T47D breast cancer cells switch from ER/HER to HER/c-Src signaling upon acquiring resistance to the antiestrogen fulvestrant

Tove Kirkegaard a,*, Susanne K. Hansen a, Sarah L. Larsen a, Birgit E. Reiter a, Boe S. Sørensen b, Anne E. Lykkesfeldt a

a Breast Cancer Group, Cell Death and Metabolism, Danish Cancer Society Research Center, Copenhagen, Denmark
b Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

1. Introduction

The selective estrogen receptor modulator (SERM) tamoxifen is recommended as first line endocrine therapy for premenopausal women with ER-positive breast cancer [1]. However, some patients are de novo resistant to tamoxifen and other patients will, despite an initially responsive disease, acquire resistance and disease progression will occur. Many patients with progression on tamoxifen, will, due to different mechanisms of actions, respond to treatment with the pure antiestrogen fulvestrant (ICI 182,780 or faslodex), which is a selective ER down regulator (SERD) [2]. Binding of fulvestrant to ER prevents ER dimerisation, which results in destabilisation and rapid degradation of the ER-fulvestrant complex and consequently loss of estrogen signaling [3]. Unlike tamoxifen, fulvestrant has no known agonistic effects on cancer cell growth [4] and due to the unique mechanisms of action, fulvestrant delays development of resistance compared to tamoxifen in a xenograft model [5]. The clinical efficacy of fulvestrant in patients with breast cancer progressing on tamoxifen is similar to the effect of the aromatase inhibitor anastrozole [6,7]. However, upon long-term estrogen deprivation by aromatase inhibitors, increased sensitivity to estradiol treatment has been seen in some cell models [8]. Moreover, it is known that cross talk between ER and activated growth factor receptors or their downstream kinases are able to activate ER in a non-genomic manner [9]. Thus, to prevent the action of ER in patients treated with aromatase inhibitors, combination with fulvestrant to degrade ER could be a treatment option. This approach was validated in two recently reported studies [10,11], of which only one showed superiority of the combined treatment [10]. Although combination of different endocrine therapies may be efficient for some breast cancer patients upon relapse, resistance to fulvestrant is, as for tamoxifen, inevitable for patients with advanced breast cancer. Thus, to identify novel treatment for endocrine resistant breast cancer as well as markers for treatment response, it is important to clarify the molecular mechanisms behind acquired resistance to endocrine therapies. In vitro cell model systems using human breast cancer cell lines with acquired resistance to different forms of antiestrogens are highly valuable tools to identify changes between antiestrogen sensitive and resistant breast cancer cells [12–22]. Based on the ER-positive human breast cancer cell line, MCF-7, we have established a wide selection of cell lines resistant to different endocrine therapies and explored the molecular mechanisms involved in both tamoxifen and fulvestrant resistance [12–14,21,23–28]. We observed that the expression and activation of proteins from the Human Epidermal growth factor Receptor (HER) family as well as the expression of the HER3/HER4 ligands heregulin2 and 2p were increased in the fulvestrant resistant breast cancer cell lines compared to the expression in the fulvestrant sensitive MCF-7 cell line [24]. Moreover, our antiestrogen resistant breast cancer cell...
lines had increased activation of downstream kinases involved in various signaling pathways including phosphorylation of Akt [23,25,27,28]. In accordance with the increased expression and activation of the HER receptors and their downstream signaling molecules, the cells were growth inhibited by targeting the HER receptors or signaling molecules downstream of the HER receptors [23,24,26]. Thus, based on our studies as well as others it is well recognized that growth of antiestrogen resistant breast cancer cells can switch from being driven by ER to be mediated by the HER receptors upon acquiring antiestrogen resistance [15–21,24,26,28].

The HER receptors are a family of four type-1 transmembrane receptor tyrosine kinases: EGFR/HER1, HER2, HER3 and HER4 [29,30]. In breast tumors, the expression and phosphorylation of HER1, HER2 and HER3 have been associated with a poor prognosis, in contrast to the expression of HER4 which has been linked to a better outcome [31–33]. Upon ligand activation of the HER receptors, downstream signaling pathways are activated, which leads to increased cell proliferation and reduced cell death [29]. Gene amplification or protein over expression of HER2 is seen in 15–20% of early-stage breast cancer [34,35] and is associated with a significantly shorter time to relapse and reduced overall survival [36–39]. Although trastuzumab (Herceptin; Genentech), a humanized monoclonal antibody targeting HER2, has clearly improved the clinical outcome for HER2-positive breast cancer patients in the adjuvant and advanced setting, still many patients exhibit de novo or acquired resistance [37,38]. For ER-positive breast cancer patients, amplification or over expression of HER2 reduce the sensitivity to endocrine therapy [39]. This was also demonstrated in a MCF-7 breast cancer cell line ectopically over expressing HER2 [40]. Moreover, combination of endocrine therapy and HER targeted treatment is superior to endocrine therapy alone [41]. Thus, preclinical and clinical data strongly link signaling via the HER receptors to reduced response to endocrine therapy and treatment targeting the HER receptors and their downstream signaling pathways are attractive new therapies.

c-Src belongs to the family of non-receptor tyrosine kinases consisting of 9 members [42]. c-Src is over expressed in human breast cancers [43], and is associated with reduced overall survival of breast cancer patients [44]. *In vitro* studies have shown that dual targeting of c-Src and ER completely prevents development of resistance to tamoxifen [45]. The majority of breast cancers with over expressed or activated c-Src also over express one of the HER receptors [46,47] and in HER2-positive breast cancer, activated c-Src correlates with HER2 positivity and poor prognosis [48]. c-Src can bind to HER1, HER2 and HER3 [47,49–51] and as c-Src activity seems to be required for HER2:HER3 complex formation and subsequent downstream signaling [51], targeting c-Src might be an alternative way of preventing HER-driven cell growth.

