The effects of breast cancer therapy on estrogen receptor signaling throughout the body

Droog, M.

Chapter 5

Tamoxifen-resistance: From Bench to Bedside

Marjolein Droog, Karin Beelen, Sabine C. Linn, and Wilbert Zwart

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Abstract

Although tamoxifen is a classic example of a potent targeting drug, a substantial proportion of estrogen receptor alpha positive breast cancer patients lack benefit. Over the last few decades, cell biological studies discovered many potential biomarkers aimed to predict tamoxifen sensitivity, and to guide treatment selection. Nonetheless, these biomarkers seldom face clinical introduction because the number of potential biomarkers is very large. Patient samples with clinical follow-up are a depletable resource, and laborious to obtain. Therefore, clinical scientists can only validate a fraction of the potential biomarkers in clinical material. In this review, we describe a number of 'cell biological biomarkers' for tamoxifen resistance and their possible clinical implications. This may guide the clinical scientist in choosing what potential biomarkers to test on tumour samples, and catalyse the translation of scientific discoveries into daily clinical practice of breast cancer medicine.

Abbreviations

AF, activation function; AIB1 (SRC3) , amplified in breast cancer 1; AKT, RAC-alpha serine/threonine-protein kinase; AP-2γ, Activating Protein 2γ; ER, estrogen receptor; ERBB2, the gene that encodes HER2; ESR1, the gene that encodes ERα; FOXA1, forkheadbox protein A1; GATA3, GATA-binding protein; GRIP1 (SRC2), glucocorticoid receptor-interacting protein 1; HER2, human epidermal growth factor receptor 2; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NCOA (SRC), nuclear receptor coactivator; PBX1, pre-B-Cell Leukemia Homeobox 1; PI3K, phosphoinositide 3-kinase; SRC, steroid receptor coactivator; TIF-2 (SRC 2), transcriptional mediators/intermediary factor 2; XBP1, x-box-binding protein 1.

Introduction

Breast cancer is the most common malignancy in women, with annually over 1.5 million newly diagnosed cases and 400,000 deaths[1]. It is a heterogeneous disease with multiple subtypes that are traditionally defined through standard pathological criteria. These include cell morphology, invasive phenotype and well-established clinical markers, like estrogen receptor (ER)α, progesterone receptor, and human epidermal growth factor receptor (HER)2 levels. Over a decade ago, gene expression arrays identified distinct gene expression patterns in these subtypes[2,3]. These mRNA expression patterns gave rise to the now well-known breast cancer subtypes luminal A, luminal B and HER2, each with their own, more or less distinct pathological criteria and treatment options[4].

The vast majority of all breast cancers (75%) fall within the luminal subtype, which is positive for, and driven by, ERα. Such tumours are typically low grade, occur predominantly in postmenopausal women, and have a relatively favourable prognosis. Apart from chemotherapeutic treatment, ERα-positive breast cancer patients generally receive endocrine agents to prevent disease recurrence.

Tamoxifen and aromatase inhibitors represent the majority of anti-hormonal agents that are used to treat ERα-positive breast cancer patients[5,6]. Tamoxifen, a small molecule inhibitor, competes with estrogen to bind ERα. In breast cells, this competition prevents the formation of an ERα-mediated transcription complex, blocking estrogen-driven tumour cell proliferation[7]. In contrast, aromatase inhibitors deplete serum estrogen levels in postmenopausal patients because they block the aromatase enzymes, which convert testosterone into estrogen[8].

Despite endocrine treatment, a significant proportion of patients who receive adjuvant endocrine treatment still develop a recurrence[9,10]. This implies that the treatment was unsuccessful in these patients, who may have benefited from a different drug. Even though cross-resistance to other ERα inhibiting drugs might occur in some patients, other patients who relapse on one kind of endocrine treatment can still respond to another[10-12]. Therefore, in order to choose the right drug for the right patient, clinicians need to identify additional biomarkers, apart from ERα, to predict if a patient will benefit from a given drug.

Gradually, due to the emergence of new technologies, it is becoming more apparent that tumour specific characteristics exist that affect the sensitivity to ERα-targeted therapy. Identification of these characteristics, and subsequent translation into a clinical test that accurately predicts drug sensitivity, is of great value in the clinic because it enables a tailored-treatment of each individual breast cancer patient. Because multiple mechanisms have been described for tamoxifen resistance *in vitro,* the

question arises which of these cell biological markers are likely to have clinical validity (i.e. the potential to categorise subgroups of patients with differential drug sensitivity[13], and should therefore be tested in clinical samples?

Translation of these cell biological findings into a clinical test that predicts tamoxifen sensitivity is until now rarely successful; a pubmed search on the criteria 'tamoxifen resistance', 'cell', and 'breast cancer' resulted in 997 hits. In addition, if these biomarkers are tested on clinical samples, these studies can suffer from methodological flaws like mixing prognosis with prediction[14]. It is also important to recognise the difference between cell lines and patients, and understand the pros and cons of cell line studies as highlighted in Table 1. Sadly, the vast majority of potential biomarkers may never be tested on clinical samples, simply due to their massive numbers. We will therefore focus our review on promising cell biological biomarkers that give an indication of endocrine treatment resistance on the basis of *in vitro* type of analyses.

A cell biological biomarker for resistance associates with unresponsiveness to ERα-inhibition (like tamoxifen treatment or hormonal deprivation) of breast cancer cell lines. An overview of all criteria and definitions for the different types of markers is shown in Table 2. In the discussion of these cell biological biomarkers, we focus on those that are promising, and likely to have clinically validity when analysed in clinical tumour samples. In order to define 'promising', we determined the following criteria that the biomarker has to meet:

Table 1. Advantages and disadvantages for the use of breast cancer cell lines

Advantages	Disadvantages
Very straightforward to maintain	Prone to undergo geno- and phenotypic drift during continuous culture
Able to replicate perpetually	Selection for subpopulations of clones that have a growth advantage
Contain high levels of homogeneity	Derived from one patient, results can therefore not be generalised
Replaceable from frozen stocks should contamination occur	Artefacts are often observed due to tissue culture conditions
Often well annotated	Out of tissue-context, without interactions with extracellular matrix, basal compartment or infiltrating immune cells

Table 2: Definitions of different types of biomarkers

Term	Definition
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [138].
Prognostic biomarker	Biomarker that forecasts the likely course of the disease irrespective of treatment [138]. It provides information on 'who to treat'.
Predictive biomarker	Biomarker that is objectively measured and evaluated as an indicator of (in)sensitivity for a particular drug [138]. It provides information on 'how to treat'.
Predictive biomarker for endocrine treatment	A tumour characteristic that is objectively measured and evaluated as an indicator of (in)sensitivity for endocrine treatment in hormone-receptor positive breast cancer. It provides information on 'how to treat hormone receptor-positive breast cancer'.
Predictive cell biological biomarker for hormone receptor positive breast cancer cells	A characteristic of Estrogen Receptor-driven breast cancer cells that correlates with (un)responsiveness to endocrine treatment or hormonal deprivation of breast cancer cell lines with respect to Estrogen Receptor-activity and cell proliferation.

1. Associate with unresponsiveness to ERα inhibition of cell proliferation *in vitro*, in several studies from multiple labs;
2. Provide insights in the molecular mechanisms in which the biomarker is involved;
3. Be verified in multiple model systems, different cell lines, or murine models.

If multiple hits from within the same biological pathway are identified as potential biomarkers, the functionality of such a pathway could be considered as a promising biomarker. In addition, if the marker itself is druggable, this would open possibilities for novel intervention methods and drug target discovery.

Although some novel and potentially powerful biomarkers directly fulfil all these criteria, other markers included in this review only comply with part of the issues that are raised above. Tumour–stroma interactions and immune cell infiltration possibly influence breast cancer cell behaviour, metastatic outgrowth, and possibly response to treatment[15-17]. In this review we will exclusively discuss cell intrinsic signalling cascades of the tumour cell. We exclude details regarding immune cell infiltration and tumour–stroma interactions, even though we acknowledge the importance of these processes in breast cancer biology.

