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Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant

Thomas Frogne · Rikke V. Benjaminsen · Katrine Sonne-Hansen ·
Boe S. Sorensen · Ebba Nexo · Anne-Vibeke Laenholm · Louise M. Rasmussen ·
David J. Riese II · Patricia de Cremoux · Jan Stenvang · Anne E. Lykkesfeldt

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Abstract Seven fulvestrant resistant cell lines derived from the estrogen receptor α positive MCF-7 human breast cancer cell line were used to investigate the importance of epidermal growth factor receptor (ErbB1-4) signaling. We found an increase in mRNA expression of EGFR and the ErbB3/ErbB4 ligand heregulin2 (hrg2) and a decrease of ErbB4 in all resistant cell lines. Western analyses confirmed the upregulation of EGFR and hrg2 and the downregulation of ErbB4. Elevated activation of EGFR and ErbB3 was seen in all resistant cell lines and the ErbB3 activation occurred by an autocrine mechanism. ErbB4 activation was observed only in the parental MCF-7 cells. The downstream kinases pAkt and pErk were increased in five of seven and in all seven resistant cell lines, respectively. Treatment with the EGFR inhibitor gefitinib

preferentially inhibited growth and reduced the S phase fraction in the resistant cell lines concomitant with inhibition of Erk and unaltered Akt activation. In concert, inhibition of Erk with U0126 preferentially reduced growth of resistant cell lines. Treatment with ErbB3 neutralizing antibodies inhibited ErbB3 activation and resulted in a modest but statistically significant growth inhibition of two resistant cell lines. These data indicate that ligand activated ErbB3 and EGFR, and Erk signaling play important roles in fulvestrant resistant cell growth. Furthermore, the decreased level of ErbB4 in resistant cells may facilitate heterodimerization of ErbB3 with EGFR and ErbB2. Our data support that a concerted action against EGFR, ErbB2 and ErbB3 may be required to obtain complete growth suppression of fulvestrant resistant cells.

T. Frogne · R. V. Benjaminsen · K. Sonne-Hansen ·
L. M. Rasmussen · J. Stenvang · A. E. Lykkesfeldt (✉)
Department of Tumor Endocrinology, Institute of Cancer
Biology, Danish Cancer Society, Strandboulevarden 49,
2100 Copenhagen, Denmark
e-mail: al@cancer.dk

B. S. Sorensen · E. Nexo
Department of Clinical Biochemistry, Aarhus University
Hospital, Aarhus, Denmark

A.-V. Laenholm
Department of Pathology, Odense University Hospital,
Odense, Denmark

D. J. Riese II
Department of Medicinal Chemistry and Molecular
Pharmacology, Purdue University and Purdue Cancer Center,
West Lafayette, IN, USA

P. de Cremoux
Department of Tumor Biology, Institute Curie, Paris, France

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Gefitinib · Autocrine stimulation

Introduction

The selective estrogen receptor (ER) modulator tamoxifen has been first line endocrine therapy for ER positive breast cancer patients for more than 25 years, but patients with advanced disease and initially responsive tumors will eventually develop resistance [1]. Treatment with the steroidal antiestrogen fulvestrant has proven effective upon progression on tamoxifen therapy, both in vitro and in vivo [2–4] and is now approved for second-line treatment after tamoxifen or aromatase inhibitors. Fulvestrant shows several superior qualities compared to tamoxifen; it is devoid of agonistic activity and strongly downregulates the ER protein. Furthermore, fulvestrant binds the ER with a

higher affinity, suppresses ER regulated genes more strongly and is more potent than tamoxifen in inhibiting ER driven cell growth [5]. As for tamoxifen treatment of advanced breast cancer, resistance will inevitably occur also for fulvestrant. Clarification of the molecular changes associated with the resistant growth is urgently needed to find targeted treatment to resistant tumor cells and treatments that can inhibit or delay the emergence of resistance.

We have established fulvestrant resistant cell lines by treating MCF-7 cells with 100 nM fulvestrant and after several months, resistant cell lines were obtained from single colonies of cells. The resistant cell lines are grown in presence of fulvestrant, but upon withdrawal of fulvestrant, the resistant phenotype is stable and the cells still express ER α , although at a reduced level [6]. Analysis of global gene expression in tamoxifen or fulvestrant resistant MCF-7 cell lines has shown that tamoxifen resistant cells primarily alter expression of genes downstream of ER. In contrast, fulvestrant resistant cells were independent of ER signaling and displayed a strong remodeling of gene expression, including activation of compensatory growth promoting signals [7]. Thus, resistance to fulvestrant may originate from altered expression of several genes, all resulting in the resistant phenotype but with different origin. The molecular details underlying acquired resistance to fulvestrant are only beginning to emerge, but at least six proteins have been associated with growth of fulvestrant resistant cell lines: EGFR/ErbB2 and Erk [7, 8], Akt [9], NF κ B [10] and β -catenin [7].

The observed changes in fulvestrant resistant breast cancer cells indicate a switch from growth promoted by the ER to dependence on growth factor receptor signaling, particularly via the ErbB receptors. The ErbB system comprises four receptors and numerous activating ligands; at least 11 ErbB ligands have been reported [11, 12]. Upon ligand binding the receptors dimerize, become phosphorylated and hence activated, resulting in regulation of a variety of cellular processes including cell proliferation and survival. Akt and Erk represent downstream signaling molecules, frequently found activated by the ErbB system [11]. Expression of EGFR, ErbB2 and ErbB3 in breast tumors is associated with ER negativity and poor survival, whereas tumors overexpressing ErbB4 had increased survival [13]. Also, several of the ErbB ligands have been shown to be implicated in breast cancer and in antiestrogen resistance, including TGF α , hrg1 and amphiregulin [14–16]. Many anti-ErbB agents have been developed and several are currently used in the clinic [17]. The best documented inhibitors are the anti-ErbB2 antibody trastuzumab (HerceptinTM) and the kinase inhibitor gefitinib. However, both drugs show limited effect in the clinic and good markers for response to gefitinib have not been established [18]. Recent reports have suggested that this could be due to lack of inhibition of ErbB3, and ErbB3

has been suggested to be a marker for resistance to EGFR-targeted therapy [19, 20].

The work presented here is an assessment of the ErbB receptors, ligands and the downstream kinases Akt and Erk in seven fulvestrant resistant cell lines. We show that all seven resistant cell lines have increased activation of EGFR, ErbB3 and Erk, and decreased activity of ErbB4 and also possess an increased amount of mRNA and protein of the ErbB3/ErbB4 ligand hrg2. By treatment with the EGFR inhibitor gefitinib, neutralizing antibodies to ErbB3 or the Erk inhibitor U0126, we show growth dependence upon active signaling from EGFR, ErbB3 and Erk in fulvestrant resistant cell lines. Our data indicate that fulvestrant in combination with therapies targeting ErbB3 and EGFR will be required for treatment of fulvestrant resistant cells.

