The kinome associated with estrogen receptor-positive status in human breast cancer

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Abstract
Estrogen receptor alpha (ERα) regulates and is regulated by kinases involved in several functions associated with the hallmarks of cancer. The following literature review strongly suggests that distinct kinomes exist for ERα-positive and -negative human breast cancers. Importantly, consistent with the known heterogeneity of ERα-positive cancers, different subgroups exist, which can be defined by different kinome signatures, which in turn are correlated with clinical outcome. Strong evidence supports the interplay of kinase networks, suggesting that targeting a single node may not be sufficient to inhibit the network. Therefore, identifying the important hubs/nodes associated with each clinically relevant kinome in ER+ tumors could offer the ability to implement the best therapy options at diagnosis, either endocrine therapy alone or together with other targeted therapies, for improved overall outcome.

Introduction
The idea of personalized approaches to therapy in breast cancer based on the molecular nature of the tumor can be traced back to the late 1960s and early 1970s, when it was discovered that some but not all human breast tumors express estrogen receptors (ERs; Jensen et al. 1971). This heralded the beginning of an era of targeted therapies for breast cancer, identified the first biomarker used clinically to predict the biological behavior of breast cancer, and established the beginnings of understanding molecular mechanisms by which the ovarian hormone, estrogen, drives the growth and survival of the majority of human breast cancers (Jensen & Jordan 2003), at least initially. Inhibiting the activity of ERs with the antiestrogen tamoxifen was the first targeted therapy in breast cancer.

Although the knowledge that female hormones were involved in breast cancer and the hormonal/endocrine therapy was used for breast cancer at least in the form of ablation surgery such as ovariectomy dates back well over a century, the identification of ERs in breast cancers provided a molecular mechanism and rationale for the use of hormonal therapies (Jensen & Jordan 2003). This led directly to the development of the successful modern endocrine therapies such as tamoxifen and other selective estrogen receptor modulators (SERMs), which bind to the ERs and induce conformational changes that modify and in some cases inactivate the ERs (Jensen & Jordan 2003). The newer endocrine therapies, the aromatase inhibitors (AIs), which inhibit the aromatase enzyme, eliminate the production of estrogen and therefore inhibit estrogen’s proliferative action (Goss et al. 2011).

It was evident from the start that not all ER+ breast tumors were created equal. Although a little more than
70% of all breast tumors express ER, only about half of patients with ER+ tumors respond to tamoxifen. Therefore, ER+ breast cancers exhibit heterogeneity associated with prognosis and treatment outcomes. The first step to resolve this heterogeneity came from the idea that the measurement of a downstream target of estrogen-dependent ER signaling such as the progesterone receptor (PR) would increase confidence that the pathway was intact (Horwitz et al. 1975). This increased the accuracy of treatment prediction, but was obviously still imprecise as some 20–30% of ER+/PR+ tumors are de novo resistant to the endocrine therapies. Furthermore, initial response to endocrine therapies is often followed by acquired resistance despite the continued expression of ER (Encarnacion et al. 1993, Bachleitner-Hofmann et al. 2002).

The next significant insight into the heterogeneity of ER+ breast cancer came with the identification of HER2 (ERBB2) amplification. Approximately 20% of all breast cancers have amplified, overexpressed HER2, and 40–50% of these will also be ER+. Interestingly, ER+/HER2+ tumors are more likely to be resistant to endocrine therapy, in particular tamoxifen, thus providing important insight into the relevance of crosstalk of signaling pathways initiated at the level of the plasma membrane with many aspects of the ER signaling pathway. This may cause ligand-independent activation of ER signaling and hormone therapy resistance.

The molecular detailing that has become possible through the Human Genome Project and new high-throughput/high-content technologies in the last decade initially established five intrinsic molecular subgroups (Sorlie et al. 2003) of which three were significantly populated with ER+ tumors. Most recently, at least ten molecular subgroups of human breast cancer have been described (Curtis et al. 2012) and eight of these appeared to be significantly populated with ER+ tumors (Curtis et al. 2012). Associated with these studies has been the frequent identification of kinases, either mutated and/or structurally altered in large cohorts of breast tumors (Banerji et al. 2012, Curtis et al. 2012, Shah et al. 2012, Stephens et al. 2012). Some of these have also been found frequently altered and associated with AI sensitivity (Ellis et al. 2012).

