2 (pT202/pY204, 9101) (New England Biolabs, Hertfordshire, UK), ERα (ID-5) (DAKO Corp, Ely, UK) and β-actin (AC-15) (Sigma, UK). These antibodies were selected as they have been demonstrated to be mono-specific and do not cross-react with other family members.

RT-PCR

Total RNA was isolated from the four cell lines using an RNA isolator kit (Tri Reagent, Sigma, Poole, UK) and 1 μg was reverse transcribed using standard conditions as described previously [12]. Resultant cDNA samples were amplified using specific primers for progesterone receptor (PgR) and β-actin (house-keeping positive control), respectively, and conditions were optimised as described previously [12]. Briefly, an initial denaturing step of 95^{o}C for 2 minutes was followed by a set number of cycles of 94 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds. PCR products were separated on a 3% w/v agarose gel, containing ethidium bromide, visualised by UV illumination. The primers used for ErbB3, ErbB4, PgR and β-actin were as reported previously [12].

Cell Proliferation

Cell monolayers were grown for 7 days in wRPMI supplemented with 5% FCS in the presence of either fulvestrant (100 nM), fulvestrant and gefitinib (1 μM; a kind gift from AstraZeneca, Macclesfield) , fulvestrant and trastuzumab (100 nM; a kind gift from Roche Diagnostics, Penzburg), HRGβ1 (10 ng/ml), a combination of these agents or the appropriate vehicle control. Cell population growth was then evaluated by means of trypsin dispersion of the cell monolayers and cell number was measured using a Coulter counter (Luton, UK). All experiments were performed in triplicate.

Immunocytochemistry

Experimental Cell Culture

The 4 cell lines were grown on sterile 3-aminopropyltriethoxysilane-coated coverslips at 1×10^4 cells/cm^2 in wRPMI supplemented with 5% FCS for 7 days in the presence

of either fulvestrant (100 nM) alone, HRGβ1 (10 ng/ml) alone, a combination of the two agents or the appropriate vehicle control. The coverslips were then washed with PBS and fixed immediately according to the immunocytochemical assay to be performed. The Ki67 assay used in these studies followed the protocol previously described [33].

Assessment

Immunostaining for each assay was assessed by two personnel using a dual-viewing attachment to an Olympus BH-2 light microscope. Percentage of cells positively stained for nuclear Ki67 was determined using a minimum evaluation of 2000 cells per coverslip.

Statistics

For immunocytochemical analysis, comparisons of % nuclear positivity values were determined using the Mann-Whitney 'U' test for non-parametric data. For growth studies, overall differences between control and treatment groups in all 4 cell lines were determined by one-way analysis of variance. Direct comparisons between control and treatment effects were assessed using a post-hoc t-test with either the Tamhane or the Bonferroni adjustment factor for unequal and equal variances, respectively. Differences were considered significant at the $P \leq 0.05$ level.

Results

Anti-oestrogenic activity of fulvestrant in four ER-positive breast cancer cell lines

Western blotting analysis revealed that treatment of MCF-7, T47D, BT474 and MDAMB361 cell lines with fulvestrant, at a concentration of 100 nM for 7 days, resulted in a substantial reduction in ERα protein levels in all four cell lines. The reduction was most apparent in MCF-7 and T47D cells with expression of ER being virtually abolished, however, ERα expression was still clearly apparent, although greatly reduced, in fulvestrant-treated BT474 and MDAMB361 cells (Figure 1A). Loss of ERα expression in response to fulvestrant treatment was associated with reductions in progesterone receptor (PgR) mRNA and cyclin D1 protein expression in all four cell lines indicative of a reduction in ERα transcriptional activity (Figure 1B). Cell proliferation was also significantly reduced by fulvestrant treatment, in a concentration-dependent manner, in all four cell lines with this pure anti-oestrogen, with the maximal concentration of 100 nM, significantly reducing growth by ~90% in MCF-7 and ~80% in MDAMB361 ($P<0.001$, n=3 for both cell lines; Figure 1C) and~60% in T47D and ~50% in BT474 cells ($P<0.01$, n=3 for both cell lines; Figure 1C). These falls in cell number were mirrored by similar reductions in nuclear Ki67 immunostaining across the four cell lines with 100 nM fulvestrant significantly lowering nuclear Ki67 positivity from 97±1.2% to 55±4.5% in MCF-7 cells ($P<0.01$, n=3), 80±2.2% to 41±3.6% in T47D cells ($P<0.001$, n=3), 38±2.2% to 13±1.3% in BT474 cells ($P<0.001$, n=3) and 82±0.9% to 38±1.8% in MDAMB361 cells ($P<0.001$, n=3) (Table 1, Supplementary figure S1 in Additional file 1).

Induction of ErbB3 and ErbB4 receptor expression and signalling by fulvestrant

Expression of ErbB3 protein was observed across the panel of cell lines, however, ErbB4 protein was only detected in T47D, BT474 and MDAMB361 cell lines (Figure 2A). The expression profile for both ErbB receptors was highly heterogenous across the four cell lines. In the ErbB2-low expressing MCF-7 and T47D cell lines, a 7 day fulvestrant treatment, at a concentration of 100 nM, promoted ErbB3 protein but not mRNA expression in both cell lines (Figure 2B and C). ErbB4 mRNA and protein expression increased in response to fulvestrant in MCF-7 cells, however, in T47D cells ErbB4 mRNA and protein expression was clearly reduced in response to treatment with this pure antioestrogen. (Figure 2B and C). In the ErbB2-positive BT474 and MDAMB361 cell lines, ErbB3 mRNA and protein expression appeared unchanged following treatment with fulvestrant whilst ErbB4 protein, but not mRNA, expression was clearly enhanced in response to this antihormone. (Figure 2B and C). In addition to promoting ErbB3 and ErbB4 receptor protein expression fulvestrant treatment also enhanced basal phosphorylation of both ErbB receptor family members in all four cell lines (Figure 2C). Two ErbB4 tyrosine (Y) phosphorylation sites, Y1284 and Y1056, were examined with increased levels of Y1284 phosphorylation being seen across all four cell lines following fulvestrant treatment and increased Y1056 phosphorylation being observed in the ErbB2-low but not overexpressing cell lines in response to the antihormone (Figure 2C). This enhanced basal ErbB3 and ErbB4 activity was also associated with increased basal phosphorylation of ERK1/2 in the four cell lines, with the antihormone having no effect on total ERK1/2 expression (Figure 2C). A differential effect of fulvestrant on AKT activity was observed in the ErbB2-low compared to ErbB2-over-expressing cell lines with fulvestrant promoting basal AKT phosphorylation in MCF-7 and T47D cells but causing if anything a small

reduction in phosphorylation of this signalling element in BT474 and MDAMB361 cell lines (Figure 2C).

Fulvestrant sensitizes ER-positive cell lines to heregulin β1

Effect of HRGβ1 in the absence of fulvestrant

In the MCF-7 and T47D cell lines, HRGβ1 induced a concentration-dependent activation of ErbB receptors and associated downstream signalling elements, with phosphorylation of both ErbB3 and ErbB4, ERK1/2 and AKT clearly apparent following exposure to 1-10 ng/ml HRGβ1 (Figure 3A). Interestingly, HRGβ1 phosphorylated ErbB4 predominantly at Y1056 in these cells with little effect on Y1284 being observed (data not shown). Activation of these signalling pathways by HRGβ1 was associated with a trend towards increased proliferation of MCF-7 but not T47D cells (Figure 3B). Thus, 10 ng/ml HRGβ1 caused a 20-30% increase in cell numbers, however, no obvious increase in Ki67 immunostaining could be observed as nuclear expression of this protein in control MCF-7 cells was close to 100% (Figure 3B, Table 1, Supplementary figure S1 in Additional file 1). No significant change in T47D cell number was observed following HRGβ1 treatment, however a small but significant reduction in nuclear Ki67 staining from 80% to 63% was noted in response to this ligand ($P<0.05$, n=3; Figure 3B, Table 1, Supplementary figure S1 in Additional file 1).