We first describe the ERα signalling pathway as well as the key components involved in the activation of this transcription factor. We group and discuss the potential biomarkers in two different subsections; as part of the ERα transcription complex, and as the growth factor receptor signalling cascades that can influence the ERα transcription complex. In the concluding remarks, we group the novel biomarkers for their level of being 'promising' according to the criteria as defined above and discuss missing issues and possible concerns for future analyses of biomarker validation studies.

The Estrogen Receptor as a Transcription Factor, and its Pathways

Anti-estrogens target the growth-stimulating effects of estrogens on breast tumour tissue that ERα mediates. Estrogen, the natural hormone and ligand, activates ERα by binding the C-terminal activation function 2 (AF-2), which lies in the ligand-binding domain. ERα then binds the DNA where transcription is mediated by the receptor's activation function 1 (AF-1) domain, close to the N-terminus.

Apart from the classical ligand activation of ERα, it can also be activated in a ligand (estrogen)-independent fashion. A number of post-translational modifications modulate ERα, activating transcription of the ligand-independent pathway. These include the direct phosphorylation of ERα by mitogen-activated protein kinase (MAPK)[18,19], RAC-alpha serine/threonine-protein kinase (Akt)[20], p90 ribosomal S6 kinase (Rsk)[21], protein kinase A[22], and c-Src[23,24]. The signalling pathway of this ligand-independent ERα activation by posttranslational modification will be discussed in more detail later.

The activation of the ERα ultimately results in a downregulation of transcriptional repressors, anti-proliferative and pro-apoptotic genes, and an upregulation of genes involved in cell cycle progression[25], such as Cyclin D1 and Myc. A graphic representation of the ERα transcription complex can be found in Figure 1.

Upon either mode of activation, the receptor associates with the chromatin. So called 'pioneer factors' facilitate this chromatin association of ERα. A pioneer factor precedes other chromatin-binding proteins, even if this chromatin is highly inaccessible. The first described, and best-studied pioneer factor, in breast cancer biology is FOXA1. One of the helices of FOXA1 structurally mimics the linker histone H1 and H5, which bind the DNA and the core histones[26]. In this manner, FOXA1 interacts with histone H3 and H4, and renders the chromatin accessible.

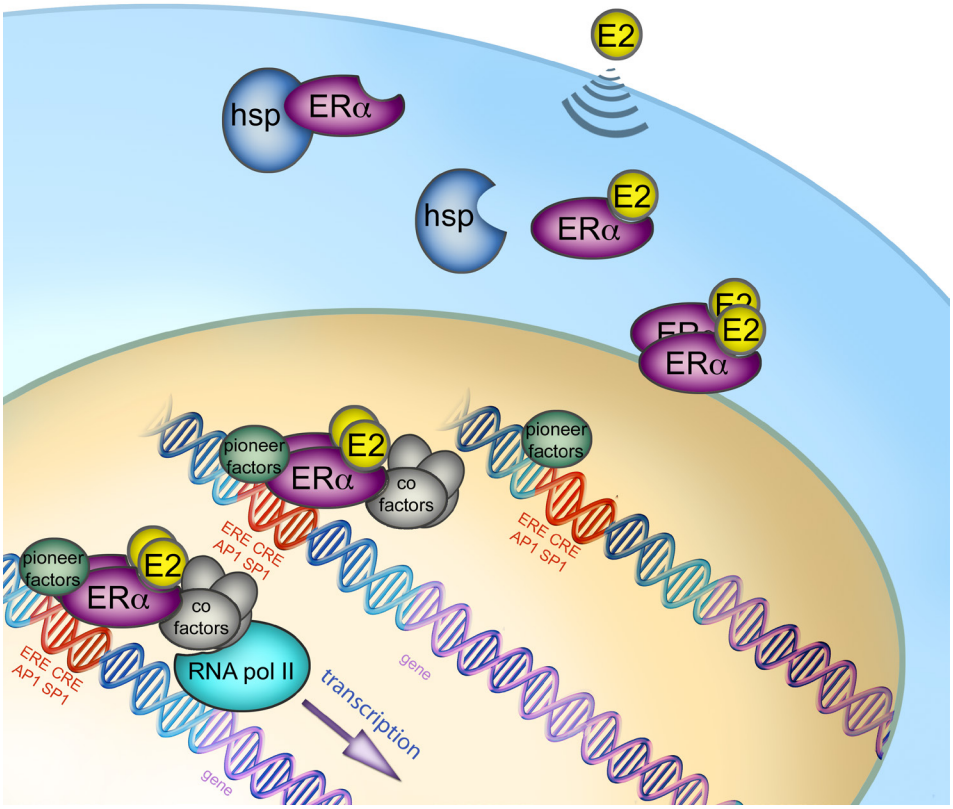

Figure 1. The estrogen receptor transcription complex. When estrogen diffuses into the cell, it binds the estrogen receptor, which leads to a dissociation from HSP90 and subsequent homodimerization. The estrogen receptor then binds the DNA at estrogen-responsive sites where it is able to recruit the transcription complex, resulting in transcription of responsive genes that are involved in cell proliferation.

As stated above, FOXA1 is an essential player in estrogen receptor biology, being crucial for ERα chromatin interactions and functional activity[27]. Since the initial reports of FOXA1 co-occupancy at ERα binding sites[28,29], other reports confirmed these findings[27] and found comparable functions for this pioneer factor in prostate cancer[30,31], enabling androgen receptor/chromatin interactions.

In breast cancer cell lines, knockdown of FOXA1 inhibits the association between ERα and the chromatin, and thereby prevents estrogen-induced gene expression[27-29]. But although ERα requires FOXA1, it is insufficient

for its complete functional activity. A cellular reprogramming into ERα responsive growth requires the expression of a second 'luminal-defining'[2,3] transcription factor, GATA3[32]. GATA3 associates to ERα binding sites in MCF-7 breast cancer cells[32], and inhibits breast cancer growth and metastasis formation[33] through a suppression of epithelial-to-mesenchymal transition[34].

Gradually, it is becoming apparent that FOXA1 is not the only ERα pioneer factor in breast cancer, but that other proteins share this function as well. AP-2γ promotes tumour cell proliferation[35] and controls estrogen-responses in breast cancer cell lines[36]. Like FOXA1 and AP-2γ, PBX1 enables ERα to associate with the chromatin, and facilitates ERα-mediated gene transcription[37]. Both PBX1 and AP-2γ share some of their binding sites with FOXA1, which synergistically influences ERα action[37,38].

ERα adopts different conformations depending on the type of ligand it binds[39,40]. Estradiol-binding activates the AF-2 domain[41], which leads to the formation of the coactivator-binding pocket of the ERα[39], where the p160 family of coactivators can bind to initiate transcription[42]. This protein family is composed of steroid receptor co-activator 1 (SRC1 or NCOA1), SRC 2 (also known as TIF-2, GRIP1 or NCOA2) and SRC3 (also known as AIB1 or NCOA3)[43]. Two ERα molecules jointly bind a single p160 protein[44], implying these proteins bind ERα in a mutually exclusive manner. And although the p160 family of coactivators share some chromatin-binding sites between them, proof of compensation upon knockdown of individual family members remains absent[45], implying that p160 family member are irreplaceable to ERα.

Through their role as protein scaffolds for the ERα complex, the p160 proteins play an essential role in subsequent transcription initiation, elongation, RNA splicing, receptor and coregulator turnover, and translation[46-49]. These p160 interactors of the ERα complex include p300 and CBP[50]. Both p300 and CBP can modify the chromatin accessibility through acetyltransferase activity, thereby regulating gene expression[51]. Other p160 interactors include histone modifiers CARM1[52,53] and JMJD2B[54,55] as well as members of the SWI/SNF complex, including BAF57[56]. Together, these proteins are essential for the ERα-complex to function properly.