So far most model studies investigating antiestrogen resistant breast cancer have been performed using the ER-positive breast cancer cell line, MCF-7. However, in order to determine if the molecular mechanisms driving growth of fulvestrant resistant MCF-7 cells are general for ER-positive breast cancer cell lines and to extend our knowledge of acquired resistance to antiestrogens, we have long-term treated another ER-positive human breast cancer cell line, T47D, with fulvestrant and established two fulvestrant resistant breast cancer cell lines. The work presented here was carried out to characterize the established T47D fulvestrant resistant breast cancer cell lines with respect to cell growth and signaling.

### 2. Materials and methods

#### 2.1. Cell lines, culture condition and reagents

The T47D cell line was originally obtained from the Human Cell Culture Bank (Mason Research Institute, Rockville, MD, USA). The cells were maintained in growth medium without phenol red (RPMI; Gibco, Invitrogen, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Perbio, Thermo Fischer Scientific, Roskilde, Denmark), 2 mM glutamax (Gibco) and 8 μg/ml insulin (Sigma–Aldrich, Copenhagen, Denmark). The fulvestrant resistant cell lines, T47D/ 

182–2 (182–2) and T47D/182–2 (182–2) were established by long term treatment with 100 nM fulvestrant (Fig. 1) and maintained in the same medium as the parental T47D cell lines plus 100 nM fulvestrant (Tocris, Avonmouth, Bristol, UK). For experiments, 2.5 × 10^6 U penicillin and 31.25 μg/l streptomycin (Gibco) were added to the growth medium. Estradiol (E2) was obtained from Sigma–Aldrich. The HER2 tyrosine kinase inhibitor, AG825 was purchased from Tocris Biosciences (Ellisville, Missouri, USA), whereas gefitinib, Ct-1033 and dasatinib were purchased from Selleck Chemicals (Munich, Germany). Stock solutions of 10^–2 M E2 and 10^–1 M fulvestrant were dissolved in 96% ethanol, whereas stock solutions of 10 mM gefitinib, 50 mM AG825, 10 mM Ct-1033 and 1 mM dasatinib were dissolved in DMSO.

#### 2.2. Quantitative real-time RT-PCR

Cells were grown to 70–80% confluence. Total RNA was purified with the QIAamp RNA blood mini kit (Qiagen, Copenhagen, Denmark) and 0.1 μg total RNA reverse transcribed with AMV reverse transcriptase according to the manufactures instructions (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using a Lightcycler 480 and the Sybr Green 1 PCR kit both from Roche (Hvidovre, Denmark). Quantification is presented as the ratio between the amount of target gene and β-actin mRNA expression in each sample, as previously described [52,53]. For primer sequences and PCR profiles see [24].

#### 2.3. Cell growth assays

The effect of fulvestrant on growth of the T47D cell line, 4 × 10^4 cells/well were seeded in 24-well multidishes in growth medium and allowed to adhere for 2 days. Cell number was determined by manually counting the cells from three wells in a Bürker–Türk chamber (day 0) and growth medium with vehicle or 100 nM fulvestrant was added to the remaining wells. Cell number was determined and medium renewed in the remaining wells every second or third day for a total of 12 days. To determine the doubling time of parental and fulvestrant resistant T47D cell lines, 3 × 10^4 cells/well were seeded in 24-well multidishes in growth medium and allowed to adhere for 1 day. At selected time points (days 0–12), cells from 3 wells were trypsinized and cell number determined by manually counting the cells in a Bürker–Türk chamber. After each count, growth medium was renewed in the remaining wells. The doubling time was calculated from the exponential part of the growth curve.

All dose response growth experiments were performed in 24-well multidishes except for the experiments with dasatinib, which were performed in 96-well multidishes. To compensate for the uneven plating efficiency, the parental T47D cells were seeded with 4 × 10^3 cells/well in growth medium and the resistant cells were seeded with 4.5 × 10^3 cells/well in growth medium with 100 nM fulvestrant in 24-well multidishes. For the growth experiments with dasatinib, the parental cells were seeded with 2300 cells/well in 96-well multidishes in growth medium, whereas the resistant cells were seeded with 2500 cells/well in 96-well multidishes in growth medium with 100 nM fulvestrant.

After 2 days growth (day 0), growth medium was renewed and media containing the compounds of interest were added. The experimental medium was replaced on day 3 and cell number determined on day 5 using a crystal violet colorimetric assay as previously described [54]. Growth experiments with E2 (1 μM) were performed with medium containing 10% newborn calf serum (NCS; Gibco), which contains very low estrogenic activity, 2 mM glutamax and 8 μg/ml insulin. Stability studies were performed with resistant cell lines which have been withdrawn from fulvestrant for 1 and 7 weeks.