In the MDAMB361 and BT474 cell lines HRGβ1 similarly promoted a concentration-dependent activation of ErbB3 and ErbB4, AKT and ERK1/2 (Figure 4A), however, in these cell lines HRGβ1 phosphorylated ErbB4 primarily on Y1284 (Figure 4A) with little phosphorylated Y1056 being observed (data not shown). BT474 cells appeared less responsive than MDAMB361 cells to HRGβ1 with respect to activation

of AKT and ERK1/2 with increased activity of both these elements only being seen in response to the highest concentration of this ligand, 10 ng/ml. Interestingly, activation of these signalling pathways had diametrically opposite effects on proliferation of the two cell lines. Thus, HRGβ1 was a modest growth suppressor of MDAMB361 cells, a 10 ng/ml concentration significantly decreasing cell number by ~20% ($P<0.01$, n=3) and significantly reducing Ki67 staining from 82±0.9% to 59±2.2% ($P<0.001$, n=3), but a growth promoter of BT474 cells with cell number significantly increasing by ~ 20% ($P<0.05$, n=3) and nuclear Ki67 positivity rising significantly from 38±2.2% to 48±2.2%, in response to 10 ng/ml of HRGβ1 ($P<0.001$, n=3; Figure 4B, Table 1, Supplementary figure S1 in Additional file 1).

Effects of HRGβ1 in the presence of fulvestrant

Following a 7 day treatment with fulvestrant the MCF-7 and T47D cell lines demonstrated an enhanced sensitivity to increasing concentrations of HRGβ1 with phosphorylation of ErbB receptors, ERK1/2 and AKT being observed at the lower concentration of 0.1 ng/ml HRGβ1 and a greater magnitude of phosphorylation being apparent in response to the higher concentrations of the ligand (Figures 3A). This enhanced signalling response to HRGβ1 was associated with recovery of cyclin D1 protein expression and enhanced proliferative activity with HRGβ1 treatment completely over-riding the growth inhibitory effects of fulvestrant in both cell lines ($P<0.001$, n=3; Figures 3B and 3C). Indeed, fulvestrant treatment converted HRGβ1 from a ligand with limited or negligible proliferative activity to one that potently and significantly promoted cell growth in both cell lines ($P<0.001$, n=3; Figure 5A). This was clearly reflected in the Ki67 immunostaining in MCF-7 cells with HRGβ1 increasing nuclear Ki67 positivity scores from 55±4.5% to 89±1.8% in MCF-7

(P<0.01, n=3), however, in T47D cells although an increase from 41±3.6% to 49±5.8% was observed in response to HRGβ1 in the presence of fulvestrant it did not reach statistical significance (Table 1, Supplementary figure S1 in Additional file 1). Enhanced sensitivity and an increased magnitude of response to HRGβ1 was also observed in MDAMB361 and BT474 cells following fulvestrant exposure with enhanced phosphorylation levels of ErbB4 and ERK1/2 being observed in both cell lines in response to increasing concentrations of this ligand (Figure 4A). HRGβ1-induced activation of ERK1/2 was less apparent in BT474 compared to MDAMB361 cells but a small increase in ERK1/2 activity in response to the lower concentrations of the ligand, particularly 0.1 ng/ml, was still apparent in the BT474 cell line, indicative of a sensitization effect of fulvestrant in these cells. Enhanced HRGβ1-induced ErbB3 phosphorylation was also seen in the MDAMB361 but not the BT474 cells in the presence of fulvestrant (Figure 4A). Interestingly, in both cell lines there was no sensitization of AKT activity in response to HRGβ1, if anything there appeared to be a reduced response to the lower concentrations of this ligand (0.1-1 ng/ml) following fulvestrant treatment which was most apparent in the BT474 cell line (Figure 4A). As a consequence of the enhanced HRGβ1-induced signalling seen in both cell lines in the presence of fulvestrant once again this ligand was able to promote recovery of cyclin D1 protein expression and potently and significantly overcome the growth inhibitory effects of the antihormone in both cell lines (P<0.001, n=3; Figure 4B and 4C). Furthermore, as was observed in the ErbB2-low expressing cell lines, fulvestrant treatment significantly enhanced the proliferative activity of HRGβ1 in BT474 cells and converted this ligand from one that suppressed growth into one that potently and significantly promoted MDAMB361 cell growth (P<0.001, n=3; Figure 5A). This ability of HRGβ1 to promote growth in both cell lines in the

presence of fulvestrant was again reflected in the Ki67 immunostaining with nuclear Ki67 positivity significantly rising from 13±1.3% to 50±1.3% in BT474 and from 38±1.8% to 71±2.2 in MDAMB361 cells, in the presence of 10 ng/ml of this ligand ($P<0.001$, n=3 for both cell lines; Table 1, Supplementary figure S1 in Additional file 1).

Effects of HRGβ1 in the presence of fulvestrant in combination with either gefitinib or herceptin

Treatment of MCF-7 cells with a combination of fulvestrant and either gefitinib (1 μM) or herceptin (100 nM) for 7 days significantly and potently reduced cell growth ($P<0.001$, n=3). Although not statistically significant there was a trend towards the combination providing a greater inhibition of cell growth compared to fulvestrant alone. However, this enhanced growth inhibitory action of the combination treatments did not achieve statistical significance when compared with the fulvestrant alone arm (Figure 5B). Importantly, HRGβ1 treatment was again capable of a partial but statistically significant increase in cell growth in the presence of both fulvestrant and herceptin ($P<0.01$, n=3) and fulvestrant and gefitinib ($P<0.01$, n=3) combination therapies in this cell line (Figure 5B).

Discussion

Antihormonal therapy has proved to be highly successful in the treatment of ER-positive breast cancer, however, resistance to these agents remains a significant clinical problem with many patients either gaining no benefit or relapsing on therapy [15]. Numerous preclinical and clinical studies have established that increased expression of two members of the ErbB receptor family, EGFR and ErbB2, plays a central role in the acquisition of resistance to antihormonal therapies [12-19]. Upregulation of EGFR and ErbB2 has been reported to be an early response to antihormone treatment in ER-positive breast cancer cell lines [2-4, 6-11], however, despite a number of preclinical findings reporting improved magnitude and duration of response with combined targeting of EGFR/ErbB2 and ER signalling [21], translation of these findings into the clinic has proved largely disappointing with a large number of patients gaining little or no benefit from such combination therapies [20]. Further studies have now revealed an array of candidate genes with potential involvement in antihormone resistance including the other members of the ErbB receptor family, ErbB3 and ErbB4. As both ErbB3 and ErbB4 have both been identified as oestrogen-suppressed/ antihormone induced genes in MCF-7 breast cancer cells [22] we have examined whether upregulation of these receptors is an early response to the pure antioestrogen fulvestrant in a panel of four ER-positive breast cancer cell lines, two HER2-overexpressing and two HER2-low expressing, and if so, what effect ligand activation of these receptors has on the acute growth inhibitory activity of this antihormone.

Fulvestrant consistently inhibited growth of all four ER-positive cell lines at day 7 with MCF-7 and MDAMB361 cells demonstrating a greater sensitivity than T47D and BT474 cells to this pure antioestrogen. In all four cell lines the blockade of

growth was associated with substantial reductions in ER protein expression with resultant decreased transcriptional activity, as evidenced by decreased expression of PgR mRNA and cyclin D1 protein expression levels. These findings clearly indicate the potent antioestrogenic activity of fulvestrant and are consistent with previous reports of fulvestrant action in ER-positive breast cancer cell lines [34-36]. However, alongside this potent acute growth inhibitory activity of fulvestrant there was also clear evidence of the inductive capacity of this agent with increased expression of ErbB3 protein expression in the MCF-7 and T47D cells and ErbB4 protein expression in MCF-7, BT474 and MDAMB361 cell lines at day 7 post antihormone treatment. Although ErbB3 and ErbB4 have previously been reported to be oestrogen-suppressed/antihormone-induced genes in MCF-7 cells [22], we believe this is the first report of acute induction of these receptors at the protein level by antihormonal therapy in a panel of HER2-low and overexpressing ER-positive breast cancer cell lines. Interestingly, there was no consistent upregulation of either ErbB receptor across the panel of cell lines, however, increased ErbB3 expression was common to the ErbB2-low expressing cells whilst enhanced ErbB4 levels was common to the two ErbB2-overexpressing cell lines. In the T47D cell line a reduction in ErbB4 mRNA and protein expression was observed following fulvestrant treatment consistent with previous reports that ErbB4 is an oestrogen-induced, and by implication an antihormone suppressed, gene in this cell line [23]. It is also important to note that the increases in ErbB3 protein expression in MCF-7 and T47D cells and ErbB4 protein expression in BT474 and MDAMB361 cells in response to fulvestrant were not mirrored by any substantial changes in mRNA expression of these receptors. Findings that are fully supported by Affymetrix gene array analysis of the expression of these ErbB receptors in the four cell lines prior to and following a 10 day fulvestrant

treatment (data not shown). This would suggest that this acute inductive response to fulvestrant is mediated predominantly by a post transcriptional/translational mechanism.