Many cofactors can also bind the AF-1 domain of the ERα, which MAPK mediates by phosphorylating the receptor[18,19]. The AF-1 and the AF-2 domains synergistically enhance the transcriptional activity of ERα, in which the p160 coactivators play a crucial role as well[57,58]. When tamoxifen binds the receptor, the conformation of the ligand-binding domain alters, which inhibits a direct physical interaction between ERα and the p160 coactivators at the AF-2 transactivation domain[39]. This prevents the formation of an active transcription complex, blocking ERα-mediated cell proliferation.

Components of the ERα Transcription Complex as Potential Biomarkers for Endocrine Therapy Response

ERα itself could be a potential biomarker. Analogous to the androgen receptor in prostate cancer[59], the ERα was thought to be mutated in breast cancer. Indeed, a single point mutation of the ERα at amino acid 351 (tyrosine for aspartate) has been described, and could lead to tamoxifen-stimulated growth of breast cancer cells[60]. However, other reports challenged the physiological relevance of this mutation[61]. The frequency of mutations is debated and may be influenced by the detection method[62].

ESR1 (the gene encoding ERα) amplification in breast cancer has been an area of much debate. Overexpressing ERα in cell lines gave rise to broad anti-estrogen resistance *in vitro*[63], supporting this mode of resistance. About 22% of all breast cancers, including benign and premalignant breast tumours were described to carry an *ESR1* amplification[64-66], which was accompanied by ERα overexpression[65-69]. However, various other studies have only reported low percentages of patients with *ESR1* amplifications in their cohorts[70-73], who could still expose a possible better prognosis. The role of tamoxifen herein remains elusive[65,66,74].

As the essential player in luminal breast cancer, the success of ERα status as a biomarker for endocrine responsiveness is limited. Although patients with the highest ERα protein expression benefit slightly more from tamoxifen compared with patients with low receptor expression, the latter group still derives substantial benefit[9]. It is likely that many potential biomarkers for anti-estrogen resistance are within the same complex as ERα because they facilitate its function.

Unlike *ESR1*, amplifications of coactivators's genes are often and consistently found. A well-known example is AIB1 (amplified in breast cancer 1), also named SRC3[75], and a member of the p160 family. SRC3 is overexpressed in a tamoxifen-resistant MCF-7 breast cancer cell line, and is required for the acquisition of EGFR-mediated tamoxifen resistance[76]. Genome-wide binding studies illustrated that the gene profile that was exclusively regulated by an SRC3-bound ERα, but not shared with the other two p160 coactivators, enabled the identification of breast cancer patients with a poor outcome after tamoxifen treatment[45]. These data suggest that the specificity of p160 coregulators can have distinct clinical implications for breast cancer patients.

Beside impacting classical hormone-receptor positive breast cancer cells, SRC3 expression also associates with the expression of the clinical marker HER2, and poor outcome after tamoxifen treatment[77]. SRC-3 competes with the transcription factor PAX2 for binding and regulation of *ERRB2* (the gene that encodes HER2), of which the outcome determines tamoxifen response in breast cells[78]. PAX2 expression associated

with improved recurrence-free survival[78]. The transcriptional regulation *ERRB2* expression could be clinically relevant because HER2 overexpression is commonly mediated through amplification of the locus[79].

In addition to SRC3, another p160 protein, SRC1, has also been implicated in endocrine resistance. Cell lines that were selected to proliferate in the absence of estrogen (mimicking aromatase inhibitor resistance), exposed increased levels of SRC1[80]. In addition, SRC1-positive tumours associated with a reduction in disease-free survival in aromatase inhibitor-treated patients [80] as well as in tamoxifen-treated patients[81]. However, the predictive value of SRC1 has never been shown in the context of a randomized clinical trial (where treatments were compared in patients with similar clinical parameters without them receiving this treatment)[14]. SRC1 overexpression studies failed to induce tamoxifen resistance or hormone unresponsiveness in cell lines[22,82], indicating high SRC1 levels are insufficient for endocrine resistance induction.

In addition to their relevance in ERα-mediated transcription, the p160-ERα interaction surface has also been studied for its druggability. Peptides that are also present in the ERα-binding surface of p160 coactivators, could directly prevent the interaction between ERα and p160 proteins by competitive inhibiton, and thus inhibit ERα action[83]. This could open the possibilities for novel drug development strategies based on the chemical features of the p160-ERα-binding interface[84], a strategy that has already proven successful in highly analogous androgen receptor inhibition studies[85].

Before their recognition as key-role players in ERα-mediated transcription, many factors had already been categorised as luminal A-defining genes[2,3]. These include FOXA1 and GATA3. Immunohistochemical data illustrated that FOXA1 expression levels associate with ERα and progesterone receptor levels in breast tumours[86,87].

Tamoxifen-resistant derivatives of MCF-7 cells, which retained their ERα dependence, required FOXA1 for ERα/chromatin-binding and cell proliferation[88]. Overexpression of FOXA1 in ERα-transfected cell lines render the receptor capable to interact with the chromatin and sensitive to tamoxifen treatment, which then inhibits cell proliferation[27,32]. FOXA1 expression associates with a favourable prognosis[89-92], also in ERα negative patients[93], but this was unconfirmed in another report[86]. Even though a clear role for FOXA1 in anti-estrogen response prediction in ERα positive breast cancer remains absent, it might be a promising drug target in hormonal breast cancer treatment[88,90] due to its role in estrogen response and its tissue specific expression profile[94].

As stated above, FOXA1 is not unique in its role as pioneer factor in ERα biology because this function is shared with AP-2γ and PBX1.

Overexpression of pioneer factor AP-2γ was found to associate with a poor survival of breast cancer patients and poor outcome after endocrine agents, and *in vitro* data illustrated that endocrine-resistant cell lines have elevated levels of AP-2γ[95]. PBX1 associated with an unfavourable outcome of ERα-positive breast cancer patients upon tamoxifen treatment[37]. Whether this poor outcome is related to a more aggressive breast cancer or unresponsiveness of these tumours to tamoxifen needs to be

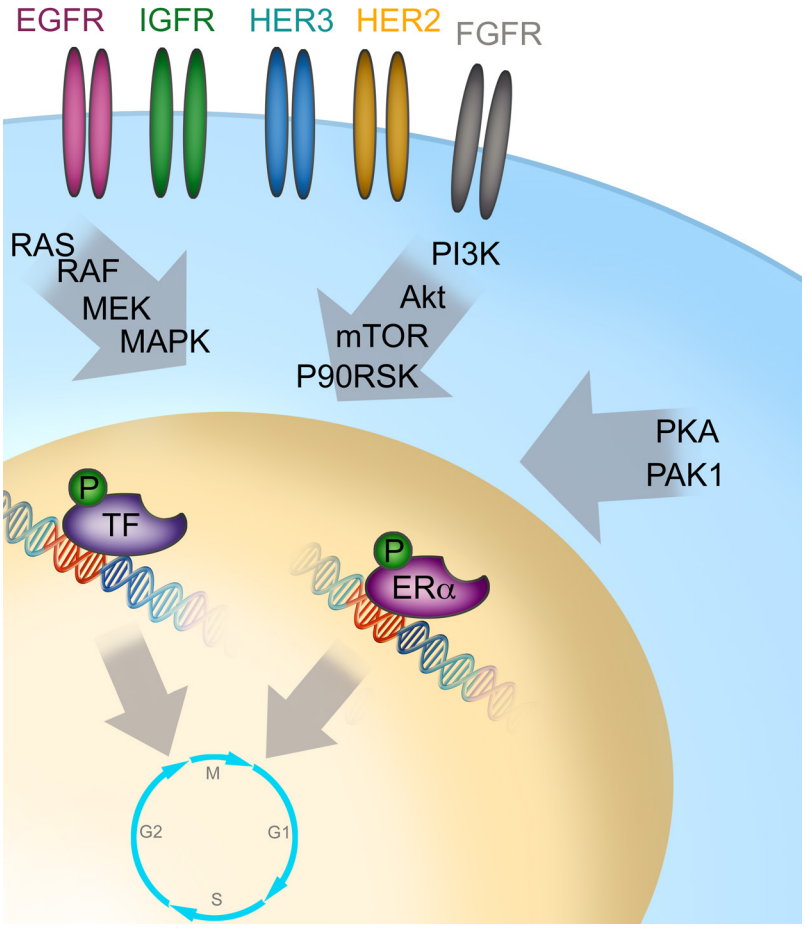

Figure 2. Growth factor receptor signalling cascades that share a common mode of downstream kinase activation; the MAPK and PI3K pathway. Upon ligand binding, the transmembrane growth factor receptors initiate a downstream phosphorylation cascade that will activate the MAPK and PI3K pathways. Along with PKA and PAK1, these pathways can lead to a phosphorylation of the estrogen receptor and other transcription factors, which leads to a cell cycle progression.

elucidated. Similar to FOXA1, AP-2γ and PBX1 could be interesting drug targets.