Materials and methods

Cell lines, culture conditions and reagents

The MCF-7 cell line was obtained from the Human Cell Culture Bank, Mason Research Institute, Rockville, MD and adapted to grow in phenol red free DMEM/F12 (1:1) (Life Technologies) supplemented with 1% FCS, Glutamax 2,5 mM and 6 ng/ml insulin (Novo Nordisk). The fulvestrant resistant cell lines MCF-7/182^R-1 (182^R-1), MCF-7/182^R-6 (182^R-6), MCF-7/182^R-7 (182^R-7), MCF-7/164^R-1 (164^R-1), MCF-7/164^R-4 (164^R-4), MCF-7/164^R-5 (164^R-5) and MCF-7/164^R-7 (164^R-7) were established as described earlier [6] and were maintained in growth medium supplemented with 100 nM fulvestrant. These conditions for MCF-7 and the resistant cell lines are from hereon designated growth medium. Stock solutions of 0.1 mM fulvestrant (FaslodexTM, Tocris) were dissolved in 96% ethanol. Gefitinib (IressaTM) was obtained from AstraZeneca. Recombinant Hrg1 β was from R&D systems (396-HB/CF) and was added for 15 min. at 10 ng/ml, which is equivalent to app. 1.25 nM (an 8 kDa peptide). Recombinant GST-NRG2 β (hrg2 β) was produced by Dr. Riese as described earlier [21] and we used 50 nM of this hrg2 β fusion protein.

Quantitative real-time RT-PCR (qPCR)

Total RNA was purified with a kit from Qiagen. 0.1 μ g total RNA were used for reverse transcription according to the suppliers' instructions (Applied Biosystems) with a primer consisting of 16 dT residues. For measurement of mRNA levels under basal growth conditions, all cell lines were seeded in 6-well plates and grown in their respective growth media and then harvested at 70–80% confluence.

All PCR reactions were performed using a Lightcycler instrument and the Sybr Green I real time PCR kit both from Roche and quantifications are presented as the ratio between the amount of target gene and the amount of β -actin mRNA in each sample, as previously described [22]. For primer sequences and PCR profiles please see [23] (ErbB1-4), [22] (hrg1-4) or [24] (EGF, TGF α , HB-EGF, betacellulin, amphiregulin and epiregulin).

Western blot (WB) analysis

For measurements under basal growth conditions, all cell lines were seeded in 6-well plates and grown in their respective growth media and harvested at 70–80% confluence. For experiments with gefitinib, the Erk inhibitor U0126 (#9903, Cell Signaling) or the ErbB3 neutralizing antibody Ab5 (see Table 1), the cells were treated for the last 24 h before harvest. Whole cell extracts were generated by lysing the cells on ice in RIPA buffer (100 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 0.5% sodiumdeoxycholate, 0.1% SDS and 1 mM EDTA) with the addition of 1 mM DTT, 1 mM NaF, 10 mM β -glycerophosphate, 100 μ M Na₃VO₄, 150 μ M PMSF and one tablet/10 ml complete mini protease inhibitor cocktail (Roche). 10–30 μ g of total protein per sample determined by Bio-Rad protein assay kit were separated on 3–8% Tris-Acetate, 4–12% Bis-Tris (Nupage from Invitrogen) or 15% SDS-PAGE gels under reducing conditions. The proteins were transferred to an Immobilon-P membrane (Millipore) and immunostaining was performed by blocking the membrane in TBS containing 5% dry-milk, 0.2% FCS and 0.1% Tween-20. Incubation with primary antibodies in blocking buffer was done overnight at 4°C. All antibodies are presented in Table 1. The enhanced chemiluminescence (ECL+) detection system (Amersham) was used to visualize the protein bands, and then detected using a CCD camera (LAS-1000, Fuji) or by exposure to Hyperfilm (Amersham). The antibodies were removed from the membrane in stripping buffer (62.5 mM Tris-HCl, 100 mM β -mercaptoethanol and 2% (w/v) SDS, pH 6.7), washed and then reprobbed with the next antibody.

Immunohistochemistry (IHC)

Paraffin-embedded cell pellets were obtained by harvesting with trypsin, fixation in Lilliss fluid (VWR, #10276) and subsequent dehydration in a histokinette. A paraffin embedded block was made by insertion of one 2 mm core from each cell pellet. Sections at 5 μ m from this block were prepared for IHC with antibody against pEGFR, pErbB3 and total ErbB4 (see Table 1). Heat induced antigen retrieval was performed by microwave oven in T-EG buffer (10 mM Tris, 0.5 mM EGTA, pH 9) for

Table 1 Antibody list

| Antigen | Assay & dilution | Company & catalog number |
|---------------------------------|----------------------|--------------------------|
| EGFR | WB 1:10.000 | Fitzgerald, 20-ES04 |
| p ¹¹⁷³ EGFR | IHC 1:200 | Cell Signaling, 4407 |
| ErbB2 | WB 1:2000 | Neomarkers, MS-730 |
| p ¹²⁴⁸ ErbB2 | WB 1:250 | Neomarkers, MS-1072 |
| ErbB3 | WB 1:2000 | Neomarkers, MS-201 |
| p ¹²⁸⁹ ErbB3 | WB 1:500 IHC 1:500 | Cell Signaling, 4791 |
| ErbB3 | Growth 10 μ g/ml | Neomarkers, MS-303 (Ab5) |
| ErbB4 | IHC 1:250 | Neomarkers, RB-9045 |
| ErbB4 | WB 1:500 | Cell Signaling, 4795 |
| ErbB4 | IP 10 μ g/mg | Neomarkers, RB-274 |
| pTyr | WB 1:1000 | Cell Signaling, 9411 |
| Hrg2 | WB 1:1000 | Abnova, H9542 |
| Erk1/2 | WB 1:2000 | Cell Signaling, 9102 |
| p ^{202/204} Erk1/2 | WB 1:1000 | Cell Signaling, 4377 |
| Akt | WB 1:2000 | Cell Signaling, 9272 |
| p ⁴⁷³ Akt | WB 1:500 | Cell Signaling, 9271 |
| p ^{21/9} GSK3/ β | WB 1:1000 | Cell Signaling, 9331 |
| p ^{221/227} RSK1/2 | WB 1:2000 | R&D systems, AF892 |
| Hrg2 α | WB 1:250 | Dunn et al. [28] |
| Hrg2 β | WB 1:100 | Dunn et al. [28] |
| Hsp70 | WB 1:400.000 | Neomarkers, MS-482 |
| Anti-mouse Ig | WB 1:2000 | DAKO, P0448 |
| Anti-rabbit Ig | WB 1:2000 | DAKO, P0260 |
| Anti-sheep Ig | WB 1:2000 | DAKO, P0163 |
| Anti-mouse Ig (following IP) | WB 1:2500 | ABR, SA1-100 |

The antibodies used in this study are denoted by their respective antigen, application (assay and dilution) and the supplier and their catalog number. In the column designated “antigen”, the numbers in superscript denote the specific phospho-site recognized by this antibody. These phospho-sites have all been shown to correlate with activation of the protein. WB: western blot, IP: immunoprecipitation, IHC: immunohistochemistry

15 min. The slides were incubated with primary antibody for 60 min at room temperature and as detection system for the primary antibody the AdvanceTM HRP (DAKO) was used. The immunostaining was performed using the automated immunostainer Tech-mate 500 (DAKO) and known positive and negative controls were included.

Immunoprecipitation (IP)

Whole cell lysates were generated as for western analysis, however by pooling one 6 well plate/IP sample. The IP lysates were pre-cleared by gentle rotation with 20 μ l agarose beads (SantaCruz, #sc-2003)/mg protein for 1 hour at 4°C. The beads were pelleted and the supernatant was incubated overnight at 4°C with primary antibody and

20 μ l beads. The antibody for ErbB4 was used as noted in Table 1. After IP, the samples were washed four times in PBS and western blotting was performed using an antibody against phosphorylated tyrosine (pTyr). To visualize the protein bands we used a cross-absorbed secondary antibody (see Table 1) and super signal west dura ECL (Pierce, #34075). The membrane was then stripped and reprobed with the antibodies used for western analysis of ErbB4. As negative control we substituted the ErbB4 IP antibody with a pool of total IgG from non-immunized rabbits (DAKO, X0936).