Although research into the role of ErbB receptor signalling in breast cancer has focused primarily on EGFR and ErbB2, it is becoming increasingly clear that both ErbB3 and ErbB4 also have important roles to play in this disease. ErbB3 has been identified as a key partner for ErbB2, with this receptor heterodimer being identified as an oncogenic unit in breast cancer cells [37], whilst overexpression of ErbB4 has also been shown to promote growth of human breast cancer cells [38,39]. Furthermore, blockade of ErbB3 expression, using an artificial transcription factor E3, inhibits breast cancer cell growth and targeted downregulation of ErbB4, using either ribozymes or siRNA, can reduce growth of MCF-7 and T47D cell lines both *in vitro* and *in vivo* [23, 40]. Both receptors have also been shown to be overexpressed in breast cancer, however, their prognostic significance remain a subject of debate. High expression of ErbB3 has been shown to positively associate with tumour size, recurrence, metastasis and significantly reduced patient survival [41-46]. However, ErbB3 receptor status has also been reported to have a positive prognostic value associating with an ER-positive phenotype and longer disease-free survival for breast cancer patients [45, 47, 48]. Similarly, an association between expression of ErbB4 and a favourable clinical outcome has been reported in some clinical studies [41,45, 48, 49], whilst other researchers have suggested that ErbB4 expression might be a marker of a poorer outcome in some breast cancer patients [43, 50]. These discrepancies are probably due to the fact that the function of these receptors is highly reliant on their localisation and the relative expression levels of other ErbB receptor family members within the tumour cells. For example, both ErbB3 and ErbB4 have

been found to localise not only at the membrane but also within the nucleus in breast cancer cells [51, 52]. The role of nuclear ErbB3 remains unclear, however, levels are higher in non-malignant versus malignant breast epithelial cells suggesting it may have potential anti-proliferative activity [51]. In contrast, ErbB3 localised at the membrane can heterodimerize with other ErbB family members, principally ErbB2, in response to ligand stimulation to potently promote breast cancer cell growth [37]. In the case of ErbB4, nuclear expression of the intracellular domain of this receptor, generated by the proteolytic actions of tumour necrosis factor-α converting enzyme and γ-secretase following ligand binding, promotes ER-driven cell growth [23, 52], whilst cytosolic/membrane localisation of ErbB4 has been shown to inhibit growth and promote apoptosis in breast cancer cells [53-55]. This varied function according to ErbB receptor expression and localisation may also go some way to explain the differential effect of heregulin on growth of the four breast cancer cell lines we observed in the present study. In MCF-7 and BT474 cells HRGβ1 promoted cell growth, whilst this ligand had little effect on T47D cell proliferation and actually suppressed growth of MDAMB361 cells. A possible explanation for this is that the antiproliferative activity of ErbB4 may be more prominent in the T47D and MDAMB361 cell lines as HRGβ1-induced activation of this receptor, relative to ErbB3, was far greater in these cells when compared to the other two cell lines.

In addition to enhancing total protein expression levels, fulvestrant treatment also promoted basal ErbB3 and ErbB4 phosphorylation in all four cell lines, indicative of enhanced signalling through these ErbB receptors. Indeed, the enhanced level of ErbB3/4 phosphorylation in MCF-7 and T47D was associated with increased activation of both the MAPK/ERK1/2 and PI3K/AKT signalling pathways, whilst in the BT474 and MDAMB361 cell lines it was associated with increased ERK1/2 but

not AKT activity possibly reflecting the continued dominant role of ErbB2, a key recruiter of the MAPK pathway, in these cells. This induction of ErbB3/4 signalling by fulvestrant, as previously suggested for EGFR/ErbB2, may provide these cells with a further input into signalling pathways that could potentially allow cells to survive the initial action of the antihormone and ultimately provide a resistance mechanism [5]. Indeed, this is supported by our finding that fulvestrant treatment enhanced the ability of HRGβ1 to stimulate signalling via ErbB3/4 resulting in this ligand potently over-riding the suppression of cyclin D1 protein expression and the blockade of growth by fulvestrant in all four cell lines. Importantly, following fulvestrant treatment HRGβ1 was converted from a ligand having quite differential effects on cell growth into one that consistently and potently promoted proliferation in all four cell lines examined. Interestingly, the mechanisms by which HRGβ1 overcame fulvestrant action were subtly different in the HER2-low and the HER2-overexpressing cell lines. In the MCF-7 and T47D cells fulvestrant selectively enhanced the ability of HRGβ1 to phosphorylate ErbB4 at Y1056, a PI3K-p85 recruitment site [56], and promote AKT signalling activity whereas in the BT474 and MDAMB361 cells it was the HRGβ1-induced ErbB4 Y1284 SHC recruitment site [56] and ERK1/2 phosphorylation that was selectively augmented by antihormonal treatment. The differential ErbB4 phosphorylation and downstream pathway recruitment may reflect the expression of alternative ErbB4 isoforms in these cells, with the CYT-2 isoform, that lacks the Y1056 PI3K binding consensus site, potentially being preferentially expressed in the ErbB2-overexpressing cell lines [57]. It is also possible that such differences may also arise from distinct heterodimerization partners binding ErbB4 in response to HRGβ1 in the ErbB2-low and -overexpressing cell lines following fulvestrant treatment. Studies are currently on-going to examine

these possibilities. In MCF-7 cells it seems the likely heterodimerization partner for ErbB4 is ErbB3 as blockade of either EGFR with gefitinib or ErbB2 with herceptin was without effect on the ability of HRGβ1 to promote cell growth. Importantly, these findings also indicate that HRGβ1 signalling via ErbB3/4 can provide a potent resistance mechanism to such combination therapies and may provide an explanation for the disappointing results from clinical studies examining the combination strategy of antihormones alongside an anti-EGFR/ErbB2 agent [20, 21]. These findings are identical to those of Sonne-Hansen and colleagues who similarly demonstrated that HRGβ1 can over-ride the growth inhibitory effects of fulvestrant in combination with either cetuximab, trastuzumab or pertuzumab in MCF-7 cells [58]. This group went on to show that combining fulvestrant with a pan-ErbB inhibitor effectively prevented HRGβ1-induced growth in this cell line further emphasising the key findings of the present study, that ErbB3 and ErbB4 have the potential to play a central role in the development of resistance to this antihormonal therapy [58]. However, it should also be noted that these studies were all performed in a single cell line and further studies in the other ER-positive cell lines are required to fully support this proposition.

Conclusions

These current findings demonstrate that targeting ER signalling with the pure antioestrogen fulvestrant can both suppress and induce gene and protein expression in ER-positive ErbB2-low and -overexpressing breast cancer cell lines. As expected fulvestrant was a potent growth inhibitor in all four of these ER-positive breast cancer cell lines through its ability to suppress proliferation-related genes such as cyclin D1. However, the simultaneous induction of ErbB3 and ErbB4 also provided a mechanism for these cells, when in a HRGβ1-enriched environment, to promote re-expression of

cyclin D1 and ultimately drive resistant cell growth. What is more fulvestrant treatment converted HRGβ1 from a ligand with both growth promoting and suppressive activity, depending on cell type, into one that consistently and potently promoted cell growth, regardless of ErbB2 status. Thus, although antihormones, such as fulvestrant, may have potent acute growth inhibitory activity in ER-positive breast cancer cells their ability to rapidly induce and sensitize cells to growth factors, such as heregulins, may serve to reduce and ultimately limit their inhibitory activity. Indeed, such a rapid induction of these proliferative genes may provide a mechanism of de novo endocrine resistance in situations where heregulin expression in the tumour microenviroment is high.

Abbreviations

HRGβ1 = heregulin beta1; FCS = foetal calf serum; EGFR = epidermal growth factor receptor; ER = oestrogen receptor; E2 = oestradiol; MAPK = mitogen-activated protein kinase; ERK1/2 = extracellular-signal regulated kinase ½; PI3K = phosphatidylinositol 3-kinase; NRG = neuregulin; wRPMI = phenol red-free Roswell Park Memorial Institute, PBS = phosphate-buffered saline; TBS = Tris-buffered saline; PCR = polymerase chain reaction; PgR = progesterone receptor; Y = tyrosine.

Competing interests

IRH, JMWG and RIN are in receipt of funding from AstraZeneca, HEF is funded by a BBSRC AstraZeneca CASE studentship and RIN is also a member of an advisory board for AstraZeneca. The remaining authors declare that they have no competing interests.