Apart from its essential role for ERα biology and as luminal-defining factor[2,32], the presence of GATA3 was identified in a small study as a predictor for hormone responsiveness in breast cancer[96,97]. However, analysis of GATA3 in a larger series of ERα-positive patients did not show a difference in outcome, both in patients treated with, or without tamoxifen[98].

XBP-1 is a direct transcriptional target of E2-stimulated ERα, and is one of the key-defining genes for luminal A breast cancer[2,3]. XBP-1 is a CREB/ATF transcription factor that acts during the unfolded protein response (a cellular stress response related to the endoplasmatic reticulum), and inhibits apoptosis . A spliced XBP-1 variant (XBP-1S) results in a larger transcript, and associates with poor outcome after adjuvant tamoxifen treatment[99].

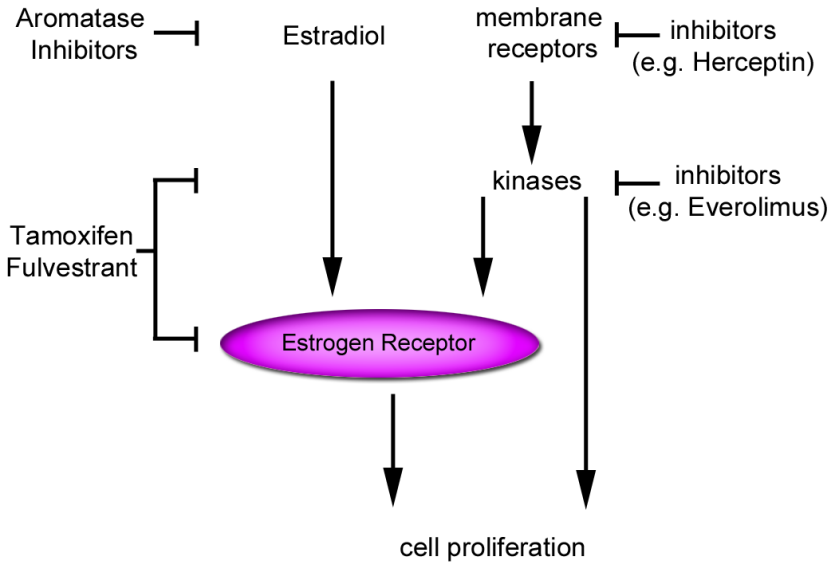

Figure 3. Possible therapeutic interventions to estrogen receptor- and growth factor-mediated cell proliferation. The estrogen receptor activity can be blocked by inhibiting ligand binding or preventing its phosphorylation through upstream kinases. The inhibition of ligand binding can be achieved by estrogen-depletion (using aromatase inhibitors), a direct competion with estrogen-binding to the receptor (using tamoxifen) or through degradation of the estrogen receptor (using fulvestrant). The second approach to block estrogen receptor activation is by preventing the estrogen receptor to be phosphorylated. Additionally, this will also prevent the phosphorylation of other transcription factors that might otherwise lead to cell cycle progression. This may be achieved by drugs that block growth factor receptor activation (such as Herceptin), but also through an inhibition of any step in the PI3K or MAPK pathway, e.g. mTOR (Everolimus) or ERK inhibitors.

Also RARA is a direct ERα-responsive gene[100], which encodes retinoic acid receptor alpha (RAR-α). RARα is a protein that binds the genomic regions that are shared with ERα[101,102]. One study claimed ERα and RAR-α competed in binding[101], whereas a different study claimed RAR-α functions as a coactivator for ERα activation [102]. The last study showed supporting clinical data, where patients whose tumour expressed high RAR-α levels, showed favourable outcome, suggesting a functional ERα pathway.

Growth Factor Receptor Signalling Pathways Influence the ERα Transcription Complex

Endocrine resistance can rely on other mechanisms than the ligand-dependent ERα pathway. Activation of receptor tyrosine kinase pathways, including HER2, EGFR, IGFR and FGFR, can cause endocrine therapy resistance. Each receptor has its own designated ligand, a mitogen that triggers the cell to proliferate. Upon binding of these mitogens, the relevant receptor undergoes conformational changes that cause (hetero) dimerisation[103]. In doing so, the dimerised receptors are activated, autophosphorylated, and cause downstream kinase activation[104-106]. These growth factor receptor signalling cascades share a common mode of downstream kinase activation pathways (Figure 2); the MAPK and phosphoinositide 3-kinase (PI3K) pathway. Both kinase pathways share a common feature of stimulating cell proliferation.

In addition to the direct growth-stimulatory effects of these transmembrane receptors, their downstream kinase pathways also directly influence ERα activity through phosphorylation of its residues. The kinases expose a clear specificity for distinct ERα-phosphorylation sites, each with their own effects. Because numerous review papers have discussed these phosphorylation events on ERα extensively[107,108], we will only focus on two ERα phosphorylation events (serine 118 an serine 305) that affect the binding of ERα with coactivators in the presence of tamoxifen[109] because these might have strong translational and clinical potential.

One of ERα's residues that is directly targeted by the MAPK pathway is serine 118[19]. This amino acid can also be phosphorylated through multiple other signaling cascades, including PAK1[110] and CDK7[111]. MAPK-induced ERα phosphorylation involves a ligand-independent receptor activation, whereas CDK7-induced phosphorylation of serine 118 is a direct consequence of ligand-activation[111]. The functional implication of ERα serine 118 phosphorylation on endocrine sensitivity might thus be dependent on the upstream signaling cascade.

Another ERα-residue that can be phosphorylated is serine 305, which lies within a domain that is important for the interaction with coregulatory proteins. The phosphorylation of ERαS305 can trigger the phosphorylation

of ERαS118[110]. Phosphorylation of ERαS305 alters the conformation of ERα depending on the ligand it binds[22,109], which influences the orientation between ERα and coactivators[109], and stimulates activation.

Components of the Growth Factor Receptor Signaling Pathways as Potential Biomarkers for Endocrine Therapy Response

Each level of regulation within the membrane-initiated receptor tyrosine kinase pathway could provide possible biomarkers for endocrine response, and therapeutic intervention. Many examples exist for tamoxifen resistance in cell line models in regard to receptor tyrosine kinase activation. This link was first recognized in MCF-7 cells that were treated with epidermal growth factor (EGF), which decreased ERα and progesterone receptor expression, and decreased response to antiestrogens[112].

In mouse models, tamoxifen stimulated tumour growth from HER2-overexpressing-transfected MCF-7 cells, although estrogen depletion therapy remained effective[113]. Because ERα still needs a ligand to be activated in such a setting, aromatase inhibitor treatment might be superior in similar tumours. A clinical study however, was unable to show benefit from aromatase inhibitors versus tamoxifen in HER2 positive cases[114]. Possibly, these HER2 overexpressing tumours lost their dependency on ERα signaling for growth.

Endocrine resistance can also involve the insulin-like growth factor (IGF) pathway. In this pathway, insulin, IGF-I, and IGF-II, activate the IGF-I receptor (IGF-IR). *In vitro* data show an increased sensitivity to the proliferative effects of IGF-I in a tamoxifen-resistant MCF-7 cells[115,116]. Breast cancer cell lines become unresponsive to antiestrogens upon IGF-1R overexpression in response to IGF-1 ligand stimulation, involving active MAPK and PI3K pathways[117]. Moreover, cross-talks between the IGF-IR and the EGFR via c-SRC activation exists, which mediates tamoxifen-resistant cell proliferation[118].