Growth experiments

All cell lines were seeded in 24-well plates in growth medium and allowed to adhere for one or two days. At start of the experiment (day 0), experimental medium containing either vehicle (0.1% DMSO), or increasing concentration of gefitinib or U0126 was added. Medium was renewed after 3 days, and cell number determination was performed at day 5 with a colorimetric assay as described previously [9]. Each experiment was performed in triplicate. A two-sample equal variance *t*-test was used to test for statistically significant effects of the treatments ($P < 0.05$). The same setup was used for the experiments with the ErbB3 neutralizing antibody (Ab5).

Conditioned media

MCF-7, 164^R-5 and 164^R-7 cells were seeded in T-150 flasks and grown in their respective growth media. When cells reached 70% confluence, fresh medium was added for 24 h, collected and concentrated (100 \times) by ultrafiltration using a membrane with a molecular weight cut-off of 20 kDa (iCONTM Concentrator, Pierce). MCF-7 cells grown in standard growth medium were treated for 15 min. with the concentrated, conditioned medium, and cell lysates for western blot analysis were obtained as described. As negative control we used our regular growth medium (incl. serum), which was concentrated in parallel to the conditioned medium.

FACS analysis

All cell lines were seeded in 6-well plates and grown in their respective growth medium for 3–4 days. The cells were treated with vehicle (0.1% DMSO), gefitinib or U0126 for 48 h before harvest by trypsination followed by resuspension in PBS containing 1% FCS and then washed twice in PBS without FCS. We adjusted each sample to contain app. 10⁶ cells followed by addition of 70% ethanol and incubation at minus 20°C for at least 24 h. Pretreatment with RNase A (25 μ g/ml, #R4642 from Sigma) was

performed before staining with propidium iodide (50 μ g/ml, #P4170 from Sigma). The fluorescence signal from 10,000 cells in each sample was measured using a Beckson Dickinson FACS sorter and a quantitative measurement of the cell cycle distribution was obtained using the ModFit software. A two-sample equal variance *t*-test was used to test for statistically significant effects of the treatments ($P < 0.05$).

Results

Expression of the ErbB receptors in fulvestrant resistant breast cancer cell lines

We have measured the expression level of all four ErbB receptors at the mRNA, total protein and activated phosphorylated protein level. All ErbB receptors were expressed in the parental MCF-7 cells, but the level of EGFR mRNA was extremely low as also described in a previously published work [25]. Figure 1a shows mRNA levels of the four ErbB receptors in seven fulvestrant resistant sublines expressed relative to the level in parental MCF-7 cells. We found between 2.5 and 5 fold upregulation of EGFR mRNA in five of the resistant cell lines, while the 182^R-1 and 164^R-5 cells showed only a minor increase in EGFR mRNA level. In all fulvestrant resistant cell lines, a strong downregulation of ErbB4 mRNA was observed. In contrast to EGFR and ErbB4, no major differences were observed for the expression of the ErbB2 and ErbB3 mRNA.

In general, the total protein level of the four receptors correlated with the mRNA levels, as studied by western blots of whole cell extracts (Fig. 1b). A large collection of antibodies was tested to detect the extremely low level of EGFR protein and only the 20-ES04 antibody recognized protein in MCF-7 cells. Two protein bands were observed and the lower band corresponds to EGFR, whereas the upper band is presumably ErbB2, which is expressed at significantly higher amounts. The EGFR protein level was increased in all resistant cell lines and the highest protein levels were observed in the five resistant cell lines with the highest mRNA levels. The Erb4 protein level was markedly reduced in all resistant cell lines, while no major change was observed for the protein levels of ErbB2 and ErbB3. Importantly, we found upregulation of pErbB3 in all seven resistant cell lines, with the highest levels in 164^R-5 and 164^R-7 (Fig. 1b). The pErbB2 levels in the resistant cells were equal to or reduced compared to the MCF-7 cell line. Due to the low expression level of the EGFR, we were unable to detect the activated form of the EGFR by western blotting either with the use of commercially available phosphospecific antibodies or by

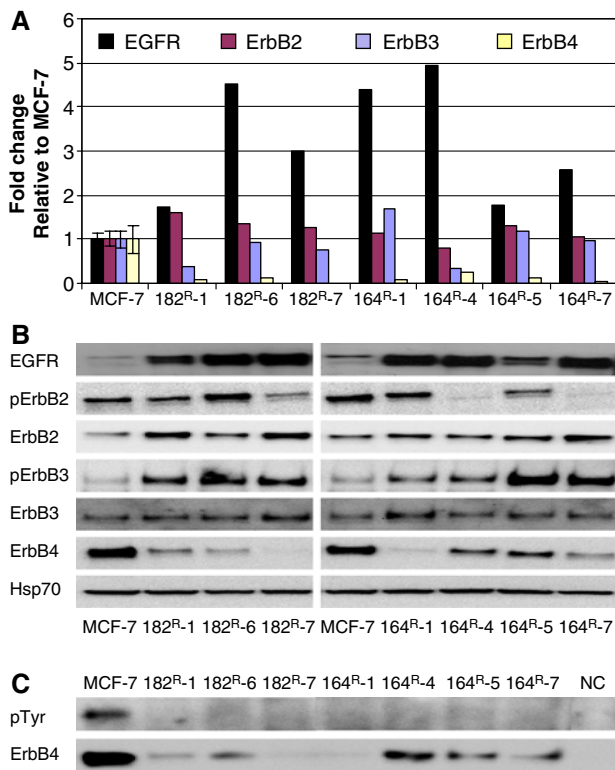


Fig. 1 ErbB receptor expression and activation. **(a)** Real-time RT-PCR quantification of EGFR, ErbB2, ErbB3 and ErbB4 mRNA levels in MCF-7 and seven fulvestrant resistant cell lines grown in standard growth medium. As the data are presented relative to MCF-7, four independent measurements were performed on this cell line and standard deviations are shown. For the resistant cell lines, two independent determinations were done and a representative result is shown. **(b)** Western blots showing total levels of EGFR, ErbB2, ErbB3 and ErbB4 and phosphorylated levels of ErbB2 and ErbB3 from MCF-7 and the resistant cell lines grown in their standard growth medium. Hsp70 serves as loading control. Two independent experiments were performed and similar results were obtained. **(c)** Immunoprecipitation of ErbB4 from MCF-7 and resistant cell lines grown in their standard growth medium. The IP membrane was analyzed for phospho-tyrosine, stripped and reprobed for total ErbB4 protein. Negative control (NC) was done on MCF-7 cell lysate using total IgG from non immunized rabbits. Two independent experiments were performed and similar results were obtained

immunoprecipitation. ErbB4 was immunoprecipitated and the activated form of ErbB4 detected with antibodies to phosphorylated tyrosine (Fig. 1c). Only the parental MCF-7 cells possessed a measurable amount of ErbB4 tyrosine phosphorylation.