Authors' contributions

IRH conceived the study, participated in its design and execution and drafted the manuscript. LG and JMK carried out the western blotting studies and HEF performed the RT-PCR analysis. RM and DB performed all cell cultures and carried out the growth studies. JMWG carried out the immunocytochemistry and with RIN participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the manuscript.

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References

1 Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL: **Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells.** *Endocr Relat Cancer* 2003, **10**:179-186.

2. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS. **Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype**. *Endocrinology* 2003, **144**:4562-4574.

3. Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. **Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells.** *Cancer Res* 2004, **64**:1522-1533.

4. Gee JM, Shaw VE, Hiscox SE, McClelland RA, Rushmere NK, Nicholson RI. **Deciphering antihormone-induced compensatory mechanisms in breast cancer and their therapeutic implications.** *Endocr Relat Cancer* 2006, **13** Suppl 1:S77-88.

5. Gee JMW, Stone A, McClelland RA, Hiscox SE, Hutcheson IR, Jordon NJ, Fiegl HM, Widschwendter M, Shaw VE, Barrow D, Nicholson RI. **The dark side of antihormonal action in breast cancer.** *In: Therapeutic resistance to anti-hormonal drugs in breast cancer: New molecular aspects and their potential as targets.* Edited by: Hiscox S, Gee J and Nicholson RI, Springer Publishing, London. 2009: 63-84.

6. Bates NP, Hurst HC. **An intron 1 enhancer element mediates oestrogen-induced suppression of ERBB2 expression**. *Oncogene* 1997, **15:** 473–481.

7. Newman SP, Bates NP, Vernimmen D, Parker MG, Hurst HC. **Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer.** *Oncogene* 2000, **19:** 490–497.

8. Yarden RI, Wilson MA, Chrysogelos SA. **Estrogen suppression of EGFR expression in breast cancer cells: a possible mechanism to modulate growth.** *Journal of Cellular Biochemistry* 2001, **81:** 232–246.

9. Wilson MA, Chrysogelos SA **Identification and characterization of a negative regulatory element within the epidermal growth factor receptor gene first intron in hormone-dependent breast cancer cells.** *Journal of Cellular Biochemistry* 2002, **85:** 601–614.

10. Gee JM, Harper ME, Hutcheson IR, Madden TA, Barrow D, Knowlden JM, McClelland RA, Jordan N, Wakeling AE, Nicholson RI. **The antiepidermal**

growth factor receptor agent gefitinib (ZD1839/Iressa) improves anti-hormone response and prevents development of resistance in breast cancer *in vitro.* *Endocrinology* 2003, **144:** 5105–5117.

11. Hurtado A, Holmes KA, Geistlinger TR, Hutcheson IR, Nicholson RI, Brown M, Jiang J, Howat WJ, Ali S, Carroll JS. **Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen.** *Nature* 2008, **456**:663-6.

12. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE & Nicholson RI. **Elevated levels of epidermal growth factor receptor/c-ErbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells**. *Endocrinology* 2003, **144:** 1032–1044.

13. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, Weiss H, Rimawi M, Schiff R. **Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function.** *Cancer Res* 2008, **68**: 826-33.

14. Nicholson RI, Gee JM, Knowlden JM, McClelland RA, Madden T-A, Barrow D, Hutcheson IR. **The biology of antihormone failure in breast cancer.** *Breast Cancer Res Treat* 2003 **80**: S29-S34.

15. Nicholson RI, Hutcheson IR, Jones HE, Hiscox SE, Giles M, Taylor KM, Gee JM. **Growth factor signalling in endocrine and anti-growth factor resistant breast cancer.** *Rev Endocr Metab Disord*. 2007, **8**: 241-253.

16. Nicholson RI, McClelland RA, Finlay P, Eaton CL, Gullick WJ, Dixon AR, Robertson JF, Ellis IO, Blamey RW. **Relationship between EGF-R, c-ErbB2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity.** *Euro J Cancer* 1993, **29A**: 1018-1023.

17. Nicholson RI, McClelland RA, Gee JMW, Manning DL, Cannon P, Robertson JF, Ellis IO, Blamey RW. **Epidermal growth factor receptor expression in breast cancer: Association with response to endocrine therapy.** *Breast Cancer Res Treat* 2004, **29**:117-125.

18. Gee JM, Robertson JF, Gutteridge E, Ellis IO, Pinder SE, Rubini M & Nicholson RI. **Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer.** *Endocrine-Related Cancer* 2005, **12** (Suppl 1): S99–S111.

19. Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, Schiff R, Osborne CK & Dowsett M. **Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase.** *Journal of Clinical Oncology* 2005, **23:** 2469–2476.

20. Normanno N, Morabito A, De Luca A, Piccirillo MC, Gallo M, Maiello MR, Perrone F. **Target-based therapies in breast cancer: current status and future perspectives**. *Endocr Relat Cancer* 2009, **16**: 675-702.

21. Johnston SR. **New strategies in estrogen receptor-positive breast cancer.** *Clin Cancer Res* 2010, **16**: 1979-1987.

22. Revillion F, Pawlowski V, Lhotellier V, Louchez MM, Peyrat JP. **mRNA expression of the type I growth factor receptors in the human breast cancer cells MCF-7: regulation by estradiol and tamoxifen.** *Anticancer Res* 2003, **23**: 1455-1460.

23. Zhu Y, Sullivan LL, Nair SS, Williams CC, Pandey AK, Marrero L, Vadlamudi RK, Jones FE. **Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells**. *Cancer Res* 2006, **66**: 7991-7998.

24. Hayes NV, Gullick WJ. **The neuregulin family of genes and their multiple splice variants in breast cancer.** *J Mammary Gland Biol Neoplasia* 2008, **13**: 205-214.

25. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA. **Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors.** *Proc Natl Acad Sci U S A.* 1989, **86**: 9193-9197.

26. Tang CK, Perez C, Grunt T, Waibel C, Cho C, Lupu R. **Involvement of heregulin-beta2 in the acquisition of the hormone-independent phenotype of breast cancer cells.** *Cancer Res* 1996, **56**: 3350-3358.

27. Frogne T, Benjaminsen RV, Sonne-Hansen K, Sorensen BS, Nexo E, Laenkholm AV, Rasmussen LM, Riese DJ 2nd, de Cremoux P, Stenvang J, Lykkesfeldt AE. **Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant.** *Breast Cancer Res Treat* 2009, **114**: 263-275.

28. Ghayad SE, Vendrell JA, Larbi SB, Dumontet C, Bieche I, Cohen PA. **Endocrine resistance associated with activated ErbB system in breast cancer cells is reversed by inhibiting MAPK or PI3K/Akt signaling pathways.** *Int J Cancer* 2010, **126**: 545-562.

29. Liu B, Ordonez-Ercan D, Fan Z, Edgerton SM, Yang X, Thor AD. **Downregulation of ErbB3 abrogates ErbB2-mediated tamoxifen resistance in breast cancer cells.** *Int J Cancer* 2007, **120**: 1874-1882.

30. Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG. **Outcome and human epidermal growth factor receptor (HER) 1-4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling.** *Breast Cancer Res*. 2004, 6: R246-R251.

31. Folgiero V, Avetrani P, Bon G, Di Carlo SE, Fabi A, Nisticò C, Vici P, Melucci E, Buglioni S, Perracchio L, Sperduti I, Rosanò L, Sacchi A, Mottolese M, Falcioni R. **Induction of ErbB-3 expression by alpha6beta4 integrin contributes to tamoxifen resistance in ERbeta1-negative breast carcinomas.** *PLoS One* 2008, **3**: e1592.

32. Guler G, Iliopoulos D, Guler N, Himmetoglu C, Hayran M, Huebner K. **Wwox and Ap2gamma expression levels predict tamoxifen response.** *Clin Cancer Res* 2007, **13**: 6115-6121.

33. Albanell J, Codony-Servat J, Rojo F, Del Campo JM, Sauleda S, Anido J, Raspall G, Giralt J, Roselló J, Nicholson RI, Mendelsohn J, Baselga J. **Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments.** *Cancer Res* 2001, **61**: 6500-6510.

34. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, McCue BL, Wakeling AE, McClelland RA, Manning DL, Nicholson RI. **Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer.** *J Natl Cancer Inst* 1995, **87**: 746-50.