ER and its many coactivators are regulated by phosphorylation as well as other post-translational modifications (PTMs; Rowan et al. 2000, York et al. 2010, Le Romancer et al. 2011, Zhang et al. 2013). The discovery that the ER and its coactivators are substrates of several kinases (enzymes causing phosphorylation of specific substrates), which are regulated by signaling pathways frequently mutated or structurally altered in breast cancer (Yamnik et al. 2009, Yamnik & Holz 2010, Murphy et al. 2011), as well as the demonstration that a clinically relevant phosphorylation profile of ERs can be identified in human breast cancer (Skliris et al. 2010a), suggests that kinases and/or phosphatases associated with ER+ breast cancer could provide a wealth of potential drug targets to complement existing endocrine therapies or generate new endocrine therapies. The following is a review of kinases that have been identified as associated with ER status in breast tumors or those that have been implicated in the regulation of estrogen signaling and/or modifying sensitivity to estrogen and its antagonists in breast cancer.

**Kinases identified as mutated or structurally altered in breast cancer in large breast cancer cohorts**

Genome-wide analyses of large cohorts of breast cancer cases are providing detailed, comprehensive analyses of genomic aberrations in breast cancer at a population level. Some studies have also provided data concerning their impact on clinical characteristics. These studies have shown that many of the frequently altered (amplified, fused, deleted, or mutated) genes encode kinases. Some of these frequently altered genes, such as HER2, were previously known but others, such as MAP3K1 and its substrate MAP2K4, have not previously been identified to have functional roles in breast cancer. However, given that enzymes, kinases in particular, have proven to be clinically efficacious therapeutic targets, a wealth of data has now been generated not only to understand the complex biology of the disease but also to identify new treatments for specific cohorts.

The recently published METABRIC cohort of breast cancers, in which 2000 individual breast cancers were interrogated, identified ten integrative clusters, each with distinct molecular characteristics associated with clinical outcome (Curtis et al. 2012). Often, altered genes encoding kinases and phosphatases dominate individual clusters (Table 1). Furthermore, the original intrinsic subtypes, in particular the ER+ luminal A and luminal B (Perou et al. 2000, Ignatiadis & Sotiriou 2013) subtypes, have been further subdivided due to the METABRIC study (Curtis et al. 2012, Dawson et al. 2013). Therefore, a brief description of this follows as it relates to clusters that have significant ER+ components.

**METABRIC integrative cluster 1 (IntClust1), representing 7% of breast cancer (Curtis et al. 2012, Dawson et al. 2013), contains predominantly ER+ tumors with luminal B features and is characterized by amplification of the**
phosphorylation of coactivators such as SRC1 and/or another kinase, PAK1, known to affect ER

11q13/14 characterizes this cluster and high expression of (Dawson characteristics of both luminal A and luminal B subtypes receptors (Proia 2003, Holm 2006), possibly by modulating

Integrative cluster 2 (IntClust2), representing 4% of breast cancers, is predominantly ER+ group with both luminal A and luminal B characteristics (Dawson et al. 2013). Tumors of this group show amplification of the chromosome 8p12 locus and are genomically unstable with an intermediate prognosis. Less than 20% of tumors in this group have PIK3CA mutations, which is low compared to other ER+ groups. The gene for FGFR1 is located in the chromosome 8p12 region. FGFR1 amplification and overexpression have been directly linked to endocrine therapy resistance (Turner et al. 2010, Balko et al. 2012). FGFR1 also regulates several kinases such as PI3K and AKT, which are known to phosphorylate ERx and its coactivators to enhance the ligand-independent activity of ERx as well as to enhance the agonist activity of tamoxifen (Wu et al. 2005, Le Romancer et al. 2011).

IntClust6, representing 4% of breast tumors, is another predominantly ER+ group with both luminal A and luminal B characteristics (Dawson et al. 2013). Tumors of this group show amplification of the chromosome 8p12 locus and are genomically unstable with an intermediate prognosis. Less than 20% of tumors in this group have PIK3CA mutations, which is low compared to other ER+ groups. The gene for FGFR1 is located in the chromosome 8p12 region. FGFR1 amplification and overexpression have been directly linked to endocrine therapy resistance (Turner et al. 2010, Balko et al. 2012). FGFR1 also regulates several kinases such as PI3K and AKT, which are known to phosphorylate ERx and its coactivators to enhance the ligand-independent activity of ERx as well as to enhance the agonist activity of tamoxifen (Wu et al. 2005, Le Romancer et al. 2011).

IntClust7 (10% of breast tumors) and IntClust8 (15% of breast tumors) are mainly ER+ groups and while similar in many ways tumors in IntClust8 display higher genomic instability, with an associated inferior prognosis than tumors in IntClust7 (Dawson et al. 2013). Interestingly, both again have a high frequency of PI3K (PIK3CA) mutations. IntClust7 has a high frequency of MAP3K1 mutations and IntClust8 has the highest frequency of MAP2K4 mutations, a downstream target of MAP3K1.