Activated EGFR, activated ErbB3 and total ErbB4 were detected by immunohistochemical analysis (Fig. 2a–c). MCF-7 cells did not display detectable staining of the activated EGFR or ErbB3, whereas membrane staining was observed for both activated EGFR and ErbB3 in the seven fulvestrant resistant cell lines. A significantly more intense membrane staining for ErbB4 was observed in MCF-7 cells compared to resistant cells.

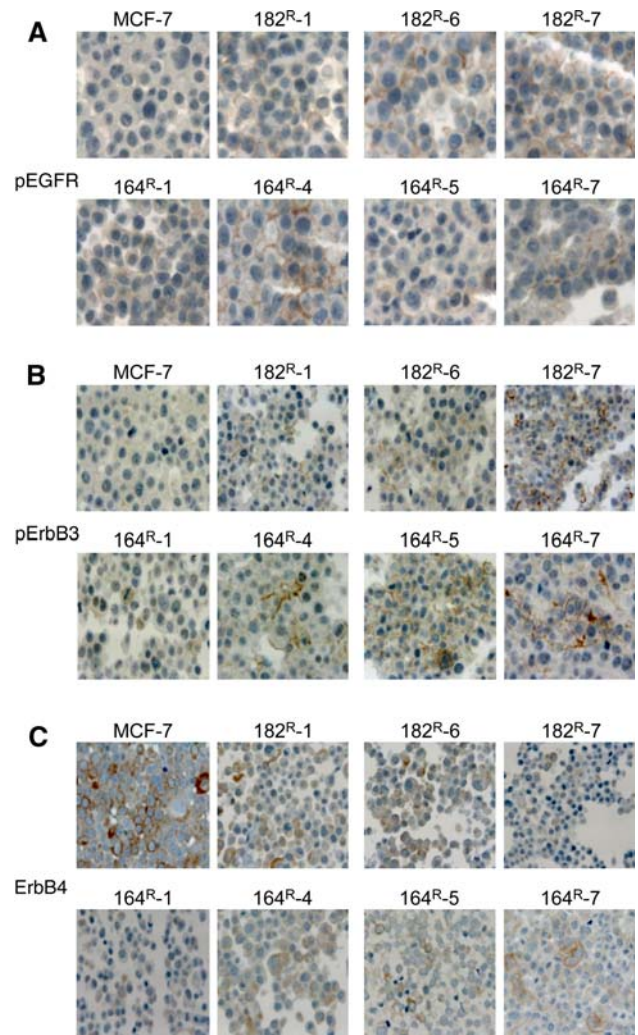


Fig. 2 Immunohistochemical determination of pEGFR, pErbB3 and total ErbB4. Immunohistochemical staining showing **(a)** pEGFR; **(b)** pErbB3 and **(c)** total amount of ErbB4 in MCF-7 and resistant cell lines grown in their standard growth medium. All stainings were done simultaneously using sections from a paraffin block with all cell lines present as 2 mm cores from paraffin-embedded cell pellets. The pictures are representative of two independent experiments

Expression of the ErbB downstream signaling kinases Erk, RSK, Akt and GSK3

Erk and Akt are classical ErbB downstream targets, and we measured the level of phosphorylated Akt and Erk, and their substrates GSK3 and p90RSK (RSK), respectively (Fig. 3). A considerable and corresponding increase in pErk and its downstream activated substrate pRSK was observed in all resistant cell lines, while increased pAkt and pGSK3 were seen in five of the seven resistant cell lines. Thus, increased activation of Erk and/or Akt occurs in the fulvestrant resistant cell lines.

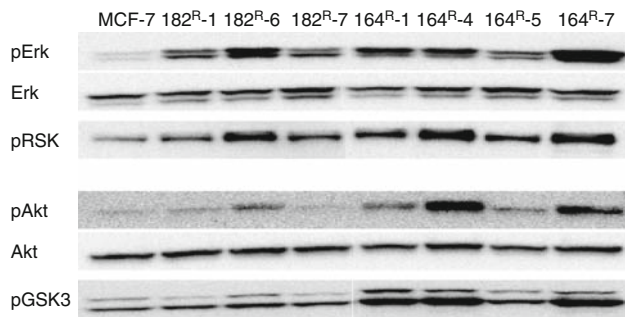


Fig. 3 Activation of the kinases Erk, Akt, RSK and GSK3. Western blots showing total and phosphorylated levels of the kinases Erk and Akt and their substrates RSK and GSK3, respectively. Total proteins were extracted from MCF-7 and the resistant cell lines grown in their standard growth medium. Total levels of Akt and Erk serve as loading control. Two independent experiments were performed and comparable results obtained

Effect of the EGFR inhibitor gefitinib on growth, cell cycle distribution, ErbB phosphorylation and downstream signaling

Based on results from our screening, we selected the four 164^R cell lines and our 182^R-6 cell line for experiments with gefitinib, thereby encompassing all observed changes in ErbB signaling, including increased expression of pEGFR, pErbB3, pAkt, pGSK3, pErk, pRSK and also severely reduced ErbB4 activation (see Figs. 1a, b, 2a and 3). To determine whether ErbB receptor activation is involved in growth of resistant cell lines in presence of fulvestrant, we treated the cells with 1, 5 and 10 μ M gefitinib (Fig. 4a). At 1 μ M gefitinib, which is presumed to preferentially target the EGFR, MCF-7 cell growth was not affected, whereas 4 of the 5 resistant cell lines were significantly growth inhibited by approximately 30%, suggesting that EGFR activation is involved in antiestrogen resistant cell growth. At higher concentrations, also MCF-7 cell growth was affected but the resistant cell lines were more sensitive. To further assess the effect of gefitinib, we performed cell cycle analysis of all cell lines treated with 1 or 5 μ M gefitinib for 48 h (Fig. 4b). Again, the MCF-7 cells were not affected by 1 or 5 μ M gefitinib and treatment with 5 μ M gefitinib resulted in only a minor increase in the G1 phase fraction. When the fulvestrant resistant cells received the same treatment, we observed a significantly stronger increase in G1 and concomitant decrease in the S phase fraction for the 5 μ M gefitinib treatment. Overall, gefitinib is a more potent inhibitor of both cell proliferation and cell cycle progression into S phase in the fulvestrant resistant cell lines.

The EGFR expression is extremely low in our cell lines and we were not able to detect pEGFR by western analysis, thus also unable to demonstrate inhibition of basal pEGFR expression with 1 μ M gefitinib. However, when we

overexpress the EGFR in our MCF-7 cells and treat them with the EGFR ligands TGF α or HB-EGF we see an induction in pEGFR levels and importantly, this increase in phosphorylation is completely prevented by treatment with 1 μ M gefitinib (data not shown). Based on these experiments and the inhibition of pErk expression by 1 μ M gefitinib (Fig. 4), we believe that 1 μ M gefitinib inhibits basal pEGFR expression in our cell lines. We measured the activated form of the other ErbB family members in cells, which were treated for 24 h with vehicle, 1 or 10 μ M gefitinib (Fig. 4c). We found that the phosphorylation of ErbB2 in MCF-7 cells was slightly reduced by 1 μ M gefitinib and more severely with 10 μ M. In the resistant cell lines, gefitinib had a small effect on ErbB2 phosphorylation in the 182^R-6, the 164^R-1 and the 164^R-4 cell lines. In contrast, 1 μ M gefitinib exerted a reproducible reduction in pErbB3 expression in four of five resistant cell lines and close to complete inhibition of pErbB3 expression was observed with 10 μ M, whereas the total ErbB3 level remained unchanged. A strong downregulation of pErk was observed in all cell lines already at 1 μ M gefitinib, whereas pAkt was unaffected, except in the 164^R-1 cell line (Fig. 4d).