35. Nicholson RI, Gee JM, Manning DL, Wakeling AE, Montano MM, Katzenellenbogen BS. **Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen-sensitive and -resistant experimental and clinical breast cancer.** *Ann N Y Acad Sci* 1995, **761**: 148-63.

36. McClelland RA, Manning DL, Gee JM, Anderson E, Clarke R, Howell A, Dowsett M, Robertson JF, Blamey RW, Wakeling AE, Nicholson RI. **Effects of short-term antiestrogen treatment of primary breast cancer on estrogen receptor mRNA and protein expression and on estrogen-regulated genes.** *Breast Cancer Res Treat* 1996, **41**: 31-41.

37. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. **The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation.** *Proc Natl Acad Sci U S A.* 2003, **100**: 8933-8938.

38. Junttila TT, Sundvall M, Lundin M, Lundin J, Tanner M, Härkönen P, Joensuu H, Isola J, Elenius K. **Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells.** *Cancer Res* 2005, **65**: 1384-1393.

39. Määttä JA, Sundvall M, Junttila TT, Peri L, Laine VJ, Isola J, Egeblad M, Elenius K. **Proteolytic cleavage and phosphorylation of a tumor-associated ErbB4 isoform promote ligand-independent survival and cancer cell growth**. *Mol Biol Cell* 2006, **17**: 67-79.

40. Tang CK, Concepcion XZ, Milan M, Gong X, Montgomery E, Lippman ME. **Ribozyme-mediated down-regulation of ErbB-4 in estrogen receptor-**

positive breast cancer cells inhibits proliferation both in vitro and in vivo. *Cancer Res* 1999, **59**: 5315-5322.

41. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. **Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer**. *J Pathol* 2003, **200:** 290–297.

42. Travis A, Pinder SE, Robertson JF, Bell JA, Wencyk P, Gullick WJ, Nicholson RI, Poller DN, Blamey RW, Elston CW, Ellis IO. **C-ErbB-3 in human breast carcinoma: expression and relation to prognosis and established prognostic indicators.** *Br J Cancer* 1996, **74**: 229-233.

43. Bieche I, Onody P, Tozlu S, Driouch K, Vidaud M, Lidereau R. **Prognostic value of ERBB family mRNA expression in breast carcinomas.** *Int J Cancer* 2003, **106:** 758–765.

44. Lemoine NR, Barnes DM, Hollywood DP, Hughes CM, Smith P, Dublin E, Prigent SA, Gullick WJ, Hurst HC. **Expression of the ERBB3 gene product in breast cancer**. *Br J Cancer* 1992, **66**: 1116–1121.

45. Koutras AK, Fountzilas G, Kalogeras KT, Starakis I, Iconomou G, Kalofonos HP. **The upgraded role of HER3 and HER4 receptors in breast cancer**. *Crit Rev Oncol Hematol*. 2010, **74**: 73-78.

46. Chiu CG, Masoudi H, Leung S, Voduc DK, Gilks B, Huntsman DG, Wiseman SM. **HER-3 overexpression is prognostic of reduced breast cancer survival: a study of 4046 patients.** *Ann Surg*. 2010, **251**: 1107-1116.

47. Lee Y, Cho S, Seo JH, Shin BK, Kim HK, Kim I, Kim A. **Correlated expression of ErbB-3 with hormone receptor expression and favourable clinical outcome in invasive ductal carcinomas of the breast**. *Am J Clin Pathol* 2007, **128:** 1041–1049.

48. JKnowlden JM, Gee JM, Seery LT, Farrow L, Gullick WJ, Ellis IO, Blamey RW, Robertson JF, Nicholson RI. **c-ErbB-3 and c-ErbB-4 expression is a feature of the endocrine responsive phenotype in clinical breast cancer**. *Oncogene* 1998, **17:** 1949–1957.

49. Suo Z, Risberg B, Kalsson MG, Willman K, Tierens A, Skovlund E, Nesland JM. **EGFR family expression in breast carcinomas. c-ErbB-2 and c-ErbB-4 receptors have different effects on survival.** *J Pathol* 2002, **196**: 17–25.

50. Lodge AJ, Anderson JJ, Gullick WJ, Haugk B, Leonard RC, Angus B. **Type 1 growth factor receptor expression in node positive breast cancer: adverse prognostic significance of c-ErbB-4.** *J Clin Pathol* 2003, **56**: 300-304.

51. Offterdinger M, Schöfer C, Weipoltshammer K, Grunt TW. **c-ErbB-3: a nuclear protein in mammary epithelial cells**. *J Cell Biol* 2002, **157:** 929–939.

52. Ni CY, Murphy MP, Golde TE, Carpenter G. **gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase**. *Science* 2001, **294**: 2179-81.

53. Sartor CI, Zhou H, Kozlowska E, Guttridge K, Kawata E, Caskey L, Harrelson J, Hynes N, Ethier S, Calvo B, Earp HS 3rd. **Her4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells**. *Mol Cell Biol* 2001, **21:** 4265–4275.

54. Naresh A, Long W, Vidal GA, Wimley WC, Marrero L, Sartor CI, Tovey S, Cooke TG, Bartlett JM, Jones FE. **The ERBB4/HER4 intracellular domain 4ICD is a BH3-only protein promoting apoptosis of breast cancer cells**. Cancer Res 2006, **66**: 6412–6420.

55. Chen X, Levkowitz G, Tzahar E, Karunagaran D, Lavi S, Ben-Baruch N, Leitner O, Ratzkin BJ, Bacus SS, Yarden Y. **An immunological approach reveals biological differences between the two NDF/heregulin receptors, ErbB-3 and ErbB-4.** *J Biol Chem* 1996, **271**: 7620-9.

56. Cohen BD, Green JM, Foy L, Fell HP. **HER4-mediated biological and biochemical properties in NIH 3T3 cells. Evidence for HER1-HER4 heterodimers.** *J Biol Chem* 1996, **271**: 4813-8.

57. Elenius K, Choi CJ, Paul S, Santiestevan E, Nishi E, Klagsbrun M. **Characterization of a naturally occurring ErbB4 isoform that does not bind or activate phosphatidyl inositol 3-kinase.** *Oncogene* 1999, **18**: 2607-15.

58. Sonne-Hansen K, Norrie IC, Emdal KB, Benjaminsen RV, Frogne T, Christiansen IJ, Kirkegaard T, Lykkesfeldt AE. **Breast cancer cells can switch between estrogen receptor alpha and ErbB signaling and combined treatment against both signaling pathways postpones development of resistance.** *Breast Cancer Res Treat* 2010, **121**: 601-13.

Table 1. Effects of fulvestrant, HRGβ1 or a combination of the two treatments on immunocytochemically-determined nuclear Ki67 positivity in MCF-7, T47D, BT474 and MDAMB361 cells

	% nuclear Ki67 positivity			
	control	**fulvestrant**	**HRGβ1**	**fulvestrant + HRGβ1**
MCF-7	97±1.2	55±4.5***	90±0.4	89±1.8††
T47D	80±2.2	41±3.6***	63±2.7”	49±5.8
BT474	38±2.2	13±1.3***	48±2.2””	50±1.3†††
MDAMB361	82±0.9	38±1.8***	59±2.2***	71±2.2†††

Nuclear positivity percentage values are expressed as mean ± standard deviation of the assessment of six fields of view per coverslip in triplicate experiments.*** P<0.001 cf. control, ** P<0.01 cf. control, * P<0.05 cf. control, ††† P<0.001 cf. fulvestrant, †† P<0.01 cf. fulvestrant. HRGβ1, heregulin beta1.

Figure legends

Figure 1: Effect of a 7 day incubation of MCF-7, T47D, BT474 and MDAMB361 cells with either fulvestrant (100 nM) or vehicle control (ethanol) on (A) ER protein and (B) PgR mRNA (upper bands), β-actin (lower bands) and cyclin D1 protein expression. Data are representative of at least three separate experiments. β-actin protein expression was also assessed for all Western blotting studies (not shown) to confirm equivalent sample loading. (C) Effects of increasing concentrations of fulvestrant (0.01-1 μM) on the basal growth of MCF-7, T47D, BT474 and MDAMB361 cells on day 7 from initial treatment. The results are expressed as mean±SEM of triplicate wells and are representative of three separate experiments. * $P<0.05$ vs control (0), ** $P<0.01$ vs control (0), *** $P<0.001$ vs control (0).