The structural alterations usually found in MAP3K1 and MAP2K4 are deletions and mutations associated with loss of function (Teng et al. 1997, Pham et al. 2013), suggesting that the pathways they regulate have

Table 1 Kinases and phosphatases altered in ER+-dominated breast cancer clusters from METABRIC (Curtis et al. 2012, Dawson et al. 2013)

<table>
<thead>
<tr>
<th>METABRIC integrative clusters</th>
<th>Kinases or phosphatases identified</th>
<th>Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IntClust1</td>
<td>Ribosomal protein S6 kinase 1 (RPS6KB1) and protein phosphatase 1D (PPM1D)</td>
<td>Amplification/overexpression</td>
</tr>
<tr>
<td>IntClust2</td>
<td>PAK1</td>
<td>Mutation</td>
</tr>
<tr>
<td>IntClust3</td>
<td>PIK3CA, MAP3K1</td>
<td>Mutation</td>
</tr>
<tr>
<td>IntClust5</td>
<td>PIK3CA, MAP3K1, HER2, PI3K, MAP3K1</td>
<td>Mutation</td>
</tr>
<tr>
<td>IntClust6</td>
<td>PIK3CA, FGFR1, PI3K, MAP3K1</td>
<td>Mutation</td>
</tr>
<tr>
<td>IntClust7</td>
<td>PI3K, MAP2K4</td>
<td>Mutation</td>
</tr>
<tr>
<td>IntClust9</td>
<td>Regulatory subunit B of protein phosphatase 2 alpha (PPP2R2A)</td>
<td>Deletion</td>
</tr>
</tbody>
</table>

17q23 region. Two genes in this amplicon associated with increased expression are ribosomal protein S6 kinase beta 1 (RPS6KB1 and p70S6K1) and protein phosphatase 1D (PPM1D). Interestingly, expression of both of these genes has been shown to be regulated by estrogen in ER+ breast cancer cells at the transcriptional level (Han et al. 2009, Yammnik et al. 2009, Yammik & Holz 2010). In addition, the protein product of RPS6KB1 (p70S6K1) has been shown to phosphorylate ERx (Yammik & Holz 2010) and under IGF1 stimulation p70S6K1 can be co-immunoprecipitated with ERx in ER+ breast cancer cells (Becker et al. 2011). PPM1D/Wip1 is known for its p53 inhibitory effects and, in ER+ MCF7 cells, has been shown to be regulated by estrogen and to increase the transcriptional activity of ERx (ESR1) as well as other steroid hormone receptors (Proia et al. 2005), possibly by modulating phosphorylation of coactivators such as SRC1 and/or p300/CPB (Proia et al. 2006).

Integrative cluster 2 (IntClust2), representing 4% of breast tumors, is also dominated by ER+ tumors with characteristics of both luminal A and luminal B subtypes (Dawson et al. 2013). Amplification of chromosome region 11q13/14 characterizes this cluster and high expression of another kinase, PAK1, known to affect ERx phosphorylation and activity (Wang et al. 2002, Mazumdar & Kumar 2003, Holm et al. 2009, Kok et al. 2010) is associated with its amplification in this region. A high frequency (~50%) of PI3K catalytic subunit p110α (PIK3CA) mutations is also observed in this group.

ER+ tumors with luminal A characteristics predominate integrative cluster 3 (IntClust3), which represents 15% of all breast tumors (Dawson et al. 2013). Tumors in this cluster tend to have few structural alterations, low genomic instability, and usually an excellent prognosis. Interestingly, they also have the highest frequency of PIK3CA mutations (~58%). As well, similar to other clusters dominated by ER+ tumors, a high level of MAP3K1 mutations (~15%) is observed in IntClust3.

IntClust5 represents 10% of all breast tumors and is characterized by HER2 amplification with ~42% of them also expressing ERx (Dawson et al. 2013). Approximately 30% of tumors in this cohort also have PIK3CA mutations. ER+ tumors with overexpressed HER2 are more likely to be resistant to endocrine therapies. One reason for this is thought to be the constitutive activation of several kinases downstream of HER2, such as ERK1/2, PI3K, and AKT, all of which can phosphorylate ERx and several ER coactivators to enhance the ligand-independent activity of ERx as well as to enhance the agonist activity of tamoxifen (Wu et al. 2005, Le Romancer et al. 2011).
tumor-suppressor function. Steroid hormone receptor signaling, including estrogen and androgen receptors, shows crosstalk with MAP3K1 in some systems, and it is the ER+ tumors, in particular luminal A type, that have the highest level of mutation and/or deletion of the MAP3K1/MAP2K4 pathway (Pham et al. 2013). This pathway has been implicated in cell death pathways, which under normal conditions is important for mammary gland involution, hence its loss of function in many ER+ breast cancers may in part be responsible for the dissociation of estrogen-induced proliferative and survival pathways from growth-inhibiting differentiation pathways (Pham et al. 2013). This pathway has been described as having a molecular switch type function between survival and cell death, possibly dependent on cell-type background, subcellular localization, and caspase 3 activity (Pham et al. 2013). Increased Erz expression combined with a decreased activity of the MAP3K1/MAP2K4 pathway may, in part, underlie the change in the balance of estrogen signaling regulation of survival/proliferation vs cell cycle inhibition that is often associated with differentiation.