Effect of the Erk inhibitor U0126 on growth, cell cycle distribution and pErk

The results with gefitinib treatment suggest that activation of Erk by the EGFR is important for growth of the fulvestrant resistant cell lines. In accordance, pErk was upregulated in all fulvestrant resistant cell lines (see Fig. 3) and we therefore investigated the effect of Erk inhibition on growth of the fulvestrant resistant cell lines. Growth studies (Fig. 5a) and cell cycle analyses (Fig. 5b), were performed, using 1, 5 or 10 μ M of the Erk inhibitor U0126. The results strongly resembled those of the gefitinib treatments although inhibition of the 164R-5 cell line only reached statistical significance at 5 μ M U0126 in the growth assay. By western blotting of pErk, we verified that U0126 inhibited the Erk activation (Fig. 5c).

Treatment with neutralizing antibodies to ErbB3

The increased level of pErbB3 but not total ErbB3 expression in all resistant cell lines indicates that resistant cells may produce ligands, which activate ErbB3. In order to test this and to measure whether ligand activation of ErbB3 contributes to resistant cell growth, we performed experiments with an antibody Ab5, which has been described to block ErbB3 activation by inhibition of ligand binding [26, 27]. Firstly, we confirmed that Ab5 treatment (10 μ g/ml) inhibited basal pErbB3 expression in both MCF-7 and 164^R-5 cells, when the cells were grown in

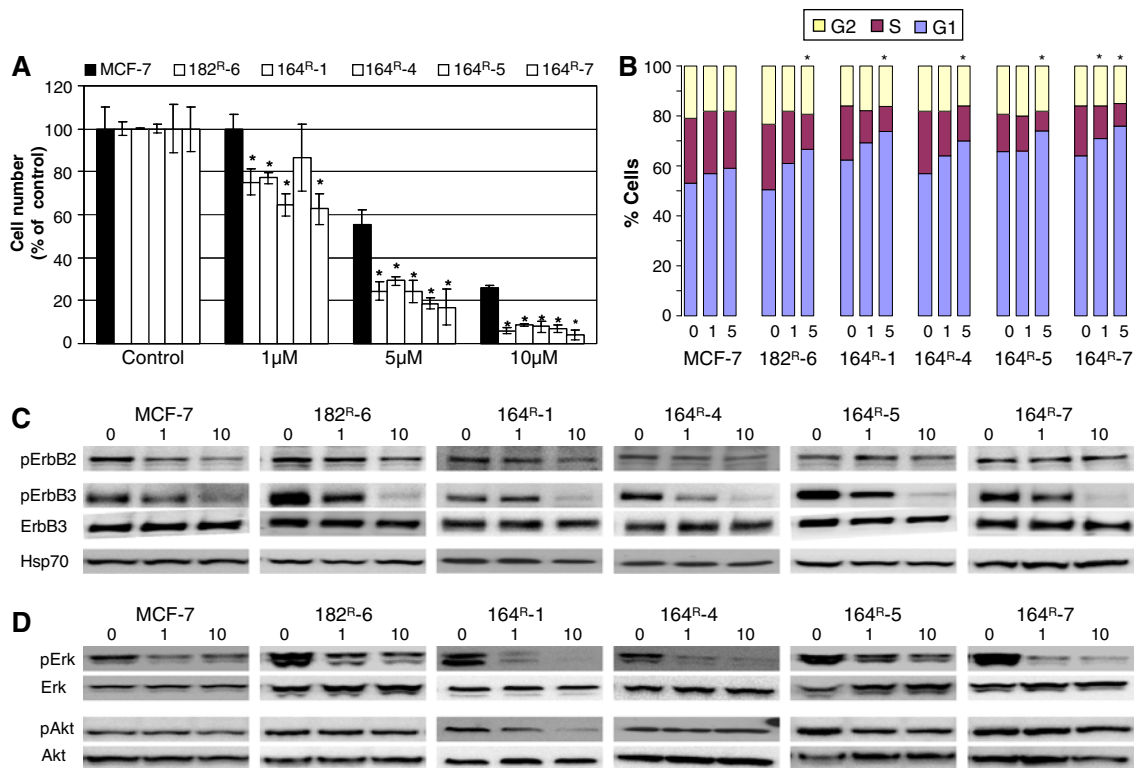


Fig. 4 Effect of gefitinib on growth and cell cycle distribution and on ErbB2, ErbB3, Erk and Akt phosphorylation. **(a)** Cell numbers of MCF-7 and five resistant cell lines were determined after five days of gefitinib treatment. Cell number is expressed relative to its own vehicle treated control. Between three and six independent experiments were performed with reproducible results and a representative experiment is shown. The error bars are standard deviations. Statistical comparison of MCF-7 and the resistant cell lines was done for cultures receiving treatment with the same gefitinib concentration, and statistical significances ($P < 0.05$) are denoted by *. **(b)** Cell cycle distribution was assessed after 48 hours of treatment with gefitinib using FACS analysis. Each bar shows the distribution of the three cell cycle phases in response to treatment with 1 or 5 μM gefitinib for 48 h. The figure is showing a representative result and at least three independent experiments with

reproducible results were performed. Statistically significant differences between MCF-7 and the resistant cell lines receiving the same treatment are denoted by *. **(c)** Western blots showing phosphorylated levels of ErbB2 and total and phosphorylated levels of ErbB3 in MCF-7 and five resistant cell lines. Hsp70 is loading control. All cell lines were grown in their standard growth medium for 3 or 4 days followed by incubation with vehicle or gefitinib for 24 h. It should be mentioned that standard growth medium for the resistant cell lines includes 100 nM fulvestrant. Three independent experiments were performed and equivalent results were observed. **(d)** Western blots showing total and phosphorylated levels of Erk and Akt protein in MCF-7 and the five resistant cell lines grown in their standard growth medium and incubated with 1 or 10 μM gefitinib for 24 h. Three independent experiments were performed and a representative result is shown

their standard growth medium (Fig. 6). Preincubation with Ab5 for one hour followed by addition of the ligands hrg1 β as well as hrg2 β (15 min) totally blocked the strong induction of pErbB3 in both cell lines. Thus, also in this model system, the Ab5 antibody is able to inhibit activation of ErbB3, by both endogenous and exogenous ligands. Downstream, induction with hrg1 β or hrg2 β resulted in pAkt and pErk stimulation in both MCF-7 and resistant 164R-5 cells (Fig. 6). Preincubation with Ab5 for one hour before addition of ligands, totally blocked the induction of pAkt in both cell lines. In contrast, the pErk induction by hrg2 β was not affected by Ab5 treatment, and the hrg1 β mediated induction of pErk was only partially inhibited with Ab5 antibodies. Interestingly, no inhibition of the basal levels of pAkt or pErk was observed with Ab5 treatment.