Figure 2: (A) ErbB3 and ErbB4 protein expression in MCF-7, T47D, BT474 and MDAMB361 cells maintained in phenol-red-free RPMI medium containing 5% FCS. (B) RT-PCR analysis of ErbB3 and ErbB4 mRNA expression and (C) Western analysis of total and phosphorylated ErbB3, ErbB4, AKT and ERK1/2 protein expression in MCF-7, T47D, BT474 and MDAMB361 cells prior to and following treatment with fulvestrant (100 nM) for 7 days. β-actin was used as a loading control (not shown for RT-PCR).

Figure 3: (A) Effect of increasing concentrations of HRGβ1 (0.1-10 ng/ml for 5 minutes) on total and phosphorylated ErbB3, ErbB4, AKT and ERK1/2 protein expression in MCF-7 and T47D cells, maintained for 7 days in the presence of either fulvestrant (100 nM) of vehicle control (ethanol). (B) Cyclin D1 protein expression in

MCF-7 and T47D cells maintained for 7 days in the presence of either fulvestrant or vehicle control and subsequently exposed to either HRGβ1 or vehicle control for 5 minutes. β-actin was used as a loading control. (C) Effect of HRGβ1 (10 ng/ml) on growth of MCF-7 and T47D cells maintained for 7 days in the presence of either fulvestrant or vehicle control (ethanol). The results are expressed as mean±SEM of triplicate wells and are representative of three separate experiments. ** P<0.01 vs control, *** P<0.001 vs control, ††† P<0.001 vs fulvestrant

Figure 4: (A) Effect of increasing concentrations of HRGβ1 (0.1-10 ng/ml for 5 minutes) on total and phosphorylated ErbB3, ErbB4, AKT and ERK1/2 protein expression in BT474 and MDAMB361 cells, maintained for 7 days in the presence of either fulvestrant (100 nM) of vehicle control (ethanol). (B) Cyclin D1 protein expression in BT474 and MDAMB361 cells maintained for 7 days in the presence of either fulvestrant or vehicle control and subsequently exposed to either HRGβ1 or vehicle control for 5 minutes. β-actin was used as a loading control. (C) Effect of HRGβ1 (10 ng/ml) on growth of BT474 and MDAMB361 cells maintained for 7 days in the presence of either fulvestrant (100 nM) or vehicle control (ethanol). The results are expressed as mean±SEM of triplicate wells and are representative of three separate experiments. * P<0.05 vs control, *** P<0.001 vs control, ††† P<0.001 vs fulvestrant

Figure 5: (A) Effect of HRGβ1 (0.1-10 ng/ml) on growth of MCF-7, T47D, BT474 and MDAMB361 cells maintained for 7 days in the presence of either fulvestrant (100 nM) or vehicle control (ethanol). * P<0.05 vs control (0), ** P<0.01 vs control (0), *** P<0.001 vs control (0). (B) Effect of HRGβ1 (10 ng/ml) on growth of MCF-7, T47D, BT474 and MDAMB361 cells maintained for 7 days in the presence of

fulvestrant alone (100 nM), fulvestrant in combination wither either gefitinib (1 μM) or trastuzumab (100 nM) or appropriate vehicle control. The results are expressed as mean±SEM of triplicate wells and are representative of three separate experiments. *** P<0.001 vs no treatment, †† P< 0.01 vs fulvestrant + herceptin, ††† P< 0.001 vs fulvestrant + gefitinib.

Additional files

Additional file 1: Supplementary Figure S1. Effect of HRGβ1 (10 ng/ml) on nuclear Ki67 immunostaining in MCF-7, T47D, BT474 and MDAMB361 cells maintained for 7 days in the presence of either fulvestrant (100 nM) or vehicle control (ethanol).

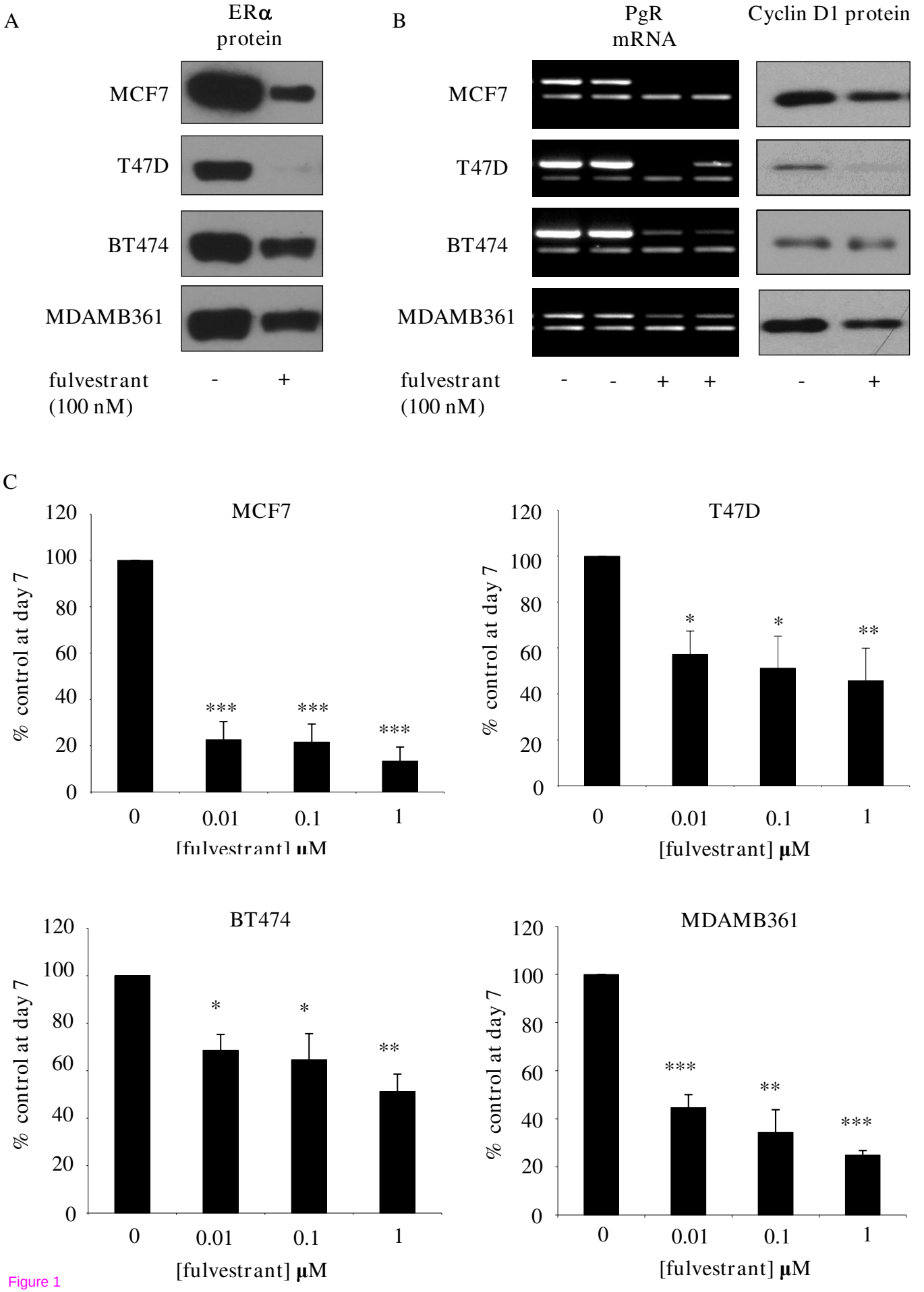
A
ERα
protein
MCF7
T47D
BT474
MDAMB361
fulvestrant
(100 nM)
-
+
B
PgR
mRNA
Cyclin D1 protein
MCF7
T47D
BT474
MDAMB361
fulvestrant
(100 nM)
- - + + - +
C
MCF7
T47D
BT474
MDAMB361
% control at day 7
120
100
80
60
40
20
0
***
*
**
0
0.01
0.1
1
[fulvestrant] µM