#### 2.4. Western blot analysis

Western analyses were performed with cells lysed in RIPA buffer (100 mM NaCl, 20 mM Tris–HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with 1 mM DTT, 1 mM NaF, 10 mM β-glycerophosphate, 100 mM NaVO<sub>4</sub>, 150 μM PMSE and 1 tablet/10 ml RIPA complete mini protease inhibitor cocktail (Roche). T47D cells grown for 4 days with 100 nM NCS with or without 1 nM E2 were used to explore the expression of ER and estrogen regulated proteins. To investigate the effect of fulvestrant on expression of ER, estrogen regulated proteins, HER receptors and downstream signaling molecules, T47D cells were grown in standard growth medium with 5% FBS in the presence or absence of fulvestrant for 4 days. For expression analysis of HER receptors and their signaling molecules in parental and resistant T47D cells, the cells were grown in standard growth medium (T47D) or in growth medium with 100 nM fulvestrant (resistant cell lines) until 70–80% confluence. To investigate the effect of HER inhibitors on HER receptor signaling, T47D cells and fulvestrant resistant T47D cells, grown in their standard growth medium until 70% confluence, were treated for 1 h with Ct-1033 (1 μM), gefitinib (1 and 5 μM) or AG825 (10 and 30 μM).

Protein concentrations in cell lysates were determined with BioRad Protein Assay kit (Bio-Rad Laboratories, Copenhagen, Denmark), 15–20 μg total protein were separated on 4–15% Tris–HCl or 3–8% Tris–Acetate resolving criterion gels (Bio-Rad) separated on 4–15% Tris–HCl or 3–8% Tris–Acetate resolving criterion gels (Bio-Rad). Protein concentrations in cell lysates were determined with BioRad Protein Assay kit (Bio-Rad Laboratories, Copenhagen, Denmark). The cells were grown to 70–80% confluence. Total RNA was purified with the QIAamp RNA blood mini kit (Qiagen, Copenhagen, Denmark) and 0.1 μg total RNA was reverse transcribed with AMV reverse transcriptase according to the manufactures instructions (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using a Lightcycler 480 and the Sybr Green 1 PCR kit both from Roche (Hvidovre, Denmark). Quantification is presented as the ratio between the amount of target gene and β-actin mRNA expression in each sample, as previously described [52,53]. For primer sequences and PCR profiles see [24].
and transferred to an ethanol-activated Immobilon-P membrane (Millipore, Bedford, MA, USA). To prevent non-specific binding, membranes were blocked in TBS containing 5% dry-milk, 5% PBS and 0.2% Tween-20 (blocking buffer) for 2–3 h at RT. Incubation with the primary antibody was performed overnight at 4 °C followed by 1 h incubation with species-specific peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). Protein bands were visualized by enhanced chemiluminescence (ECLPLUS detection system; GE Healthcare, Hilleroed, Denmark) and detected by a CCD camera (LAS-1000, Fujiﬁlm). In order to detect multiple proteins, the antibodies were removed from the membrane by incubation for 15 min in Reblot Plus Western Blot Antibody Stripping Solution (Millipore) and blocked for 2 × 5 min in blocking buffer before incubation with antibody. All antibodies used in the study are listed in Table 1.

2.5. Immunoprecipitation

To investigate dimerization partners for HER2 and HER3, parental and fulvestrant resistant T47D cells were grown under standard conditions in T75 culture flasks and whole cell lysates generated by lysis in cell lysis buffer from Cell Signaling Technology (Cat no: 9803). Immunoprecipitation was performed with Invitrogen’s kit with magnetic dynabeads. Briefly, 25 μl beads were pelleted by the magnet, washed and incubated for 1 h with 1 μg of HER2 (Thermo Fisher Scientiﬁc; Ab-17) or HER3 antibody (Thermo Fisher Scientiﬁc; MS-201) at 4 °C by gentle rotation. 1 mg of cell lysates were added to the pelleted and washed beads and the samples incubated overnight at 4 °C by gentle rotation. The beads were pelleted, washed and the immunoprecipitated proteins released with the sample buffer. Western analyses were performed using antibodies against HER1 (Cell Signaling Technologies; 4405), HER2 (Dako; A0485), HER3 (Santa Cruz Biotechnology, TX, USA; SC-285), HER4 (Cell Signaling Technologies; 4795) and c-Src (Cell Signaling Technologies; 2109). The experiments were performed three times.

2.6. Gene silencing with small interfering RNA (siRNA)

A pool of 5 different HER2-targeting small interfering RNAs (siRNAs) (obtained from Sigma–Aldrich) and scrambled sequence (non-targeting) control pool siRNA (D-001810) from Dharmacon (Lafayette, CO, USA) were used. Cells were transfected with 600 nM HER2 siRNA in 100 μl nucleofector solution V (Cell Line Nucleofector Kit V) or scramble siRNA using a Nucleofector device both from Amaxa (Lonza, Cologne, Germany) according to the manufacturer’s instructions. Transfected cells were seeded in 6- and 24-well plates in standard growth medium to measure protein expression and cell growth, respectively. Medium was replaced at day 1 and the cell number in 24-well plates were determined on day 1, 2, 3 and 6 using a crystal violet colorimetric assay as previously described [54]. Three days after transfection, cells grown in 6-well plates were harvested in RIPA buffer and subjected to western analysis.