In MCF-7 xenografts, EGFR and HER2 increased when tumors became tamoxifen-resistant. These tumors also showed increased phosphorylation of IGF-IR, which can interact with both EGFR and ERα, while total IGF-IR levels remained the same[119]. Classic ERα-gene targets however, remained suppressed, implying tamoxifen stimulates a subset of genes in these tumors, while blocking expression of another subset of genes. Gefitinib, an EGFR inhibitor, improved tamoxifen response and delayed acquired resistance, but lacked effect on estrogen-stimulated growth, whereas tamoxifen did. This study suggests that tamoxifen functions like estrogen on some genes, while working agonistically on other genes that should be targeted with a second drug.

Another contributor to endocrine resistance is the fibroblast growth factor receptor 1 (FGFR1). In response to FGF2, activation of the MAPK-PI3K-AKT signaling pathways increased in FGFR1-amplified breast cell lines that turned tamoxifen resistant[120]. In breast cancer samples, 32% showed high FGFR4 mRNA levels compared with the total population[121]. High FGFR4 mRNA levels predicted poor clinical benefit and shorter progression-free survival for patients with recurrent tamoxifen-treated breast cancer[122].

Because all receptor tyrosine kinase family members share a downstream activation of the PI3K/ mammalian target of rapamycin (mTOR)/ Akt pathway, this common denominator provides a promising target for novel therapeutics. In response to long-term estrogen depletion, cell lines showed activation of mTOR substrates (P70S6K and P85S65), as well as PI3K substrates (AKT), and became hormone independent, which reversed upon mTOR and PI3K inhibition[123]. In agreement, tumors that developed aromatase inhibitor resistance also associated with the PI3K/AKT/mTOR pathways[124]. Dual inhibition of the mTOR and ERα pathways, by simultaneous treatment of the mTOR inhibitor Everolimus and aromatase inhibitor, induced cell death *in vitro*[125], and improved progression-free survival in patients who previously received aromatase inhibitors only[126].

Activation of Akt and the downstream mTOR pathway can also lead to tamoxifen resistance[20,127,128]. A phase II trial showed benefit from the addition of an mTOR inhibitor to tamoxifen in patients with metastatic breast cancer who progressed after an aromatase inhibitor[129]. The question remains how to identify patients with hormone receptor-positive breast cancer who will benefit from (the addition of) PI3K/Akt/mTOR inhibition, and whether a selection of patients might even benefit from these drugs in the adjuvant setting or as first line therapy in metastatic disease. Determination of the clinical predictive validity of biomarkers of the PI3K/Akt/mTOR pathway for hormonal therapy resistance is therefore important.

As described above, MAPK- and PAK1-induced ERα phosphorylation on ERαS118 associate with endocrine resistance *in vitro*[19,110], whereas ligand activation causes CDK7-induced ERαS118 phosphorylation[111]. ERαS118 phosphorylation associates with a benefit from tamoxifen in pre-menopausal breast cancer patients[130], which indicates a ligand-dependent ERα pathway. In contrast, the association between ERαS118 phosphorylation and tamoxifen sensitivity remains unclear in postmenopausal patients because this was only evaluated in series of patients who were all treated with tamoxifen, and showed inconsistent results[131-133]. However, ERαS118 phosphorylation levels increased after patients developed a relapse following tamoxifen treatment[132], suggesting a positive selection of these cells at the metastatic site. It is likely that MAPK or PAK1 induces ERαS118

phosphorylation in these postmenopausal patients since estrogen levels decrease after menopause. Thus, cell biological data suggest that ERαS118 phosphorylation should be interpreted differently depending on upstream kinases, which may possibly be loosely deducted from menopausal status.

In response to stimulation, PKA[22] and PAK1[134] induced phosphorylation of ERαS305, which led to tamoxifen resistance. This phosphorylation arrests the ERα in an alternative conformation[22]. It furthermore induces the formation of an active transcription complex, chromatin remodeling, RNA polymerase II recruitment, and gene activation[109]. Numerous clinical studies validated these cell biological observations, by showing that ERαS305 phosphorylation associated with tamoxifen resistance, irrespective of the patients's postmenopausal status[135-137].

A biomarker should always be chosen while taking the available upstream pathway data into account. This is illustrated by an active PAK1 that phosphorylates Serine 305 on ERα, leading to phosphorylation of ERαS118, and induces tamoxifen resistance that depends on[110]. These data show that ERα and transmembrane growth factor receptor pathways intertwine in their functional activities, which present many possible options for therapeutic interventions (Figure 3).

Concluding Remarks

The search for cell biological biomarkers for breast cancer has resulted in hundreds of studies, describing proteins, genes, and posttranslational modifications that could be involved in endocrine response. Not all proteins that look promising in cell line models however, will translate into clinically applicable tools and so the next challenge lies in the decision of which of these biomarkers to test in the clinic. In this review, we focused on proteins that play a role in (un)responsiveness to endocrine treatment *in vitro*, and in multiple studies, and where at least some form of clinical data is available to illustrate the marker's usefulness in the clinic. Clearly, not all cell biological potential biomarkers for breast cancer treatment outcome have been discussed in this review, and many potential biomarkers are yet to be discovered and verified in clinical studies. We listed the most promising cell biological biomarkers in Table 3.

On all levels of ERα biology, promising biomarkers can be identified. FOXA1 is of considerable interest, due to the large body of evidence from both *in vitro* and *in vivo* studies as well as the potential druggability of the protein. The same line of reasoning can be applied for the p160 coregulators SRC1 and AIB1, in conjunction with the p160-ERα interface as a possible druggable interaction. Coactivators of ERα could possibly also function as biomarkers for endocrine responsiveness or drug targets because these coactivators are essential for ERα activation. Subtle

variations in their expression levels or transcriptional modification status could directly influence endocrine response. If the clinical validity of these biomarkers for tamoxifen resistance would be established, these markers could potentially be used to guide treatment decisions. In addition, these potential novel biomarkers could pave the way for novel drug development strategies for patients with an otherwise poor outcome.

ERα phosphorylation status appears to be a reliable readout for endocrine responsiveness, dependent on the phosphorylation site. ERαS305 phosphorylation was predictive for resistance to tamoxifen irrespective of the menopausal status of the patient. For ERαS118 phosphorylation, a benefit from tamoxifen was shown in the premenopausal situation, but remains unresolved in the postmenopausal setting. All and all, current literature suggests that the pathological properties of a tumour must be viewed in context of the clinical parameters, which as a combined information stream has the potency to guide endocrine treatment selection.

Regarding the growth factor receptor pathways, multiple cell line studies showed that activation of the PI3K/MAPK pathways resulted in tamoxifen resistance. However, the complex regulation of these pathways, with possible alterations at multiple levels, complicates the identification of a biomarker with clinical validity. Nevertheless, the increasing knowledge from cell biological studies regarding downstream activated proteins may ultimately lead to the identification of a biomarker that can predict clinical resistance.

Tailored treatment, on the basis of novel biomarkers, could co-exist with current clinical markers. This may ultimately lead to novel dual treatment options, where the pathways on which the tumor depends, will serve as dual targets. Breast cancer is above all a heterogeneous disease that contain driving pathways within the same tumour, which may very well be a key concept for the next generation of targeted endocrine treatment in breast cancer.

Table 3. Promising biomarkers for hormone receptor-positive breast cancer as determined in cell biology

Low(-) or high (+) expression indicative of endocrine sensitivity	Reproducibly found in multiple studies	Molecular mechanism	Multiple model systems	Same biological pathway as other markers	Possible drug target
FOXA1 (+)	X [27-29]	X [26,32]	X [87,91]	X	X [88]
SRC1 (-)	X [80,81]	-	X [76,81]	X	X [83,84,139]
SRC3 (-)	X [45,76-78]	X [78]	X [45,76]	X	X [83,84,139]
AP-2γ (-)	X [35,36,38,95]	X [38]	X [35,36,38,95]	X	-
PI3K/MAPK pathway activation (-)	X [115,120,121,123,128]	-	X [20,123,127,128]	X	X [126,129,140,141]
PAK1 (-)	X [110,136,137]	-	X [110,136,137]	X	-
ERαS305P (-)	X [22,40,136,137]	X [109]	X [22,136,137]	X	-
ERαS118P (+) for premenopausal patients	X [111,130,142]	-	X [130,142]	X	-
PAX2 (-)	- [78]	X [78]	-	X	-

Report contains clinical data

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References

1 Ferlay, J. et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer. Journal international du cancer 127, 2893-2917, (2010).