Figure 1

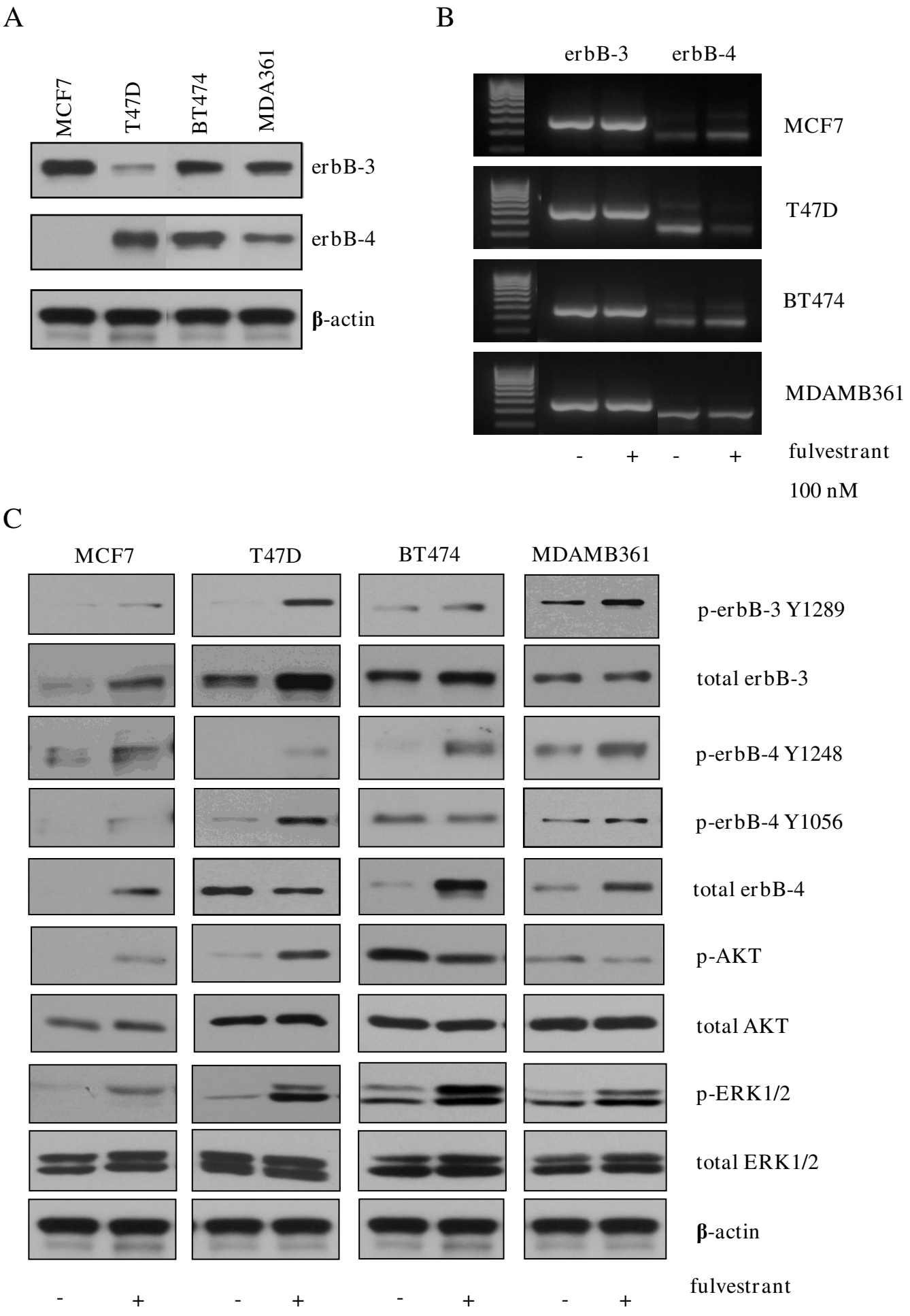
A
MCF7
T47D
BT474
MDA361
erbB-3
erbB-4
β-actin
B
erbB-3
erbB-4
MCF7
T47D
BT474
MDAMB361
- + - +
fulvestrant
100 nM
C
MCF7
T47D
BT474
MDAMB361
p-erbB-3 Y1289
total erbB-3
p-erbB-4 Y1248
p-erbB-4 Y1056
total erbB-4
p-AKT
total AKT
p-ERK1/2
total ERK1/2
β-actin
- + - + - + - +
fulvestrant
100 nM

Figure 2

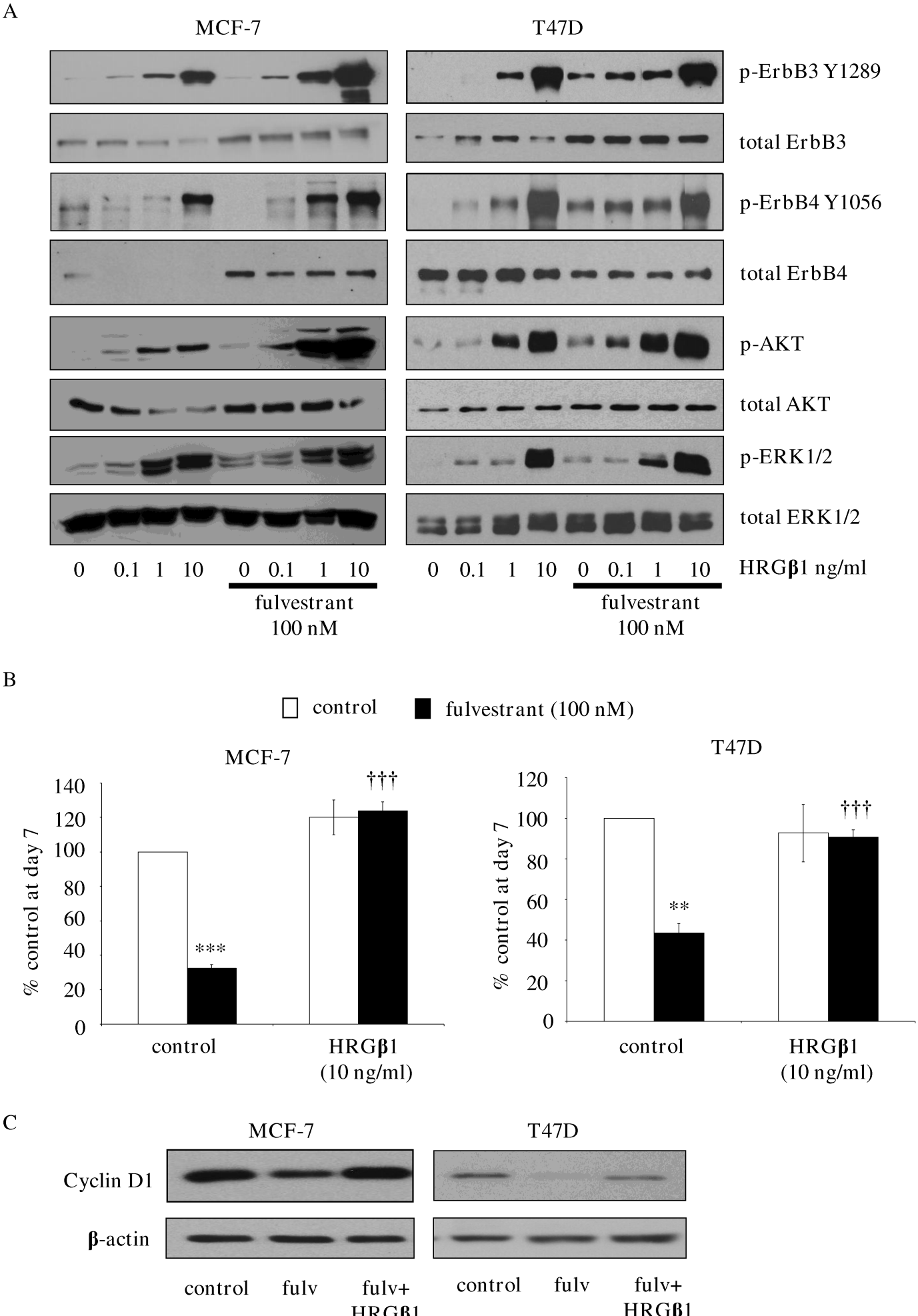
A
MCF-7
T47D
p-ErbB3 Y1289
total ErbB3
p-ErbB4 Y1056
total ErbB4
p-AKT
total AKT
p-ERK1/2
total ERK1/2
0 0.1 1 10 0 0.1 1 10
HRGβ1 ng/ml
fulvestrant 100 nM
fulvestrant 100 nM
B
control
fulvestrant (100 nM)
MCF-7
T47D
% control at day 7
***
**
†††
control
HRGβ1 (10 ng/ml)
C
MCF-7
T47D
Cyclin D1
β-actin
control
fulv
fulv+ HRGβ1