Treatment of MCF-7 cells with Ab5 did not result in modulation of growth, Fig. 7a. However, in the resistant cell line 164R-5, we observed a statistically significant growth inhibition of 20%. In another resistant cell line, the 164R-7 cell line, no statistically significant effect on cell growth was seen with the Ab5 antibody. Additional growth experiments with the Ab5 antibodies were performed in another fulvestrant resistant cell line, the 164R-1 cell line, which displayed a statistically significant growth inhibition of 20% (data not shown). In the MCF-7 cells, the low level of pErbB3 was reduced by Ab5 treatment, and in the 164R-5 and 164R-7 cells, the much higher pErbB3 level was extensively down regulated (Fig. 7b). Hence, Ab5 treatment significantly reduces the activation of ErbB3, which is presumed to occur from autocrine production of ligand. We also looked at the level of pAkt and pErk in response to

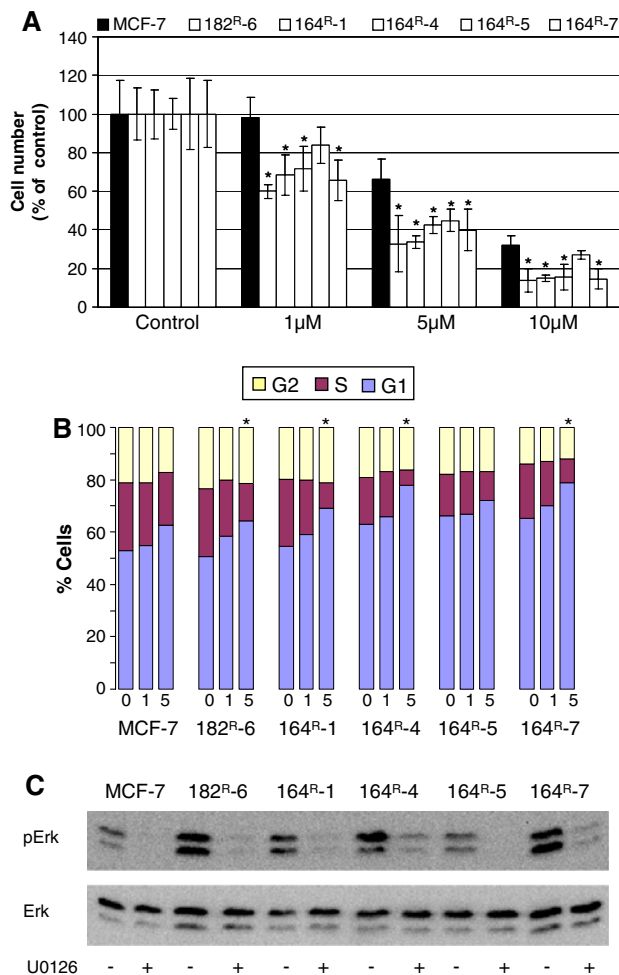


Fig. 5 Effect of the Erk inhibitor U0126 on cell growth, cell cycle distribution and Erk activation. **(a)** Growth analysis of MCF-7 and five fulvestrant resistant cell lines. Each bar represents mean cell number from triplicate wells in response to treatment for five days with increasing concentrations of U0126. At least three independent experiments were performed, each in triplicate. A representative experiment with mean and SD is shown. Statistical comparison of MCF-7 and the resistant cell lines was done for cultures receiving treatment with the same U0126 concentration, and statistical significances ($P < 0.05$) are denoted by *. **(b)** FACS analysis of cell cycle distribution after 48 h of treatment with 1 or 5 μM U0126. Each bar shows the distribution of the three cell cycle phases and a representative result of at least three independent experiments is shown. Statistically significant differences between MCF-7 cells and the resistant cell lines receiving the same U0126 treatment are denoted by *. **(c)** Western blot analysis of lysates from MCF-7 and five fulvestrant resistant cell lines, showing pErk and Erk in response to treatment with 10 μM U0126 for 24 h

Ab5 treatment (Fig. 7b), and again no effect on basal level of pAkt and pErk was observed.

Treatment with the ErbB3 blocking antibodies resulted in extensive inhibition of ErbB3 activation, with a concomitant growth inhibition in two of three antiestrogen resistant cell lines. Initial experiments combining Ab5

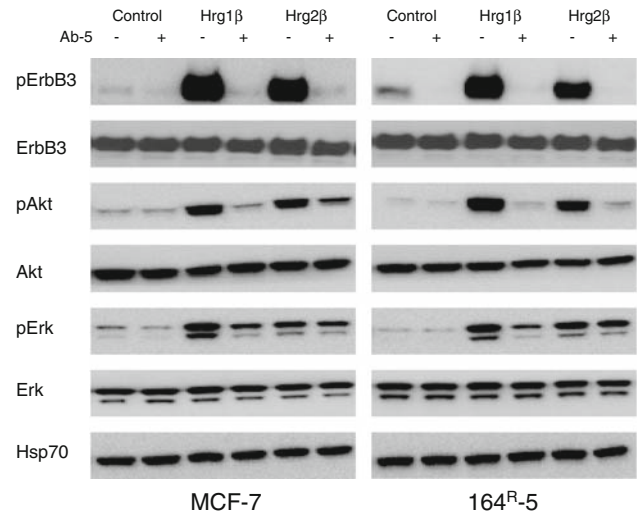


Fig. 6 Effect of the ErbB3 neutralizing antibody Ab5 on ErbB3, Akt and Erk activation. Western blots of MCF-7 and 164^{R-5} cells measuring total and phosphorylated levels of ErbB3, Akt and Erk in response to the ErbB3 neutralizing antibody Ab5 either with or without the addition of hrg1 β or GST-NRG2 β (hrG2 β). The cells were grown in standard growth medium incubated with Ab5 for 1 h followed by addition of vehicle, hrg1 β or hrg2 β for 15 min. Hsp70 is loading control. Two independent experiments were performed and similar results were obtained

treatment (10 $\mu\text{g}/\text{ml}$) with gefitinib treatment (5 μM), revealed a benefit of the combination therapy in the 164^{R-5} cell line, whereas no further reduction in cell growth of the 164^{R-7} cells was found (Fig. 7a). Gefitinib treatment (5 μM) reduced both pErbB3 and pErk expression in all three cell lines, whereas no effect on pAkt was observed (Fig. 7b). The combined treatment resulted in further pErbB3 reduction in all cell lines, but pAkt was unaffected and pErk was at the level as in cells treated with gefitinib alone.

Expression of the ErbB ligands

We performed a qPCR screening, measuring the mRNA level of 10 ErbB ligands. Analysis of the heregulin group of ligands, which binds to ErbB3 and ErbB4 showed a substantial upregulation of hrg2 mRNA in all resistant cell lines, both in the form of hrg2 α and hrg2 β (Fig. 8a). We also screened for mRNA of hrg1 α , hrg1 β , hrg3 and hrg4, but these ligands were not present in detectable amounts.