2.7. Statistics

For RT-PCR experiments and cell growth assays, two-sample unequal variance t-test followed by Bonferroni’s correction was used to calculate whether the observed differences were statistically signiﬁcant. For all experiments, a p-value less than 0.05 was considered signiﬁcant.

Table 1 Antibodies used in Western blot analysis.

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<th>Size (kDa)</th>
<th>Dilution</th>
<th>Company and catalog number</th>
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<td>170</td>
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<td>Dako, M7298</td>
</tr>
<tr>
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<td>Dako, M7297</td>
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<tr>
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3. Results

3.1. Effect of estrogen on growth and expression of ER and estrogen regulated proteins in T47D cells

Initially, the estrogen responsiveness of the T47D human breast cancer cell line was veriﬁed. As shown in Fig. 1A, growth of T47D cells in the presence of 10% estrogen poor NCS was signiﬁcantly increased by 3–4 folds after 5 days of treatment with E2. T47D cells, grown with 10% NCS, express ER and low levels of the estrogen regulated proteins; insulin-like growth factor receptor (IGF-1R) and progesterone receptor (PR). When T47D cells were treated for 4 days with E2, the expression of ER was reduced while the expression of IGF-1Rx and PR was signiﬁcantly increased (Fig. 1B).

3.2. Effect of fulvestrant on growth and signaling in T47D cells

In order to investigate the effect of the pure antiestrogen fulvestrant on growth of T47D, the cells were cultured with or without 100 nM fulvestrant for 12 days and cell number determined every 2–3 days. As seen in Fig. 1C, fulvestrant signiﬁcantly inhibited growth of T47D cells. A dose–response growth experiment was performed to conﬁrm that growth of T47D cells was signiﬁcantly inhibited in a dose-dependent manner by treatment with 0.01–100 nM fulvestrant (Fig. 1D). The expression of ER and the estrogen regulated proteins, IGF-1R and PR, were severely reduced when T47D cells were short-term (4 days) treated with fulvestrant (Fig. 1E). The protein expression of HER1 was slightly reduced, whereas a signiﬁcant decrease of HER3 and HER4 protein expression and a signiﬁcant increase in HER2 protein expression was observed after 7 days treatment with fulvestrant. Moreover, upon 7 days treatment with fulvestrant, no major effect was seen on the phosphorylation of Akt, the phosphorylation of GSK3 was increased, whereas the phosphorylation of Erk and RSK was decreased. There were no changes in the expression of the total levels of Akt, Erk, GSK3 and RSK upon treatment with fulvestrant (Fig. 1E).

3.3. Development of fulvestrant resistant T47D cell lines

To study the mechanisms behind acquired resistance to fulvestrant, T47D breast cancer cell lines resistant to fulvestrant were developed as previously described for fulvestrant resistant MCF-7
towards small colonies of very slowly dividing cells appeared in some of the wells. Individual colonies were transferred to separate wells and split in 2 after additional 3–4 weeks. After 3–4 months of treatment with fulvestrant, the cells were growth arrested after a few days of treatment. After further treatment, cell death was observed but after 2–3 days as indicated on Fig. 2B. The doubling time of the two resistant cell lines grown in the presence of fulvestrant was 38 and 39 h, and very similar to the doubling time of the parental T47D cells (36 h) grown in normal growth medium. Noteworthy, it was consistently found that the plating efficiency of the resistant cells was significantly lower than the plating efficiency observed for the parental T47D cell lines (Fig. 2B).

To investigate whether the resistant phenotype was maintained upon withdrawal of fulvestrant, the resistant cells were cultured without fulvestrant for 1 and 7 weeks before dose–response growth experiments were carried out. As shown in Fig. 2C, both 182R-1 and 182R-2 remained insensitive to fulvestrant after withdrawal of the antiestrogen for up to 7 weeks. We also investigated the expression of ER and specific estrogen regulated proteins (IGF-1R, PR-A and -B, the HER receptors, total and phosphorylated Akt (Ser473), Erk (Thr202/Tyr204), GSK3 (Ser21/Ser9) and RSK (Ser221/Ser227)) in lysates from T47D cells grown under standard conditions or with fulvestrant (fulv) for 4 days (ER and estrogen regulated proteins) or 7 days (HER receptors or signaling molecules). Hsp70 was used as loading control.