IntClust9 (7% of breast tumors) is characterized by a high frequency of PPP2R2A deletions (Dawson et al. 2013). PPP2R2A is the regulatory subunit B of protein phosphatase 2 alpha (PP2A). While IntClust9 contains a mixture of intrinsic subtypes, its ER+ members are mainly of the luminal B type, and loss of PPP2R2A expression is associated with a high mitotic index. Interestingly, PP2A also has been implicated in the regulation of Erz phosphorylation and activity (Lu et al. 2003a).

The above data underscore that distinctly altered kinase and phosphatase mutation patterns occur frequently in ER+ breast cancer subgroups with altered clinical outcome. This would support the idea that combining endocrine therapies with other targeted therapies informed by each subgroup’s distinct kinase pattern could provide better clinical outcome for breast cancer patients.

**Kinome associated with PI3K/AKT/mTOR and ER-positive breast cancer**

METABRIC and similar studies (Curtis et al. 2012, Ellis et al. 2012, Stephens et al. 2012) as well as previous smaller scale studies (Miller et al. 2010, 2011a,b) have identified the frequent structural and mutational alterations in the PI3K/AKT/mTOR pathway that occur in ER+ breast cancer. IntClust1, IntClust2, IntClust3, and IntClust5 (Dawson et al. 2013) have ~25, 50, 58, and 30% frequencies of PIK3CA mutations respectively. IntClust7 and IntClust8 also have a high frequency of PIK3CA mutations while by contrast IntClust6 has less than a 20% frequency of PI3K pathway mutations (Dawson et al. 2013).

Experimental and clinical data suggest that hyper-activation of the PI3K/AKT/mTOR pathway is associated with endocrine resistance. However, gain-of-function PIK3CA mutations in ER+ tumors are often associated with good prognosis (Miller et al. 2011a). Such apparently contradictory results may be due to complexities associated with feedforward and feedback mechanisms involved in the PI3K/AKT/mTOR pathway and other alterations co-occurring in regulators of the pathway. This latter idea is supported by the finding that most often the associations of PIK3CA mutations with good outcome in ER+ tumors are restricted to those tumors that are also HER2 negative (Fu et al. 2013).

As discussed above, the PIK3CA gene, encoding the PI3K catalytic subunit p110a, is one of the most frequently mutated genes in ER+ breast cancers (Dawson et al. 2013). Many of the mutations occur in ‘hot spots’ with the most frequent mutations being E542K, E545K (both in the helical domain), and H1047R (in the kinase domain) (Barbareschi et al. 2007). Generally, the mutations result in constitutive activation of the kinase (Di Cosimo & Baselga 2009). As mentioned above, the published data largely suggest that mutations in PIK3CA occur more frequently in ER+ breast cancer with good prognosis and may also be associated with better clinical outcome in patients treated with endocrine therapy (Di Cosimo & Baselga 2009, Miller et al. 2011a). However, this is not a universal finding (Cuorvo et al. 2014). Recent reviews have discussed this aspect in detail (Fu et al. 2013).

**AKT1** mutations, often associated with PI3K-independent constitutive activity, occur in ~4% of breast cancers and are often associated with ER+ tumors. However, the relationship of AKT expression and/or activation to outcome in breast cancer is inconsistent (Badve et al. 2010, Aleskandarany et al. 2011). Considering that the different AKT isoforms have distinct functions (Dillon & Muller 2010) and that a recent study using proximity ligation assay (PLA) to distinguish between pAKT1 and pAKT2 (Spears et al. 2012) determined the former to be associated with poor prognosis and the latter with better prognosis, it seems possible that the relative levels of each isoform may determine the final read out. Furthermore, the inability to distinguish the different pAKT isoforms may, at least in part, underlie the previous lack of consistency (Badve et al. 2010, Aleskandarany et al. 2011) around their association with breast cancer outcome.
Owing to the frequency with which components of the PI3K/AKT/mTOR pathway are altered in ER+ tumors, and the fact that the pathway is a central hub receiving growth and survival signals from many factors, the detailed molecular nature of the pathway in different ER+ tumors may be important to guide appropriate therapy options.