2 Perou, C. M. et al. Molecular portraits of human breast tumours. Nature 406, 747-752, (2000).

3 Sorlie, T. et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proceedings of the National Academy of Sciences of the United States of America 98, 10869-10874, (2001).

4 Weigelt, B. & Reis-Filho, J. S. Histological and molecular types of breast cancer: is there a unifying taxonomy? Nat Rev Clin Oncol 6, 718-730, (2009).

5 Colleoni, M. et al. Increasing steroid hormone receptors expression defines breast cancer subtypes non responsive to preoperative chemotherapy. Breast Cancer Res Treat 116, 359-369, (2009).

6 Regan, M. M. et al. Re-evaluating adjuvant breast cancer trials: assessing hormone receptor status by immunohistochemical versus extraction assays. J Natl Cancer Inst 98, 1571-1581, (2006).

7 Sutherland, R. L., Hall, R. E. & Taylor, I. W. Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells. Cancer research 43, 3998-4006, (1983).

8 Simpson, E. R. et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15, 342-355, (1994).

9 Davies, C. et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378, 771-784, (2011).

10 Vergote, I. et al. Metastatic breast cancer: sequencing hormonal therapy and positioning of fulvestrant. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 16 Suppl 2, 524-526, (2006).

11 Wang, J., Jain, S., Coombes, C. R. & Palmieri, C. Fulvestrant in advanced breast cancer following tamoxifen and aromatase inhibition: a single center experience. The breast journal 15, 247-253, (2009).

12 Yoo, C. et al. Efficacy of fulvestrant in heavily pretreated postmenopausal women with advanced breast cancer: a preliminary report. Journal of breast cancer 14, 135-139, (2011).

13 Hunter, D. J., Khoury, M. J. & Drazen, J. M. Letting the genome out of the bottle--will we get our wish? N Engl J Med 358, 105-107, (2008).

14 Beelen, K., Zwart, W. & Linn, S. C. Can predictive biomarkers in breast cancer guide adjuvant endocrine therapy? Nat Rev Clin Oncol 9, 529-541, (2012).

15 Khamis, Z. I., Sahab, Z. J. & Sang, Q. X. Active roles of tumor stroma in breast cancer metastasis. International journal of breast cancer 2012, 574025, (2012).

16 Malanchi, I. et al. Interactions between cancer stem cells and their niche govern metastatic colonization. Nature 481, 85-89, (2012).

17 Tyan, S. W. et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PloS one 6, e15313, (2011).

18 Bunone, G., Briand, P. A., Miksicek, R. J. & Picard, D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15, 2174-2183, (1996).

19 Kato, S. et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270, 1491-1494, (1995).

20 Campbell, R. A. et al. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 276, 9817-9824, (2001).

21 Joel, P. B. et al. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol 18, 1978-1984, (1998).

22 Michalides, R. et al. Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. Cancer cell 5, 597-605, (2004).

23 Arnold, S. F., Obourn, J. D., Jaffe, H. & Notides, A. C. Phosphorylation of the human estrogen receptor on tyrosine 537 in vivo and by src family tyrosine kinases in vitro. Mol Endocrinol 9, 24-33, (1995).

24 Migliaccio, A. et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15, 1292-1300, (1996).

25 Frasor, J. et al. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144, 4562-4574, (2003).

26 Cirillo, L. A. et al. Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. EMBO J 17, 244-254, (1998).

27 Hurtado, A., Holmes, K. A., Ross-Innes, C. S., Schmidt, D. & Carroll, J. S. FOXA1 is a key determinant of estrogen receptor function and endocrine response. Nature genetics 43, 27-33, (2011).

28 Carroll, J. S. et al. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 122, 33-43, (2005).

29 Laganiere, J. et al. From the Cover: Location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response. Proceedings of the National Academy of Sciences of the United States of America 102, 11651-11656, (2005).

30 Gao, N. et al. The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. Mol Endocrinol 17, 1484-1507, (2003).

31 Lee, H. J., Hwang, M., Chattopadhyay, S., Choi, H. S. & Lee, K. Hepatocyte nuclear factor-3 alpha (HNF-3alpha) negatively regulates androgen receptor transactivation in prostate cancer cells. Biochem Biophys Res Commun 367, 481-486, (2008).

32 Kong, S. L., Li, G., Loh, S. L., Sung, W. K. & Liu, E. T. Cellular reprogramming by the conjoint action of ERalpha, FOXA1, and GATA3 to a ligand-inducible growth state. Molecular systems biology 7, 526, (2011).

33 Dydensborg, A. B. et al. GATA3 inhibits breast cancer growth and pulmonary breast cancer metastasis. Oncogene 28, 2634-2642, (2009).

34 Yan, W., Cao, Q. J., Arenas, R. B., Bentley, B. & Shao, R. GATA3 inhibits breast cancer metastasis through the reversal of epithelial-mesenchymal transition. J Biol Chem 285, 14042-14051, (2010).

35 Williams, C. M. et al. AP-2gamma promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene. EMBO J 28, 3591-3601, (2009).

36 Woodfield, G. W., Horan, A. D., Chen, Y. & Weigel, R. J. TFAP2C controls hormone response in breast cancer cells through multiple pathways of estrogen signaling. Cancer research 67, 8439-8443, (2007).

37 Magnani, L., Ballantyne, E. B., Zhang, X. & Lupien, M. PBX1 genomic pioneer function drives ERalpha signaling underlying progression in breast cancer. PLoS genetics 7, e1002368, (2011).

38 Tan, S. K. et al. AP-2gamma regulates oestrogen receptor-mediated long-range chromatin interaction and gene transcription. EMBO J 30, 2569-2581, (2011).

39 Shiau, A. K. et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95, 927-937, (1998).

40 Zwart, W. et al. Classification of anti-estrogens according to intramolecular FRET effects on phospho-mutants of estrogen receptor alpha. Molecular cancer therapeutics 6, 1526-1533, (2007).

41 Kumar, V. et al. Functional domains of the human estrogen receptor. Cell 51, 941-951, (1987).

42 McKenna, N. J., Lanz, R. B. & O'Malley, B. W. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20, 321-344, (1999).

43 Xu, J. & Li, Q. Review of the in vivo functions of the p160 steroid receptor coactivator family. Mol Endocrinol 17, 1681-1692, (2003).

44 Gee, A. C., Carlson, K. E., Martini, P. G., Katzenellenbogen, B. S. & Katzenellenbogen, J. A. Coactivator peptides have a differential stabilizing effect on the binding of estrogens and antiestrogens with the estrogen receptor. Mol Endocrinol 13, 1912-1923, (1999).

45 Zwart, W. et al. Oestrogen receptor-co-factor-chromatin specificity in the transcriptional regulation of breast cancer. EMBO J 30, 4764-4776, (2011).

46 Auboeuf, D. et al. Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. Proceedings of the National Academy of Sciences of the United States of America 101, 2270-2274, (2004).

47 Onate, S. A., Tsai, S. Y., Tsai, M. J. & O'Malley, B. W. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270, 1354-1357, (1995).

48 Wu, R. C., Feng, Q., Lonard, D. M. & O'Malley, B. W. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. Cell 129, 1125-1140, (2007).

49 Yu, C. et al. An essential function of the SRC-3 coactivator in suppression of cytokine mRNA translation and inflammatory response. Mol Cell 25, 765-778, (2007).

50 McKenna, N. J. et al. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. The Journal of steroid biochemistry and molecular biology 69, 3-12, (1999).

51 Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87, 953-959, (1996).