Fig. 1. Effect of estradiol (E2) and fulvestrant on cell growth and protein expression in T47D cells. (A) T47D cells were grown for 5 days with newborn calf serum (NCS) or NCS + E2 (0.01 nM) before cell number was measured by a crystal violet colorimetric assay and expressed as percent of the NCS culture. Values (mean ± SD) are pooled data from two experiments each performed in quadruplicate measures. *designates statistical significant difference (p < 0.05) in growth between NCS and E2 treated cells. (B) Western blot showing expression of estrogen receptor (ER) and the estrogen regulated proteins; insulin-like growth factor 1 receptor alpha (IGF-1Rα) and progesterone receptor (PR; PR-A and PR-B) in T47D cells cultured for 4 days in growth medium containing NCS or NCS + E2. Actin was used as loading control. (C) T47D cells were seeded in 24-well dishes in standard growth medium. Two days after, cells in half of the wells continued growth in standard medium and cells in the other wells received 100 nM fulvestrant. At the indicated time points, cell number was determined by counting cells from 3 wells in a Bürker–Türk chamber. Pooled data from 2 independent experiments with mean ± SD are shown. *designates statistical significant differences (p < 0.05) in growth between untreated and fulvestrant treated cells. (D) T47D cells treated for 5 days with the indicated concentrations of fulvestrant. Cell number was measured by a crystal violet colorimetric assay and expressed as percentage of untreated control (designated C). Values (±SD) are pooled data from 6 experiments each performed in quadruplicate measures. *indicates statistical significant differences (p < 0.05) between control and fulvestrant treated cells. (E) Western blots showing expression of ER, the estrogen regulated proteins IGF-1R, PR-A and -B, the HER receptors, total and phosphorylated Akt (Ser473), Erk (Thr202/Tyr204), GSK3β (Ser21/Ser9) and RSK (Ser221/Ser227) in lysates from T47D cells grown under standard conditions or with fulvestrant (fulv) for 4 days (ER and estrogen regulated proteins) or 7 days (HER receptors or signaling molecules). Hsp70 was used as loading control.

cells [14] and as illustrated in Fig. 2A. Briefly, when the parental T47D cells, grown in a 24-well plate, were treated with 100 nM fulvestrant, the cells were growth arrested after a few days of treatment. After further treatment, cell death was observed but after one month of treatment, small colonies of very slowly dividing cells appeared in some of the wells. Individual colonies were transferred to separate wells and split in 2 after additional 3–4 weeks. After 3–4 months of treatment with fulvestrant, the cells were transferred to a T25 cell culture flask. During the next months, cell growth slowly increased and after 6 months of treatment with fulvestrant, the weekly split ratio was about 1:6. Two fulvestrant resistant sublines were developed and named T47D/182R-1 and T47D/182R-2. In this study the sublines will be designated 182R-1 and 182R-2.

To determine the doubling time of the parental and fulvestrant resistant T47D cells, a growth experiment was performed. The parental T47D cells were cultured in normal growth medium whereas the fulvestrant resistant cells were cultured in the presence of 100 nM fulvestrant. Cell number was determined every 2–3 days as indicated on Fig. 2B. The doubling time of the two resistant cell lines grown in the presence of fulvestrant was 38 and 39 h, and very similar to the doubling time of the parental T47D cells (36 h) grown in normal growth medium. Noteworthy, it was consistently found that the plating efficiency of the resistant cells was significantly lower than the plating efficiency observed for the parental T47D cell lines (Fig. 2B).

3.4. Expression of HER receptors and downstream signaling proteins in T47D and fulvestrant resistant breast cancer cell lines

We have previously shown that growth of the MCF-7 cells switch from being driven by ER to be mediated by the HER receptors upon acquiring resistance to fulvestrant [24,26,28]. Therefore, to explore if this mechanism was general for ER-positive breast cancer cell lines, the expression of the HER receptors and their
downstream signaling molecules was measured in T47D cells and the two fulvestrant resistant breast cancer cell lines (Fig. 3). As measured by qRT-PCR, the resistant sublines have significantly increased HER2 and significantly decreased HER4 mRNA expression whereas HER3 mRNA expression was unaltered and HER1 mRNA expression decreased (Fig. 3A). Since HER2 mRNA expression was highly increased in the resistant cell lines, we used qPCR to investigate the HER2 gene level in these cells. Similar level of HER2 DNA was observed in parental and resistant cells (data not shown). When measuring the protein expression of the HER receptors, patterns corresponding to the mRNA expression were seen (Fig. 3B). Compared to the parental T47D cells, the protein expression of HER2 was significantly increased; HER3 was unchanged or slightly increased, and HER1 and HER4 were significantly decreased in the fulvestrant resistant cell lines (Fig. 3B). Comparable level of phosphorylated HER1 was detected in parental and resistant T47D cell
Fig. 3. Expression of HER receptors and signaling molecules in parental and fulvestrant resistant T47D cells. (A) T47D, 182R-1 and 182R-2 cells were grown under their standard conditions before RNA was extracted and qRT-PCR performed. Two independent experiments were performed with quadruplicate measures and ‘designates statistical significance in mRNA expression between parental and resistant T47D cells. Pooled data with mean ± SD are shown. (B and C) Western blots showing total and phosphorylated forms of HER1 (Tyr1173), HER2 (Tyr1248), HER3 (Tyr1289), Akt (Ser473), Erk (Thr202/Tyr204), GSK3β (Ser21/Ser9) and RSK (Ser221/Ser227) in lysates from T47D, 182R-1 and 182R-2 grown under their standard conditions. Actin or Hsp70 were used as loading controls. (D) Western blots showing expression of the HER receptors in lysates from parental or fulvestrant resistant T47D cells grown under their standard conditions before RNA was extracted and qRT-PCR performed. (E and F) Western blot analysis (Fig. 5 A) showing an almost completely attenuated knockdown. The knockdown efficiency was verified by western blot analysis (Fig. 5 A) showing an almost completely attenuated knockdown. The knockdown efficiency was verified by western blot analysis. To investigate whether the HER receptors are important for growth factor receptor signaling, parental and resistant T47D cells were treated with either CI-1033 (pan-HER inhibitor) or gefitinib (inhibitor preferentially targeting HER1), AG825 (HER2 tyrosine kinase inhibitor) or CI-1033 (pan-HER inhibitor) were performed using the parental and fulvestrant resistant T47D cells (Fig. 4A–C). Treatment with increasing concentrations (1–10 μM) of gefitinib resulted in a dose–response growth inhibition to around 50% of the untreated control for both the parental and fulvestrant resistant sublines at 10 μM. Compared to the fulvestrant resistant cells, the parental T47D cells were more sensitive to low concentrations of gefitinib. At 1 μM gefitinib, the concentration described to target only HER1, growth of parental cells were 30% inhibited whereas growth of the resistant cells was only 10% inhibited compared to the untreated control (Fig. 4A). The HER2 tyrosine kinase inhibitor AG825 (5–40 μM) exerted dose dependent growth inhibition of both parental and resistant cells. As with gefitinib, the parental T47D cells were more sensitive to low concentrations of AG825 (Fig. 4B). Finally, the pan-HER inhibitor CI-1033, exhibited dose–response growth inhibitory effect on the parental and resistant cell lines (0.5–2 μM), again with a more pronounced effect on the parental cells at the low concentrations (Fig. 4C).