Kinases associated with ER status by expression analyses

Several studies have now been published in which different kinase expression patterns were found in ER+ vs other clinical subtypes of breast cancer. Using publically available RNA expression databases, Bianchini et al. (2010) identified 16 kinases that were overexpressed in ER+/HER2− breast cancer biopsy samples. These were IGF1R, STK32B, ERBB4, FGFR3, BMPR1B, MAST4, HSPP8, IKKBK, DCLK1, ERBB3, STK39, MAP3K1, PTK6, PLK2, TEX14, and MST1. Kinases uniquely overexpressed in ER− and HER2+ breast cancer subtypes were also identified. Two robust kinase clusters were recognized: a mitosis metagene cluster (12 distinct kinases) and an immune kinase cluster (15 distinct kinases), which were present in all clinical subgroups. Overexpression of kinases composing the mitosis metagene cluster was mostly found in ER− tumors and was not prognostic in this subgroup. However, ER+/HER2− tumors that presented a high mitosis kinase score were associated with a worse prognosis but showed a higher frequency of pathological complete response (pCR) to neoadjuvant chemotherapy. On the other hand, overexpression of kinases from the immune kinase cluster was associated with better survival in ER+/HER2− and HER2+ subgroups. Interestingly, the authors also observed that in ER+ tumors, many of the overexpressed kinases were transmembrane growth factor receptors, while ER− tumors mostly overexpressed intracellular kinases associated with cell proliferation (Bianchini et al. 2010).

Finetti et al. (2008) undertook a genome-wide expression analysis of a cohort of primary human breast tumors focusing mainly on the basal and luminal A intrinsic breast cancer subtypes. From this analysis, they extracted kinase genes whose differential expression was associated with a clinical outcome. Not unexpectedly, there were substantial differences in the pattern of kinase gene expression between the basal and the luminal A intrinsic subtypes. Of more interest, however, was the discovery that 16 kinases were overexpressed in most of the basal group and in a few luminal A tumors. These kinases were AURKA, AURKB, BUB1, BUB1B, CDC2 (CDK1), CDC7, CHEK1, MASTL, MELK, NEK2, Pbk, PLK1, PLK4, SRPK1, TTK, and VRK1. Many of these kinases have been previously reported to be involved in the G2 and M phases of the cell cycle (Malumbres & Barbacid 2009, Knight et al. 2010). Notably, these kinases include the 12 kinases making up the mitosis metagene cluster identified by Bianchini et al. (2010). Similarly, Finetti et al. (2008) found that overexpression of kinases from this 16-kinase signature in luminal A (ER+) tumors was associated with a poor clinical outcome. The association of mitosis and proliferation signatures with poorer prognosis in the luminal breast cancer subgroups is a consistent theme (Ribelles et al. 2013).

Unique kinases associated with ER status in breast cancer cell lines have also been reported (Michalides et al. 2002, Finetti et al. 2008, Midland et al. 2012), some of which show overlap with studies in tumors (Finetti et al. 2008, Midland et al. 2012), therefore potentially providing models of ER+ breast cancer kinomes in vitro. These data support the existence of distinct kinomes, not only associated with ER status per se, but also further define heterogeneity within ER+ breast tumors.

Kinase overexpression associated with sensitivity to endocrine therapies

There are many different kinases that when either overexpressed or underexpressed in ER+ breast cancer cells lead to altered cell growth and survival responses to both SERMs such as estrogen and tamoxifen, and Als. Many of these are listed in Table 2.

Such data underscore the importance of kinase networks, as many of the kinases listed in Table 2 converge on common hubs associated with cell growth, survival and cell death. It is not surprising that the interaction of multiple kinases with ER signaling is observed in breast cancer, as many of these kinases are key components of proliferation, survival and cell death pathways and would be expected to be regulated by estrogen, through the ER, which is the primary mitogenic/survival signal in the majority of breast cancers, at least initially (Musgrove & Sutherland 2009, Osborne & Schiff 2011).