With respect to the other ErbB ligands, the two EGFR ligands TGF α and EGF, as well as the EGFR/ErbB4 ligands HB-EGF and betacellulin were expressed in the resistant cell lines at mRNA levels comparable to the levels in MCF-7 cells, whereas mRNA expression of the last two EGFR/ErbB4 ligands in our screen, amphiregulin and epiregulin, were strongly decreased in all resistant cell lines (Fig. 8b). These results indicate that TGF α , EGF,

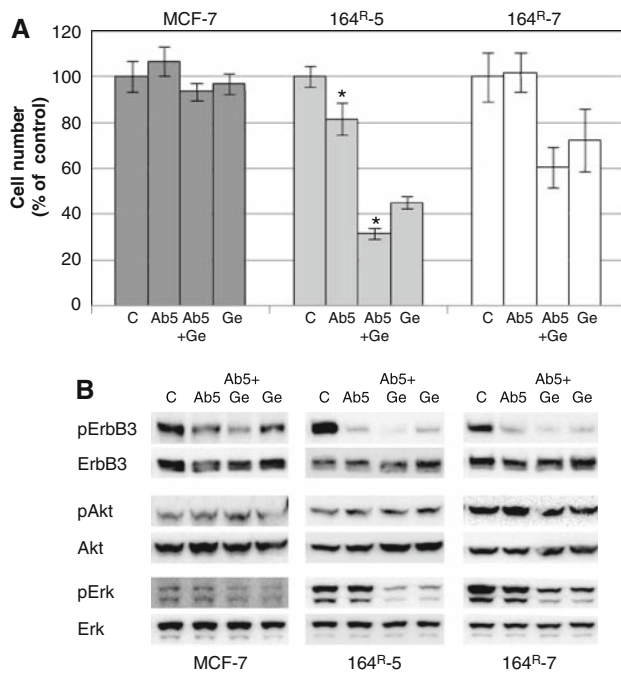


Fig. 7 Effect of Ab5 and/or gefitinib on cell growth and ErbB3, Erk and Akt activity. **(a)** Growth effect of treatment with Ab5 and/or gefitinib in MCF-7, 164^{R-5} and 164^{R-7} cells. The cells were treated with vehicle (C) 10 μ g/ml Ab5 (Ab5), 5 μ M gefitinib (Ge) or the combination of Ab5 and gefitinib (Ab5+Ge). Cell number was determined after five days of treatment and each bar represents mean cell number from triplicate wells and standard deviations are shown. Four independent experiments were performed. Statistical analysis of the difference between control and Ab5 treatment and between gefitinib and the combination of gefitinib and Ab5 was performed and significance is denoted by *. **(b)** Western blots showing total and phosphorylated levels of ErbB3, Akt and Erk in MCF-7, 164^{R-5} and 164^{R-7} cells after incubation for 24 h with the indicated concentrations of Ab5 and/or gefitinib. Two independent experiments were performed and comparable results observed

HB-EGF and also betacellulin could be responsible for activation of EGFR in the resistant cell lines.

Western analyses were performed to detect the hrg2 proteins, using antibodies against hrg2 α and hrg2 β developed by W. Gullick's laboratory [28] (Fig. 8c). Several isoforms of hrg2 exist [29, 30] and the mol. wt of full length hrg2 is expected to be approximately 85 kDa. The hrg2 α antibody recognized one protein band in the 85 kDa area and this protein band was significantly increased in 164^{R-5} cells, which also expressed the highest level of hrg2 α mRNA. The hrg2 β antibody detected two protein bands in the 85 kDa area and the lower mol. wt band was increased in all resistant cell lines, except the 164^{R-1}. Experiments are in progress to detect the cleaved hrg2 protein in cell lysates and in conditioned media.

The presumption that growth of the resistant cell lines is mediated in an autocrine manner by an increased production of growth factors being able to activate the ErbB3 is

supported by the observation that conditioned medium from two resistant cell lines, the 164^{R-5} and the 164^{R-7}, stimulated pErbB3 expression in MCF-7 cells, whereas conditioned medium from MCF-7 cells did not induce pErbB3 expression (Fig. 8d). Importantly, the negative control (designated C), which was a 100 fold concentration of our normal growth medium (including serum), was not able to significantly stimulate ErbB3 activation.

Discussion

It is well known that breast cancer progresses from ER driven to growth factor receptor driven growth. The ErbB receptors, EGFR and ErbB2, as well as the ErbB ligands TGF α , amphiregulin and heregulin1, which leads to activation of the downstream kinases Akt and Erk have all been found at increased level in endocrine resistant breast tumors and breast cancer cell lines [31–33]. Much less is known about the ErbB3 and ErbB4 receptors, and the goal of this study has been to use a large panel of fulvestrant resistant cell lines to evaluate all four receptors including their activation status and expression of the cognate ligands and thus to unravel the involvement of the ErbB receptors in fulvestrant resistant cell growth. The fulvestrant resistant cell lines have reduced expression of the ER compared to parental MCF-7 cells when grown without fulvestrant [6], and in the experiments presented in this paper in which the resistant cell lines are constantly grown in presence of fulvestrant, the ER expression is extremely low, due to destabilization of the ER upon binding of fulvestrant [34, data not shown]. Thus, whereas crosstalk between ErbB receptors and ER has been described to be an important mechanism for endocrine resistant cell growth in other model systems [35], crosstalk cannot be a major mechanism for resistant cell growth in this model system.

Several alterations in expression of the ErbB receptors were observed and the general pattern was increased activation of ErbB3 and EGFR and downregulation of ErbB4 and its activity in the fulvestrant resistant cell lines. Upregulation of EGFR in antiestrogen resistant cell lines has been shown previously [7, 8, 33], and our data demonstrating that treatment with 1 μ M gefitinib preferentially inhibits growth of fulvestrant resistant cell lines support EGFR involvement. Also, Erk activation is severely inhibited by 1 μ M gefitinib, showing that Erk and not Akt is activated by EGFR signaling in the resistant cell lines. However, the order of growth inhibition is about 30%, suggesting that EGFR activated by the available endogenous ligands may not alone drive fulvestrant resistant cell growth. Moreover, treatment with 10 μ M gefitinib is not specific for the EGFR and we observed a reduction of pErbB3 to an almost undetectable level in all cell lines,

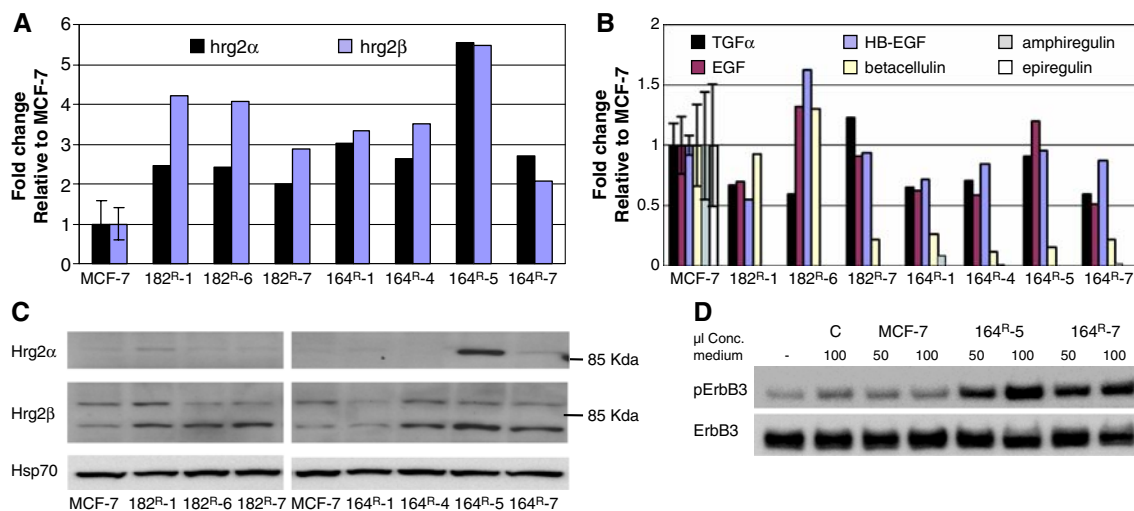


Fig. 8 ErbB ligand expression in MCF-7 and resistant cell lines. **(a)** qPCR measuring mRNA levels of the ErbB3/ErbB4 ligands hrg2 α and hrg2 β and **(b)** the EGFR/ErbB4 ligands, TGF α , HB-EGF, betacellulin, amphiregulin and epiregulin. RNA was extracted from MCF-7 and seven fulvestrant resistant cell lines grown in standard growth medium. Four independent measurements were performed on the MCF-7 cell line and standard deviations are shown. For the resistant cell lines, two independent determinations were done and similar result were observed. **(c)** Western blots showing hrg2 α and hrg2 β expression in lysates from MCF-7 and resistant cell lines grown