In order to investigate growth factor receptor signaling, parental and resistant T47D cells were treated with either CI-1033 (1 μM), AG825 (10 and 30 μM) or gefitinib (1 and 5 μM) for 1 h. Fig. 4D shows that both CI-1033 and gefitinib strongly prevented phosphorylation of HER3 and Erk and reduced phosphorylation of Akt in both parental and resistant cells. In contrast, AG825 had no effect on phosphorylation of HER3, Akt and Erk in any of the cell lines (Fig. 4D). There was no effect on the expression of total levels of HER3, Akt and Erk (Fig. 4D).

3.6. Knockdown of HER2 in parental and fulvestrant resistant T47D cell lines

Although the HER2 expression level was significantly increased in the fulvestrant resistant cell lines, only a low level of tyrosine 1248 phosphorylated HER2, similar to the level in the parental T47D cells, could be detected (Fig. 3B). Moreover, AG825 did not preferentially inhibit growth of fulvestrant resistant cells (Fig. 4B). Thus, the role of the endogenously over expressed HER2 in the fulvestrant resistant cells was investigated by siRNA-mediated knockdown. The knockdown efficiency was verified by western blot analysis (Fig. 5A) showing an almost completely knockdown of HER2 in the parental and fulvestrant resistant cells. However, as shown in Fig. 5B, siRNA-mediated HER2 knockdown had no effect on growth up to 8 days after transfection neither of the parental nor the fulvestrant resistant T47D cells.
To further investigate the role of the HER receptors in the resistant cells, we performed immunoprecipitation experiments to identify heterodimerisation partners. When precipitating HER2, none of the other HER receptors were co-precipitated in neither the parental nor the resistant cells (data not shown). In contrast, immunoprecipitation of HER3 in lysates from parental and resistant T47D cell lines, revealed co-precipitation of HER3 with HER1 and HER4 in the parental T47D cells and with HER1 and HER2 in the resistant cell lines (Fig. 6). However, compared to the high HER2 expression seen in total protein lysates, immunoprecipitation experiments revealed that only a very little amount of HER2 formed heterodimers with HER3 in the resistant cell lines. Due to low protein amounts, we were not able to detect HER1 in the input controls. But, whereas the resistant cells expressed substantially lower levels of total HER1 compared to parental T47D cells (Fig. 3B), the levels of HER1 immunoprecipitated with HER3 was

3.7. HER receptor dimers in T47D and fulvestrant resistant cell lines

To further investigate the role of the HER receptors in the resistant cells, we performed immunoprecipitation experiments to identify heterodimerisation partners. When precipitating HER2, none of the other HER receptors were co-precipitated in neither the parental nor the resistant cells (data not shown). In contrast, immunoprecipitation of HER3 in lysates from parental and resistant T47D cell lines, revealed co-precipitation of HER3 with HER1 and HER4 in the parental T47D cells and with HER1 and HER2 in the resistant cell lines (Fig. 6). However, compared to the high HER2 expression seen in total protein lysates, immunoprecipitation experiments revealed that only a very little amount of HER2 formed heterodimers with HER3 in the resistant cell lines. Due to low protein amounts, we were not able to detect HER1 in the input controls. But, whereas the resistant cells expressed substantially lower levels of total HER1 compared to parental T47D cells (Fig. 3B), the levels of HER1 immunoprecipitated with HER3 was
similar or only slightly lower in the resistant cells compared to the parental T47D cells. Thus, in the parental T47D cells, HER3 was primarily associated with HER1 and HER4, whereas HER3 in 182\(^{R-1}\) and 182\(^{R-2}\) cells was associated with HER1 and HER2.