In ER+ breast cancer, gene signatures that are based on proliferation often strongly correlate with Ki67 expression (Gao et al. 2014), a well-known marker of proliferation. Furthermore, in ER+ breast tumors in particular, it is Ki67 and not pCR that is the early correlative endpoint for predicting efficacy of both hormonal therapy or chemotherapy in ER+ breast cancer (Yerushalmi et al. 2010, Dowsett et al. 2011, Ellis et al. 2011, von Minckwitz et al. 2012, Gao et al. 2014). Recently, using
<table>
<thead>
<tr>
<th>Kinases</th>
<th>Gain of function</th>
<th>Loss of function</th>
<th>References</th>
<th>Effects</th>
<th>Clinical correlation</th>
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<td>EGFR</td>
<td>Ectopic overexpression</td>
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<td>Agthoven et al. (1992)</td>
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<td>HER2</td>
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<td>AKT</td>
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<td>Campbell et al. (2001), de Graffenried et al. (2004) and Silva et al. (2007)</td>
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<td>Yes</td>
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<td>Akt3</td>
<td>Ectopic overexpression</td>
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<td>Faridi et al. (2003)</td>
<td>E₂-independent, Tam-stimulated growth as xenograft</td>
<td>Yes</td>
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<td>Chisamore et al. (2001) and Frankel et al. (2007)</td>
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<td>Tam-resistant growth inhibition</td>
<td>Yes</td>
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<td>CRK7</td>
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<td>Tam-resistant growth inhibition</td>
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<td>Guo et al. (2010)</td>
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<td>Sukocheva et al. (2009)</td>
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<td>Yes</td>
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<td>c-ABL</td>
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<td></td>
<td>Zhao et al. (2010)</td>
<td>Sensitizes cells to Tam inhibition</td>
<td>Yes</td>
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<td>Ron receptor tyrosine kinase (MST1R)</td>
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<td></td>
<td>McClaine et al. (2010)</td>
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<td>EphA2 receptor tyrosine kinase (EPHA2)</td>
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<td>Plaza-Menacho et al. (2010)</td>
<td>Increased sensitivity to Tam resistance</td>
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<td>LMTK3</td>
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<td></td>
<td>Giamas et al. (2011)</td>
<td>Increased sensitivity to Tam resistance</td>
<td>Yes (Giamas et al. 2011)</td>
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neoadjuvant antiestrogen therapy (particularly AIs), it was observed that decreased detection of survival pathway activation markers (e.g. phosphorylated AKT and phosphorylated mTOR) in tumors after treatment, compared with pretreatment levels, was predictive of a better clinical outcome (Generali et al. 2008, 2011), suggesting that estrogen via the ER was regulating these pathways, directly or indirectly in vivo. Many preclinical models have shown that estrogen, via ER signaling, at least in part, regulates the activities of PI3K/AKT/mTOR, p38MAPK, and MAPK/ERK1/2 (Zhang et al. 2002, Lee et al. 2005, Yu & Henske 2006, Kazi et al. 2009, Maruani et al. 2012). This suggests that the use of hormonal therapies in combination with the most appropriately targeted kinase inhibitors may be more beneficial at the onset of therapy than application in a sequential fashion. Indeed, there are ongoing clinical trials evaluating the use of combination therapies with endocrine therapies as first-line approaches (Ciruelos 2014).

A major challenge of using kinase inhibitors is that kinase networks especially those associated with growth and survival are often interconnected and inhibition of one may have effects beyond the immediate targets. Adaptive reprogramming of cancer kinomes due to single kinase inhibitors has been demonstrated (Duncan et al. 2012) and an argument for targeted combination therapy approaches initially has been put forward (Stuhlmiller et al. 2014). The development of resistance to hormonal therapies most often occurs despite the continued expression of ERα (Encarnacion et al. 1993, Bachelet-Hofmann et al. 2002). In addition, many laboratory studies suggest that adaptive responses of breast cancer cells, rather than selection of existing ER-negative cells in the original heterogeneous population, frequently occur (Santen et al. 2003, Browne et al. 2013). In such cases, increased expression/activity of kinases also frequently occurs (Coutts & Murphy 1998, Santen et al. 2003, 2004), supportive of the concept that adaptive reprogramming of the breast cancer kinase, in part, underlies some mechanisms of estrogen independence and anti-estrogen resistance (Stuhlmiller et al. 2014). The challenge will be to understand this and its potential plasticity within the underlying heterogeneity of ER+ breast cancers and at the individual patient level.

**Kinases known to phosphorylate ERα**

In addition to ER signaling regulating kinase pathway expression and/or activity, ER activity can also be regulated by kinases. ERα can be phosphorylated on many residues (Fig. 1) and ERα has been identified as a substrate for several kinases with known functions in breast cancer development and progression. Dysregulation including mutation and structural alterations of kinases themselves or the signaling pathways in which they function is thought, in part, to contribute to the dysregulation of ER signaling that is associated with the development of breast cancer as well as its progression to endocrine resistance in vivo. Multiple aspects of ER activity can be affected by phosphorylation; however, the focus of the majority of studies addressing this issue has been on the transcriptional activity (Le Romancer et al. 2011) and, not surprisingly, it has been observed that differential phosphorylation of ERα (e.g. PKA activation) can alter the chromatin-binding pattern (cistrome) of the receptor, in turn altering the ERα transcriptome (de Leeuw et al. 2013). The function of phosphorylation on ER activity and that of other steroid hormone receptors have been recently extensively reviewed (Le Romancer et al. 2011, Trevino & Weigel 2013) and will not be reviewed herein.