Figure 3

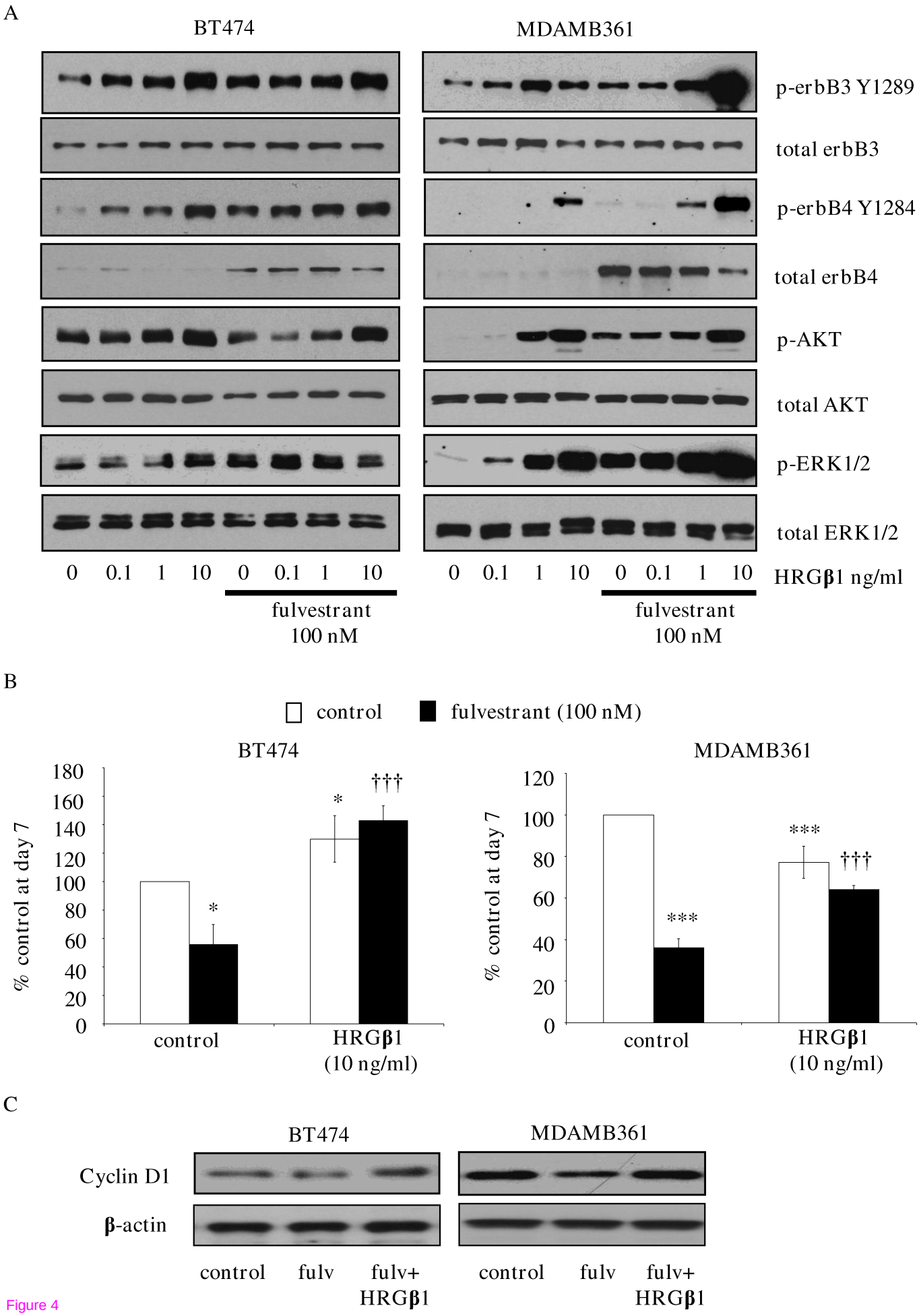
A
BT474
MDAMB361
p-erbB3 Y1289
total erbB3
p-erbB4 Y1284
total erbB4
p-AKT
total AKT
p-ERK1/2
total ERK1/2
0 0.1 1 10 0 0.1 1 10
HRGβ1 ng/ml
fulvestrant 100 nM
fulvestrant 100 nM
B
control
fulvestrant (100 nM)
BT474
MDAMB361
% control at day 7
control
HRGβ1 (10 ng/ml)
C
BT474
MDAMB361
Cyclin D1
β-actin
control
fulv
fulv+ HRGβ1

Figure 4

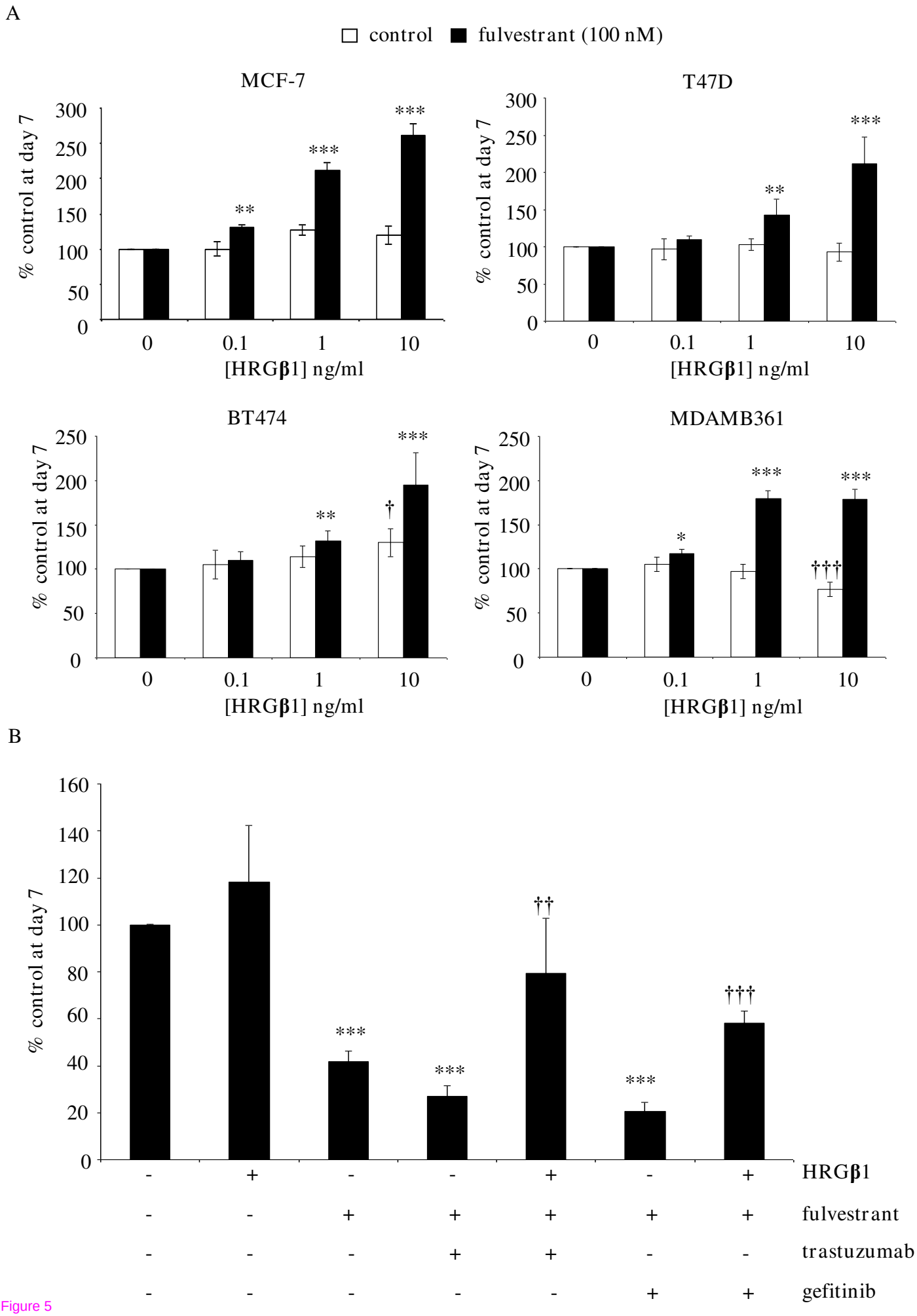
A
control
fulvestrant (100 nM)
MCF-7
T47D
BT474
MDAMB361
% control at day 7
[HRGβ1] ng/ml
0
0.1
1
10
B
HRGβ1
fulvestrant
trastuzumab
gefitinib

Figure 5

Additional files provided with this submission:

Additional file 1: Supplementary Figure 1.pdf, 217K
http://breast-cancer-research.com/imedia/8715827104737436/supp1.pdf