3.8. Importance of c-Src for growth of fulvestrant resistant cells

It has been shown that binding of c-Src to HER2 is crucial for formation of HER2:HER3 heterodimers [51]. We therefore looked for c-Src protein upon precipitation of HER2. As shown in Fig. 7A, c-Src was co-precipitated with HER2 in the two fulvestrant resistant T47D sublines but not in the parental cell line. We also detected higher expression of both phosphorylated and total levels of c-Src in the resistant sublines compared to T47D cells (Fig. 7B). Finally, 5 days dose–response growth experiments with the c-Src inhibitor dasatinib (0.05–1 \(\mu M\)) resulted in preferential and pronounced growth inhibition of the fulvestrant resistant cells (50–70%), compared to only 10–20% inhibition of growth of parental T47D cells (Fig. 7C).

4. Discussion

Research into the mechanisms of resistance to antiestrogens has demonstrated that breast cancer cell lines can switch from ER- to HER-driven cell growth upon acquiring resistance [15–21,24,26,28]. Most of these studies are based on the ER-positive and ER-dependent breast cancer cell line MCF-7. It is therefore important to determine whether the switch to HER-driven cell growth is a general mechanism for ER-positive breast cancer cells to acquire resistance to antiestrogens, or if other mechanisms are generated in other ER-positive human breast cancer cell lines. Therefore, we here present data on two new fulvestrant resistant T47D sublines, established by long-term treatment with 100 nM fulvestrant which corresponds to the blood concentration in patients treated with fulvestrant [55]. In our panel of seven fulvestrant resistant MCF-7 sublines, each derived from one isolated colony surviving fulvestrant or ICI 164,384 treatment [14], HER receptor signaling appeared to play a dominant role for growth [24,26,28]. Individual differences were observed among the cell
lines but increased expression and activation of HER1, increased level of phosphorylated HER3 and decreased level of HER4 were general findings. Increased activation of the downstream kinases Akt and Erk were also seen in the majority of the fulvestrant resistant MCF-7 sublines [23,24,26]. In contrast to MCF-7 cells, in which ER is the main driver of cell growth [24,26], we found that T47D cells utilize both ER and HER receptors for growth, as they are growth inhibited with as well antiestrogens and HER targeted therapies. The observed loss of ER expression and signaling in the fulvestrant resistant cell lines is considered to be the underlying cause for the lack of growth inhibitory effect of fulvestrant. We presume that the T47D cell line is heterogeneous and upon fulvestrant treatment, ER-negative cells may be the surviving cells which gradually acquire increased growth rate and give rise to the resistant cell lines. The observed changes in expression and signaling via the HER receptors, including significantly increased HER2 expression, decreased HER1 and HER4 expression and decreased Erk signaling indicate that HER2 could be the main driver of growth of the fulvestrant resistant T47D cell lines. To our knowledge, antiestrogen resistant T47D breast cancer cell lines have only been described in two previous studies [15,56,57] and in agreement with our finding, both studies found a switch from an ER-positive to an ER-negative phenotype. In contrast to MCF-7 cells, the switch between ER-positive and ER-negative cell lines in T47D cells could be due to differences in ER regulation as shown previously [58]. In none of the previous studies investigating resistant T47D cells, the expression level of HER2 was explored. However, an in vitro study has shown that ectopic over expression of HER2 in MCF-7 cells results in reduced response to tamoxifen [40]. This is in agreement with the clinical observation that response to endocrine therapy is reduced in ER-positive breast cancer patients with HER2-positive tumors compared to the response in HER2-negative tumors [39].

Trastuzumab has been used as targeted therapy for patients with HER2 over expressing or amplified breast cancer since 1990s [37,38,59]. Although trastuzumab has improved survival of HER2-positive breast cancer patients, still many patients do not benefit from this treatment alone. The overall response rate is only about one third of the HER2-positive metastatic breast cancer cases [38]. The established fulvestrant resistant T47D cells had over expressing of HER2 mRNA and protein, but not amplified HER2 gene, and the phosphorylation level of HER2 was similar in the parental and resistant cell lines. Targeted therapy against HER2 and HER2 knockout had no preferential inhibitory effect on the HER2 over expressing T47D fulvestrant resistant cell lines. Thus, HER2 does not seem to be the main driver of growth of these resistant cell lines.

When investigating the different heterodimers present in the T47D cell lines, we were not able to identify any heterodimers when HER2 was precipitated (data not shown). However, a small amount of HER2 was co-precipitated with HER3 in the fulvestrant resistant cell lines but not in parental T47D cells. This indicates that HER2 primarily is present as either monomer or homodimers in both parental and resistant cell lines and only a low amount of HER2 forms heterodimers in the resistant cell lines. The lack of response to HER2 targeted therapies can therefore indicate that HER2 does not contribute significantly to cell growth.