Multiple different kinases can phosphorylate the same ERα residue (e.g. serine amino acid residue 167 (S167) can be phosphorylated by AKT, p70S6K, Aurora A, and p90RSK) (Le Romancer et al. 2011). Similarly, one kinase can phosphorylate several different residues on ERα (e.g. MAPK can phosphorylate S104/106, S118, and S167) (Le Romancer et al. 2011). Many kinases known to phosphorylate ERα can also phosphorylate various

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**Figure 1**

Known phosphorylation sites on estrogen receptor α. Sites identified by stars are those that have been determined in ERα+ breast cancer cases in the Manitoba Breast Tumor Bank (MBTB) and are the basis of the P7-ERα score. The black stars are those residues which when phosphorylated are associated with a good clinical outcome in patients treated with tamoxifen. The open/white stars are those residues which when phosphorylated are associated with a poor clinical outcome in tamoxifen-treated patients (Skliris et al. 2010a). It should be noted that others have determined that S305 when phosphorylated is associated with a poor clinical outcome in tamoxifen-treated patients (Le Romancer et al. 2011) and we have found that tyrosine (Y) S537 when phosphorylated is also associated with a poor clinical outcome in tamoxifen-treated patients (Skliris et al. 2010b). These latter two results (textured stars) are consistent with our observation that phosphorylation in N-terminal residues is associated with a good clinical outcome, but phosphorylation in C-terminal residues is associated with a poor clinical outcome.
coactivators and coregulators (Wu et al. 2005), impacting ER (ESR1) transcriptional activity. This provides a potential mechanism allowing the integration of multiple pathways to regulate the biology and/or pathobiology of ERs in the mammary gland. Support for this idea, at least in breast cancer, is provided by the existence of a phosphorylation code for ERs (Skliris et al. 2010a) and at least one of its coactivators, SRC3/AIB1 (Wu et al. 2004, York et al. 2010). As ERs can undergo several other types of PTMs, e.g. acetylation, methylation, ubiquitylation, etc. (Le Romancer et al. 2011), one can envisage a broader ERs PTM or epigenetic-like code.

The identification of kinases phosphorylating ERs has been achieved using multiple methods, alone or in combination: in vitro studies using recombinant proteins, mass spectroscopy, the use of selective small-molecular-weight kinase activators or inhibitors, and overexpression or knockdown of expression using cells in culture or as xenografts. Furthermore, it is reasonable to assume, while not proving, that if ER can be directly phosphorylated by a kinase, the two proteins would directly bind under appropriate circumstances. Table 3 lists a number of kinases that have been found to undergo protein:protein interactions with ERs using different methodologies, e.g. co-immunoprecipitation (Wierer et al. 2013) and other pull-down-type assays (Kanaujiya et al. 2013), PLA (Poulard et al. 2012), yeast-2-hybrid assay (Paul et al. 2014), and fluorescence resonance energy transfer (FRET) (Zwart et al. 2007a,b). Direct interaction of ERs with kinases can occur in the cytoplasm (Poulard et al. 2012), at the plasma membrane (Poulard et al. 2012), as well as in the nucleus on chromatin (Madak-Erdogan et al. 2011, 2014, Wierer et al. 2013), with some methodologies, such as PLA, allowing the determination of the subcellular localization of any interactions that occur (Poulard et al. 2012).

There is no doubt that phosphorylation and other PTMs (Wu et al. 2007, Le Romancer et al. 2011) are important for the regulation of ER highlighted by the existence of a phosphorylation code for ERs in human breast tumors (Skliris et al. 2010a). However, it is still unclear as to which kinases and phosphatases are involved in regulating ERs in vivo. Correlations of expression and/or activity of some kinases with ER or its phosphorylated forms in human breast tumors have been reported, supporting a possible role in vivo (Murphy et al. 2004, Gee et al. 2005, Jiang et al. 2007, Shrivastav et al. 2014).

Many of the kinases that can directly phosphorylate ERs and its coregulators can also be regulated by estrogen signaling under some circumstances. Many such kinases are components of key signaling pathways involved in development/differentiation, proliferation, cell cycle, motility/invasion, and cell death. Whether or not they are regulated by estrogen or in turn regulate ERs may depend on levels of expression of both substrate and kinase, and on

Table 3 Kinases shown to interact directly with estrogen receptor α.