52 Chen, D. et al. Regulation of transcription by a protein methyltransferase. Science 284, 2174-2177, (1999).

53 Stallcup, M. R. et al. Co-operation between protein-acetylating and protein-methylating co-activators in transcriptional activation. Biochem Soc Trans 28, 415-418, (2000).

54 Kawazu, M. et al. Histone demethylase JMJD2B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PloS one 6, e17830, (2011).

55 Shi, L. et al. Histone demethylase JMJD2B coordinates H3K4/H3K9 methylation and promotes hormonally responsive breast carcinogenesis. Proceedings of the National Academy of Sciences of the United States of America 108, 7541-7546, (2011).

56 Belandia, B., Orford, R. L., Hurst, H. C. & Parker, M. G. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. EMBO J 21, 4094-4103, (2002).

57 Metivier, R., Penot, G., Flouriot, G. & Pakdel, F. Synergism between ERalpha transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 alpha-helical core and for a direct interaction between the N- and C-terminal domains. Mol Endocrinol 15, 1953-1970, (2001).

58 Zwart, W. et al. The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen. Journal of cell science 123, 1253-1261, (2010).

59 Culig, Z., Steiner, H., Bartsch, G. & Hobisch, A. Mechanisms of endocrine therapy-responsive and -unresponsive prostate tumours. Endocrine-related cancer 12, 229-244, (2005).

60 Wolf, D. M. & Jordan, V. C. The estrogen receptor from a tamoxifen stimulated MCF-7 tumor variant contains a point mutation in the ligand binding domain. Breast Cancer Res Treat 31, 129-138, (1994).

61 Anghel, S. I. et al. Aspartate 351 of estrogen receptor alpha is not crucial for the antagonist activity of antiestrogens. J Biol Chem 275, 20867-20872, (2000).

62 Barone, I. et al. Expression of the K303R estrogen receptor-alpha breast cancer mutation induces resistance to an aromatase inhibitor via addiction to the PI3K/Akt kinase pathway. Cancer research 69, 4724-4732, (2009).

63 Tolhurst, R. S. et al. Transient over-expression of estrogen receptor-alpha in breast cancer cells promotes cell survival and estrogen-independent growth. Breast Cancer Res Treat 128, 357-368, (2011).

64 Burkhardt, L. et al. Gene amplification in ductal carcinoma in situ of the breast. Breast Cancer Res Treat 123, 757-765, (2010).

65 Holst, F. et al. Estrogen receptor alpha (ESR1) gene amplification is frequent in breast cancer. Nature genetics 39, 655-660, (2007).

66 Tomita, S. et al. Estrogen receptor alpha gene ESR1 amplification may predict endocrine therapy responsiveness in breast cancer patients. Cancer Sci 100, 1012-1017, (2009).

67 Dunbier, A. K. et al. ESR1 is co-expressed with closely adjacent uncharacterised genes spanning a breast cancer susceptibility locus at 6q25.1. PLoS genetics 7, e1001382, (2011).

68 Moelans, C. B. et al. ESR1 amplification is rare in breast cancer and is associated with high grade and high proliferation: a multiplex ligation-dependent probe amplification study. Cell Oncol (Dordr) 34, 489-494, (2011).

69 Tsiambas, E. et al. Significance of estrogen receptor 1 (ESR-1) gene imbalances in colon and hepatocellular carcinomas based on tissue microarrays analysis. Med Oncol 28, 934-940, (2011).

70 Brown, L. A. et al. ESR1 gene amplification in breast cancer: a common phenomenon? Nature genetics 40, 806-807; author reply 810-802, (2008).

71 Horlings, H. M. et al. ESR1 gene amplification in breast cancer: a common phenomenon? Nature genetics 40, 807-808; author reply 810-802, (2008).

72 Reis-Filho, J. S. et al. ESR1 gene amplification in breast cancer: a common phenomenon? Nature genetics 40, 809-810; author reply 810-802, (2008).

73 Vincent-Salomon, A., Raynal, V., Lucchesi, C., Gruel, N. & Delattre, O. ESR1 gene amplification in breast cancer: a common phenomenon? Nature genetics 40, 809; author reply 810-802, (2008).

74 Nielsen, K. V. et al. Amplification of ESR1 may predict resistance to adjuvant tamoxifen in postmenopausal patients with hormone receptor positive breast cancer. Breast Cancer Res Treat 127, 345-355, (2011).

75 Anzick, S. L. et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277, 965-968, (1997).

76 Zhao, W., Zhang, Q., Kang, X., Jin, S. & Lou, C. AIB1 is required for the acquisition of epithelial growth factor receptor-mediated tamoxifen resistance in breast cancer cells. Biochem Biophys Res Commun 380, 699-704, (2009).

77 Osborne, C. K. et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95, 353-361, (2003).

78 Hurtado, A. et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. Nature 456, 663-666, (2008).

79 Slamon, D. J. et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235, 177-182, (1987).

80 McBryan, J. et al. Metastatic progression with resistance to aromatase inhibitors is driven by the steroid receptor coactivator SRC-1. Cancer research 72, 548-559, (2012).

81 Redmond, A. M. et al. Coassociation of estrogen receptor and p160 proteins predicts resistance to endocrine treatment; SRC-1 is an independent predictor of breast cancer recurrence. Clinical cancer research : an official journal of the American Association for Cancer Research 15, 2098-2106, (2009).

82 Zwart, W. et al. Resistance to antiestrogen arzoxifene is mediated by overexpression of cyclin D1. Mol Endocrinol 23, 1335-1345, (2009).

83 Carraz, M., Zwart, W., Phan, T., Michalides, R. & Brunsveld, L. Perturbation of estrogen receptor alpha localization with synthetic nona-arginine LXXLL-peptide coactivator binding inhibitors. Chemistry & biology 16, 702-711, (2009).

84 Phan, T., Nguyen, H. D., Goksel, H., Mocklinghoff, S. & Brunsveld, L. Phage display selection of miniprotein binders of the Estrogen Receptor. Chem Commun (Camb) 46, 8207-8209, (2010).

85 Vaz, B. et al. Computational design, synthesis, and evaluation of miniproteins as androgen receptor coactivator mimics. Chem Commun (Camb), 5377-5379, (2009).

86 Hisamatsu, Y. et al. Impact of FOXA1 Expression on the Prognosis of Patients with Hormone Receptor-Positive Breast Cancer. Annals of surgical oncology 19, 1145-1152, (2012).

87 Ross-Innes, C. S. et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature 481, 389-393, (2012).

88 Nakshatri, H. & Badve, S. FOXA1 as a therapeutic target for breast cancer. Expert opinion on therapeutic targets 11, 507-514, (2007).

89 Ademuyiwa, F. O., Thorat, M. A., Jain, R. K., Nakshatri, H. & Badve, S. Expression of Forkhead-box protein A1, a marker of luminal A type breast cancer, parallels low Oncotype DX 21-gene recurrence scores. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 23, 270-275, (2010).

90 Badve, S. et al. FOXA1 expression in breast cancer--correlation with luminal subtype A and survival. Clinical cancer research : an official journal of the American Association for Cancer Research 13, 4415-4421, (2007).

91 Mehta, R. J. et al. FOXA1 is an independent prognostic marker for ER-positive breast cancer. Breast Cancer Res Treat 131, 881-890, (2012).

92 Thorat, M. A. et al. Forkhead box A1 expression in breast cancer is associated with luminal subtype and good prognosis. Journal of clinical pathology 61, 327-332, (2008).

93 Albergaria, A. et al. Expression of FOXA1 and GATA-3 in breast cancer: the prognostic significance in hormone receptor-negative tumours. Breast cancer research : BCR 11, R40, (2009).

94 Bernardo, G. M. & Keri, R. A. FOXA1: a transcription factor with parallel functions in development and cancer. Biosci Rep 32, 113-130, (2012).

95 Gee, J. M. et al. Overexpression of TFAP2C in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival. J Pathol 217, 32-41, (2009).

96 Fang, S. H., Chen, Y. & Weigel, R. J. GATA-3 as a marker of hormone response in breast cancer. The Journal of surgical research 157, 290-295, (2009).