in standard growth medium. Hsp70 is loading control. Two experiments were performed and similar results obtained. Also, three additional analyses of MCF-7, 164^R-5 and 164^R-7 confirmed the presented results for these cell lines. **(d)** Western blot showing total and phosphorylated ErbB3 in lysates from MCF-7 cells treated for 15 min. with concentrated conditioned medium from MCF-7, 164^R-5 or 164^R-7. As negative control (designated C) we used our regular growth medium (incl. serum), which had been concentrated in parallel to the conditioned media

indicating that targeting also other receptors may give better growth control.

Thus, we investigated the importance of the increased activation of ErbB3 in resistant cell lines by inhibition of ligand binding by treatment with neutralizing antibodies (Ab5) to ErbB3. Firstly, we demonstrated that Ab5 could inhibit both basal and hrg1 β and hrg2 β mediated ErbB3 activation in both MCF-7 and fulvestrant resistant 165^R-5 cells. We then selected three resistant cell lines for treatment with Ab5 and in two of three resistant cell lines, Ab5 treatment resulted in a modest but statistically significant growth inhibition, showing that a highly specific inhibition of ErbB3 can indeed inhibit growth of fulvestrant resistant cell lines. Furthermore, Ab5-treatment reduced the pErbB3 expression to a very low level, indicating that the increased amount of pErbB3 was due to increased activation by endogenous ligand(s). The endogenous production of ligand was confirmed by treating MCF-7 cells with conditioned medium from two resistant cell lines, which resulted in ErbB3 activation, whereas conditioned medium from MCF-7 cells did not have any effect. Lastly, we suggest that this ligand may be hrg2 (α and β isoform), which was the only ErbB3 ligand detectable by qPCR and which was also found upregulated in the resistant cell lines at both mRNA and protein level. ErbB3 involvement in antiestrogen resistance is supported by the recent data, in

which tamoxifen resistance conferred by ErbB2 overexpression could be reverted by siRNA-mediated knockdown of ErbB3 [36]. In support of the role of ligand activated ErbB3 for antiestrogen resistance, a recent publication has demonstrated that addition of hrg1 β to a tamoxifen resistant cell line leads to ErbB3/ErbB2 heterodimerization and to decreased sensitivity to tamoxifen [37]. These data all point to ErbB3 and its cognate ligand as important mediators of antiestrogen resistant cell growth, probably acting in concert with EGFR and ErbB2. Our initial experiments designed to block both EGFR and ErbB3 revealed a more severe growth inhibition by treating 164^R-5 cells with Ab5 and gefitinib, and more experiments with combination therapy also targeting the ErbB2 are in progress.

In contrast to ErbB3, the molecular function of ErbB4 in antiestrogen resistance has not been described at all and its role in breast cancer cell lines is not yet clear. ErbB4 mRNA is downregulated by estradiol in MCF-7 cells [38] and we also found that both ErbB4 mRNA and protein were clearly estrogen downregulated in our MCF-7 cells (data not shown). Therefore, it could be expected that long-term fulvestrant treatment would act opposite and increase the ErbB4 level. On the contrary, all resistant cell lines had counteracted this direct effect of fulvestrant and displayed a strong downregulation of both ErbB4 protein and activity, indicating an antiproliferative role of ErbB4. Also, the

decreased level of ErbB4 in resistant cells may facilitate heterodimerization of ErbB3 with EGFR and ErbB2. Several groups have published that ErbB4 mediates growth inhibition and cell differentiation in the ER positive cell lines MCF-7 and T47D [39, 40], while others have found that ErbB4 promotes growth in these cell lines [41, 42]. In breast tumors, membranous ErbB4 is most often found to correlate with expression of the ER and with a more favorable prognosis both alone [43, 13, 44] and when co-expressed with ErbB3 [45].

The role for ErbB2 in growth of our resistant cell lines is not completely clear. Firstly, the level of pErbB2 is not elevated in the resistant cell lines and the gefitinib-mediated growth inhibition in the resistant cell lines is not concomitant with a general reduction of pErbB2. Secondly, we have shown that treatment of MCF-7, 182^R-6, 164^R-5 and 164^R-7 cells with 10 µg/ml of the monoclonal ErbB2 antibody trastuzumab does not have an effect on growth [46; data not shown]. However, as trastuzumab does not inhibit ErbB2 dimerisation [47], we cannot exclude a role for ErbB2 as co-receptor for the activated EGFR and ErbB3 in the resistant cell lines.

Turning to the downstream kinases, we have previously shown that active Akt is required for growth of our resistant cell lines [9]. Interestingly, the strong Ab5 mediated inhibition of the basal pErbB3 level had no effect on either Erk or Akt phosphorylation, even though the hrg1 and hrg2 mediated induction of pAkt was totally blocked by Ab5. Thus, ErbB3 activated by addition of exogenous recombinant ligand signals to Akt, whereas ErbB3 activated by endogenous ligand must signal to other yet unidentified downstream targets. A possible explanation for the inability of Ab5 and gefitinib to inhibit basal pAkt in our cell lines could be due to the fact that MCF-7 cells harbor amplification of the PIK3CA gene as well as carry the E545K mutation [48]. Also, inhibition of either the PI-3K by Wortmannin or Akt directly by the Akt inhibitor SH-6 results in downregulation of pAkt and inhibition of the fulvestrant resistant cell growth [9]. Furthermore, we found that treatment with the Erk inhibitor U0126 exerted a dose dependent and preferential growth inhibition of the resistant cell lines, supporting that the EGFR mediated activation of Erk is important for growth of the resistant cell lines.

These data are in concordance with other studies showing that activation of Akt and Erk is necessary for growth in both fulvestrant and tamoxifen resistant cell lines [8, 9, 49, 50]. Also, in tamoxifen treated patients, increased pAkt and pErk expression have been correlated to early relapse [51, 52].

In conclusion, we have demonstrated several alterations within the ErbB system upon acquiring resistance to ful-

vestrant, including activation of ErbB3, EGFR and Erk, upregulation of hrg2 and downregulation of ErbB4. The activation of ErbB3 in resistant cells occurred via production of autocrine growth factors e.g. hrg2, and abolishment of ligand binding to ErbB3 inhibited growth of resistant cells. The importance of EGFR in resistant cell growth is supported by the preferential inhibition of growth with gefitinib and with U0126. Thus, resistant cell growth involves both ErbB3 and EGFR, and the severely reduced expression of ErbB4 may be instrumental by facilitating heterodimer formation of ErbB3 with EGFR and ErbB2 in resistant cells. In order to obtain complete growth suppression of fulvestrant resistant cells, concurrent treatment with fulvestrant and compounds targeting ErbB3 and EGFR, and presumably also ErbB2, may be required.

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