<table>
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<tr>
<td>ERK1/2</td>
<td>CoIP; ChIP/reChIP</td>
<td>Madak-Erdogan et al. (2011)</td>
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<td>p70S6K1</td>
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<td>AKT</td>
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<td>PLK1</td>
<td>Co-IP; ChIP/reChIP</td>
<td>Lee &amp; Bai (2002)</td>
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Wierer et al. (2013)
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that cannot be acetylated. This in turn alters regulation of acetylated (another type of PTM) to an arginine residue. K303R changes the amino acid lysine that can be introduced new regulatory factors. The ability of the mutations or structural alterations that may eliminate or introduce new regulatory factors. The ability of the estrogen/ERα complex to activate kinases that in turn can phosphorylate ER and modify function provides a powerful feed-forward amplification system that may drive many human breast cancers. Understanding how this occurs and how it can be perturbed to drive progression to therapy resistance would provide fundamental information to inform early combination approaches, for preventing and/or treating resistant tumors.

Interestingly, although in primary breast tumors, few ERα mutations have been found, one known mutation K303R changes the amino acid lysine that can be acetylated (another type of PTM) to an arginine residue that cannot be acetylated. This in turn alters regulation of S305 phosphorylation on ERα, a site of ERα phosphorylation with established relevance in vivo and in AI resistance (Herynk & Fuqua 2004, Barone et al. 2010). There are also other examples of the co-regulation of phosphorylation with other PTMs to regulate ERα expression and/or activity. For example, c-Src can phosphorylate ERα at Y537 that in turn facilitates its interaction with E3 ubiquitin ligases such as E6AP, resulting in ubiquitin-dependent degradation of ERα (Zhou & Slingerland 2014). Most recently, however, frequent somatic mutations in ERα have been identified in metastases from ER+ breast cancer. This is more frequently associated with metastases that occur during or following endocrine therapy (Zhang et al. 1997, Robinson et al. 2013, Jeselsohn et al. 2014, Segal & Dowsett 2014). The most frequent mutation identified was at Y537 or the residue beside it, D538; therefore, affecting directly or indirectly a well-known phosphorylation site on ERα (Arnold et al. 1995a, Nettles et al. 2008, Skliris et al. 2010b) with known clinical relevance (Skliris et al. 2010b). Such data underscore the importance of ERα phosphorylation and other PTMs to breast cancer progression.

Our understanding of estrogen-regulated ERα signaling in human breast cancer cells comes from model systems that are all cancer cells. Moreover, all ER+ and ER− cell lines were originally obtained from breast cancer metastases usually pleural and ascitic effusions (Soule et al. 1973, Lippman et al. 1976). We have little if any understanding of ERα signaling in normal human breast epithelial cells, mainly due to the lack of appropriate ER+ models. This remains a large gap in our understanding, potentially limiting the development of more efficacious prevention strategies. This is especially important as increased ERα expression in normal human breast is associated with an increased cancer risk (Khan et al. 2005) and elevated ERα expression is one of the earliest alterations occurring in ‘precursors’ of breast cancer (Shoker et al. 1999, Lee et al. 2006). ERα is highly expressed in most atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive breast cancers (Shoker et al. 1999, Allred et al. 2001). However, the mechanisms leading to increased ERα expression during tumorigenesis are poorly understood. Interestingly, beside alterations in ER gene transcription (Muscat et al. 2013), altered kinase expression affecting ERα protein stability has been suggested (Henrich et al. 2003, Antoon et al. 2013) and, therefore, highlights the potential link between phosphorylation and estrogen signaling.

The challenge now is to identify those kinases and their associated networks that phosphorylate and regulate ERα function in ER+ tumors and potentially normal breast in vivo, as such information will identify the most efficacious targeted treatment approaches upfront and may inform better prevention approaches.

Conclusions

ERα regulates and is regulated by kinases involved in several functions associated with the hallmarks of cancer (Hanahan & Weinberg 2011). The presence of a phosphorylation code for ERα in breast cancers associated with clinical outcome (Skliris et al. 2010a) and the knowledge that there are also other PTMs associated with ERα (Le Romancer et al. 2011) in vivo suggest that there is an epigenetic-like code for ERα, which can regulate and is in part regulated by distinct kinomes. The literature reviewed above strongly suggests that distinct kinomes exist for ER-positive and -negative breast cancers. Even within ER+ cancers, different subgroups exist, defined by different kinome signatures, which can be correlated with clinical outcome. Strong evidence supports the interplay of kinase networks, suggesting that targeting a single node may not be sufficient to inhibit the network. Therefore, identifying the important hubs/nodes associated with each clinically relevant kinome in ER+ tumors could offer the ability to implement the best therapy options at diagnosis, either endocrine therapy alone or together with other targeted therapies for an improved overall outcome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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**Kinome associated with ER-positive status**

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