97 Parikh, P., Palazzo, J. P., Rose, L. J., Daskalakis, C. & Weigel, R. J. GATA-3 expression as a predictor of hormone response in breast cancer. Journal of the American College of Surgeons 200, 705-710, (2005).

98 Voduc, D., Cheang, M. & Nielsen, T. GATA-3 expression in breast cancer has a strong association with estrogen receptor but lacks independent prognostic value. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 17, 365-373, (2008).

99 Davies, M. P. et al. Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. International journal of cancer. Journal international du cancer 123, 85-88, (2008).

100 Roman, S. D. et al. Estradiol induction of retinoic acid receptors in human breast cancer cells. Cancer research 53, 5940-5945, (1993).

101 Hua, S., Kittler, R. & White, K. P. Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. Cell 137, 1259-1271, (2009).

102 Ross-Innes, C. S. et al. Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. Genes & development 24, 171-182, (2010).

103 Schlessinger, J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 110, 669-672, (2002).

104 Eswarakumar, V. P., Lax, I. & Schlessinger, J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 16, 139-149, (2005).

105 Schlessinger, J. Common and distinct elements in cellular signaling via EGF and FGF receptors. Science 306, 1506-1507, (2004).

106 Zhang, X., Gureasko, J., Shen, K., Cole, P. A. & Kuriyan, J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell 125, 1137-1149, (2006).

107 de Leeuw, R., Neefjes, J. & Michalides, R. A role for estrogen receptor phosphorylation in the resistance to tamoxifen. International journal of breast cancer 2011, 232435, (2011).

108 Murphy, L. C., Seekallu, S. V. & Watson, P. H. Clinical significance of estrogen receptor phosphorylation. Endocrine-related cancer 18, R1-14, (2011).

109 Zwart, W. et al. PKA-induced resistance to tamoxifen is associated with an altered orientation of ERalpha towards co-activator SRC-1. EMBO J 26, 3534-3544, (2007).

110 Rayala, S. K. et al. P21-activated kinase 1 regulation of estrogen receptor-alpha activation involves serine 305 activation linked with serine 118 phosphorylation. Cancer research 66, 1694-1701, (2006).

111 Chen, D. et al. Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. Mol Cell 6, 127-137, (2000).

112 McClelland, R. A. et al. Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). Endocrinology 142, 2776-2788, (2001).

113 Arpino, G. et al. Treatment of human epidermal growth factor receptor 2-overexpressing breast cancer xenografts with multiagent HER-targeted therapy. J Natl Cancer Inst 99, 694-705, (2007).

114 Bartlett, J. B., CL.; van de Velde, CJH.; Stocken, D.; Campbell, FM.; Hasenburg, A.; Kay, C.; Kiebeck, D.; Markopoulos, C.; Meershoek-Klein Kranenbarg, E.; Mallon, EA.; Dirix, L.; Robson, T.; Seynaeve, C.; Rea,DW. Abstract S2-4: Final Results of a Prospectively Planned Biomarker Analysis: HER1-3 as Predictive Markers of Benefit from Early Treatment with Aromatase Inhibitors Versus Tamoxifen in the TEAM Pathology Sub-Study. Cancer research 70, (2010).

115 Parisot, J. P., Hu, X. F., DeLuise, M. & Zalcberg, J. R. Altered expression of the IGF-1 receptor in a tamoxifen-resistant human breast cancer cell line. Br J Cancer 79, 693-700, (1999).

116 Wiseman, L. R. et al. Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer 29A, 2256-2264, (1993).

117 Zhang, Y. et al. Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3K/Akt signaling routes. Breast cancer research : BCR 13, R52, (2011).

118 Knowlden, J. M., Hutcheson, I. R., Barrow, D., Gee, J. M. & Nicholson, R. I. Insulin-like growth factor-I receptor signaling in tamoxifen-resistant breast cancer: a supporting role to the epidermal growth factor receptor. Endocrinology 146, 4609-4618, (2005).

119 Massarweh, S. et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. Cancer research 68, 826-833, (2008).

120 Turner, N. et al. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. Cancer research 70, 2085-2094, (2010).

121 Penault-Llorca, F. et al. Expression of FGF and FGF receptor genes in human breast cancer. International journal of cancer. Journal international du cancer 61, 170-176, (1995).

122 Meijer, D. et al. Fibroblast growth factor receptor 4 predicts failure on tamoxifen therapy in patients with recurrent breast cancer. Endocrine-related cancer 15, 101-111, (2008).

123 Miller, T. W. et al. Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. The Journal of clinical investigation 120, 2406-2413, (2010).

124 Cavazzoni, A. et al. Overcoming acquired resistance to letrozole by targeting the PI3K/AKT/mTOR pathway in breast cancer cell clones. Cancer letters, (2012).

125 Boulay, A. et al. Dual inhibition of mTOR and estrogen receptor signaling in vitro induces cell death in models of breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 11, 5319-5328, (2005).

126 Baselga, J. et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 366, 520-529, (2012).

127 Clark, A. S., West, K., Streicher, S. & Dennis, P. A. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Molecular cancer therapeutics 1, 707-717, (2002).

128 deGraffenried, L. A. et al. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. Clinical cancer research : an official journal of the American Association for Cancer Research 10, 8059-8067, (2004).

129 Bachelot, T. et al. Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 30, 2718-2724, (2012).

130 Kok, M. et al. Estrogen receptor-alpha phosphorylation at serine-118 and tamoxifen response in breast cancer. J Natl Cancer Inst 101, 1725-1729, (2009).

131 Murphy, L. et al. Phospho-serine-118 estrogen receptor-alpha detection in human breast tumors in vivo. Clinical cancer research : an official journal of the American Association for Cancer Research 10, 1354-1359, (2004).

132 Sarwar, N. et al. Phosphorylation of ERalpha at serine 118 in primary breast cancer and in tamoxifen-resistant tumours is indicative of a complex role for ERalpha phosphorylation in breast cancer progression. Endocrine-related cancer 13, 851-861, (2006).

133 Yamashita, H. et al. Low phosphorylation of estrogen receptor alpha (ERalpha) serine 118 and high phosphorylation of ERalpha serine 167 improve survival in ER-positive breast cancer. Endocrine-related cancer 15, 755-763, (2008).

134 Wang, R. A., Mazumdar, A., Vadlamudi, R. K. & Kumar, R. P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. EMBO J 21, 5437-5447, (2002).

135 Bostner, J., Skoog, L., Fornander, T., Nordenskjold, B. & Stal, O. Estrogen receptor-alpha phosphorylation at serine 305, nuclear p21-activated kinase 1 expression, and response to tamoxifen in postmenopausal breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 16, 1624-1633, (2010).

136 Holm, C. et al. Phosphorylation of the oestrogen receptor alpha at serine 305 and prediction of tamoxifen resistance in breast cancer. J Pathol 217, 372-379, (2009).

137 Kok, M. et al. PKA-induced phosphorylation of ERalpha at serine 305 and high PAK1 levels is associated with sensitivity to tamoxifen in ER-positive breast cancer. Breast Cancer Res Treat 125, 1-12, (2011).

138 Buyse, M., Sargent, D. J., Grothey, A., Matheson, A. & de Gramont, A. Biomarkers and surrogate end points--the challenge of statistical validation. Nat Rev Clin Oncol 7, 309-317, (2010).

139 McDonnell, D. P. & Wardell, S. E. The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. Current opinion in pharmacology 10, 620-628, (2010).

140 Mohseni, M. & Park, B. H. PIK3CA and KRAS mutations predict for response to everolimus therapy: now that's RAD001. The Journal of clinical investigation 120, 2655-2658, (2010).

141 Morrow, P. K. et al. Phase I/II study of trastuzumab in combination with everolimus (RAD001) in patients with HER2-overexpressing metastatic breast cancer who progressed on trastuzumab-based therapy. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 29, 3126-3132, (2011).

142 Chen, D. et al. Phosphorylation of human estrogen receptor alpha at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. Oncogene 21, 4921-4931, (2002).