Our study showed a significantly higher level of HER1 expression and Erk phosphorylation in the parental cells compared to the fulvestrant resistant T47D cells. Moreover, the finding of similar level of HER1:HER3 heterodimers in parental and fulvestrant resistant cell lines indicates that the preferential growth inhibition of parental T47D cells with gefitinib may be due to inhibition of signaling from HER1 homodimers. Thus, HER1 is important for growth of the parental T47D cells but may also, although very low expressed, play a role for growth of the fulvestrant resistant T47D cell lines.

We define antiestrogen resistance as the ability of the cancer cells to grow continuously in the presence of antiestrogens at a concentration that blocks growth of the parental cells. This was the case for the established fulvestrant resistant T47D cell lines, which were able to grow continuously with 100 nM fulvestrant with a doubling time only slightly lower than the parental T47D cells. We noticed, however, that the plating efficiency of the resistant cells was lower than for the parental T47D cell line, which could be the reason for the slightly slower growth of the resistant cell lines compared to the parental T47D cells. Since the fulvestrant resistant cell lines are maintained in growth medium with 100 nM fulvestrant, the lack of detectable ER protein in the resistant cell lines could be due to binding of fulvestrant and concomitant ER degradation. However, as the fulvestrant resistant T47D cell lines were unaffected by withdrawal of fulvestrant both in the context of cell growth and with respect to sustained loss of expression of ER and the estrogen regulated proteins IGF-1Rα and PR, the resistant cell lines may have emerged from a small population of ER-negative cells that have survived the treatment with fulvestrant. Fulvestrant treatment of parental T47D cells resulted in resistant cells with significantly increased expression of HER2, which was not due to HER2 gene amplification. The over expression of HER2 in the resistant cells could be a result from continuous exposure to fulvestrant. However, this was not the case as HER2 over expression was maintained in the resistant cell lines after withdrawal of fulvestrant. The underlying mechanism for over expression of HER2 is currently unknown. Our data demonstrate a stable ER-negative/HER2 over expressing phenotype of the fulvestrant resistant cell lines.

Over expression and/or increased activity of c-Src is seen in human breast cancers [47,60], and often together with over expression of HER2. c-Src is a mitogen-activated protein kinase (MAPK) that plays a role in cell proliferation, migration, invasion, and survival [49]. In breast cancer, c-Src is frequently over expressed and activated, and its activation is associated with poor clinical outcome [49]. Therefore, targeting c-Src has been considered as a potential therapeutic approach in breast cancer treatment [49].

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of at least one of the HER receptors [46,47]. This supports that the two families of tyrosine kinases functionally and physically interact to promote breast cancer [46,47]. In agreement with the abovementioned literature, the HER2 over expressing fulvestrant resistant T47D cell lines had increased expression and phosphorylation of c-Src and an interaction with HER2 was demonstrated by co-precipitation of c-Src with immunoprecipitated HER2. However, as knockdown of HER2 had no effect on growth of the fulvestrant resistant T47D cell lines, the role of c-Src in the resistant cell model seems to be independent of the interaction between HER2 and c-Src. It was previously published that HER2-positive cells are resistant to c-Src inhibitors [56]. In contrast to the parental cells, our ER-negative/HER2 over expressing fulvestrant resistant T47D cells were very sensitive to treatment with dasatinib. This may be explained by our finding that in spite of high HER2 expression in the resistant cells, the level of phosphorylated HER2 was not significantly increased compared to the level in the parental cells, and as documented in a recent paper, it is not the level of HER2 but the HER2 activation status which is important for resistance to c-Src inhibitors [56]. Collectively, our data suggest that c-Src plays an important role for growth of the fulvestrant resistant T47D cell lines. Like phosphorylation of c-Src, we also found increased phosphorylation of GSK3β in the fulvestrant resistant cell lines compared to the parental T47D cells. GSK3β plays a role in different signaling processes including signaling via Akt. However, as phosphorylation of Akt was unchanged in the resistant cells compared to the parental T47D cells, increased GSK3β phosphorylation must be linked to other pathways i.e. the Wnt signaling pathway [61]. Further investigations of the importance of GSK3β as well as c-Src and HER2 for signaling and growth of our fulvestrant resistant T47D cell lines are currently in progress.

In conclusion, we have demonstrated that T47D cells acquire antiestrogen resistance via other mechanisms than MCF-7 cells, emphasizing the requirement for in vitro model systems representing the different types of ER-positive clinical breast cancers. In contrast to MCF-7 cells, which remain ER-positive upon development of antiestrogen resistance, the fulvestrant resistant T47D cell lines had a stable ER-negative phenotype. The HER receptors did not appear to play a major role for growth of fulvestrant resistant T47D cells, even though the HER2 level was significantly increased. Note worthy, the intracellular kinase c-Src was a driver of growth of fulvestrant resistant T47D cells. Antiestrogen resistance may occur via activation of a variety of signaling pathways. Therefore, in vitro models are important to unravel these pathways and to find biomarkers, disclosing the signaling pathways driving growth of resistant cells. We envision that this particular model with fulvestrant resistant ER-negative/HER2 over expressing breast cancer cells may represent patients with HER2 over expressing tumors unresponsive to HER2 targeted therapies.

Conflict of interest

The authors declare that they have no conflict of interest.

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