

Crosstalk between Estrogen Receptor and Growth Factor Receptor Pathways as a Cause for Endocrine Therapy Resistance in Breast Cancer

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ABSTRACT

Data suggest that breast cancer growth is regulated by coordinated actions of the estrogen receptor (ER) and various growth factor receptor signaling pathways. In tumors with active growth factor receptor signaling (e.g., HER2 amplification), tamoxifen may lose its estrogen antagonist activity and may acquire more agonist-like activity, resulting in tumor growth stimulation. Because treatments designed to deprive the ER of its ligand estrogen will reduce signaling from both nuclear and membrane ER, aromatase inhibitors might be expected to be superior to tamoxifen in tumors with high growth factor receptor content, such as those overexpressing HER2. Recent clinical studies suggest that this is the case in humans, as trials of aromatase inhibitors show superior results compared with tamoxifen, especially in tumors overexpressing HER2. Although estrogen deprivation therapy is often effective in ER-positive breast cancer, *de novo* and acquired resistance are still problematic. Experimental models suggest that in one form of resistance to estrogen deprivation therapy, the tumor becomes supersensitive to low residual estrogen concentrations perhaps because of activation of mitogen-activated protein kinase. Such tumors respond to additional treatment with fulvestrant or even tamoxifen. On the other hand, in tumors overexpressing HER2, acquired resistance to estrogen deprivation therapy involves the loss of ER and ER-regulated genes and further up-regulation of growth factor signaling rendering the tumor hormonal therapy resistant. This process can be delayed or reversed by simultaneous treatment with growth factor pathway inhibitors. This strategy is now being tested in clinical trials.

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EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY IN BREAST CANCER

There is growing evidence that crosstalk between estrogen receptor (ER) and growth factor receptor signaling pathways, especially the epidermal growth factor receptor (EGFR) family, is one of the mechanisms for resistance to endocrine therapy in breast cancer (1–3). cErbB2 (HER2) is a member of this EGFR family of transmembrane tyrosine kinases. The family also includes HER3 and HER4 (ref. 4; Fig. 1). The role of HER4 is poorly understood. HER3 lacks a tyrosine kinase domain, and HER2 does not have a ligand to bind and activate it. These two proteins, therefore, mostly heterodimerize with another member of the family to generate the kinase cascade and downstream signals. This explains why tumors developing in transgenic mice engineered to overexpress HER2 in the ductal epithelium always overexpress HER3 as well (5). Growth factors such as epidermal growth factor (EGF), transforming growth factor α , and amphiregulin bind to the external domain of EGFR, which then induces either homo- or heterodimerization with another receptor in the family to activate the tyrosine kinase of the receptor (4). Heregulin and other ligands, on the other hand, bind to the external domain of HER3. This also initiates heterodimerization and then activation of Akt, Erk1/2 mitogen-activated protein kinase (MAPK) or other intermediates. Because HER2 does not have a ligand, it may be relatively inactive unless the cell also expresses EGFR or HER3, which can be activated by their respective ligands. HER2 is the preferred dimer partner for EGFR and HER3 because of its open conformation (6).

Activation of the EGFR/HER2 signaling pathway initiates a kinase signaling cascade that has a variety of effects on the tumor cells, including inhibition of apoptosis, stimulation of cell proliferation, enhanced invasion and cell motility, and induction of angiogenesis stimuli (Fig. 1). Cell survival and cell proliferation are mediated predominantly through the phosphatidylinositol 3'-kinase (PI3K)/Akt and the Erk1/2 MAPK pathways. These kinases are also important for ER activity in some tumors because they phosphorylate and thereby activate either ER itself or ER coregulators such as AIB1 and nuclear receptor corepressor (NCoR; refs. 2, 7–9). This phosphorylation augments the transcriptional activation potential of ER and enhances its effects on cell proliferation and survival. Working together in tumors expressing both ER and abundant HER2, these two pathways provide a strong stimulus for tumor growth and may contribute to hormonal therapy resistance.

ER IN BREAST CANCER

ER functions in the nucleus as a transcriptional regulator of specific genes (Fig. 2). The protein has a ligand-binding domain, several transcription activation domains, and a DNA-binding domain that interacts with specific regions in the promoter of

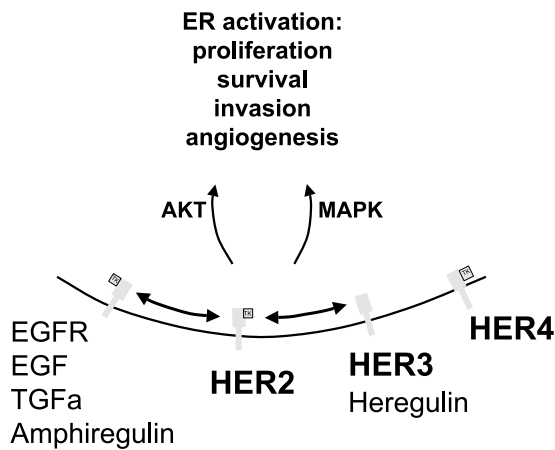


Fig. 1 EGFR family of tyrosine kinase receptors, examples of their ligands, and their tumor effects in breast cancer. Arrows, heterodimerization induced by ligand binding. HER3 lacks a TK domain. HER2 has no ligand. TK, tyrosine kinase domain.

target genes, including sites known as estrogen-responsive elements (refs. 10, 11; Fig. 2A). The binding of estrogen to ER induces phosphorylation of the receptor, alters its conformation, triggers receptor dimerization, and facilitates binding of the receptor complex to promoter regions of target genes. The recruitment of coactivators such as AIB1 (SRC3) and other proteins with acetyltransferase activity helps to unwind the chromatin allowing transcription to occur (12). By contrast, the ER conformation induced by the binding of selective estrogen receptor modulators like tamoxifen favors the recruitment of corepressors and deacetylases that inhibit transcriptional activity (2, 13). Tamoxifen displays partial agonist-antagonist activities in different tissues and cells, and these differences may be related to the milieu of the ER coactivators and corepressors in these tissues. The estrogen agonist properties of tamoxifen are enhanced in cells with increased levels of coactivators such as AIB1 or SRC1 (13).

The ER protein in the nucleus can also modify transcription of genes in other ways (Fig. 2B). Through protein-protein interactions ER can function much like a coactivator protein itself by binding to other transcription factors and recruiting acetyltransferases to complexes bound to activator protein or SP-1 sites on DNA (14). In this way, estrogen helps to regulate genes encoding proteins such as cyclin D1, insulin-like growth factor I receptor (IGF-IR), and collagenase. In total, estrogen regulates the expression of many genes important for normal cell physiology and growth of some breast tumors. Progesterone receptor (PR), PS2, the heat shock proteins, TGF- α , IGF-II, IRS1, and vascular endothelial growth factor, in addition to IGF-IR and cyclin D1, represent just a few of the many genes regulated by estrogen in target cells. Interestingly, ER can also decrease expression of many genes as well (15). This transcriptional activity of ER has been called its classical or genomic activity. From a functional perspective, a more appropriate term is nuclear-initiated steroid signaling, to differentiate these nuclear effects from recently identified ER functions that can occur very rapidly in the cell before new gene transcription takes

place and that may occur outside the nucleus or even in the cell membrane (the so-called membrane-initiated steroid signaling; ref. 16).

Studies in endothelial cells and in breast cancer cells suggest that a small pool of ER is located outside the nucleus in the cytoplasm or bound to the plasma membrane (Fig. 3; refs. 2, 17, 18). This membrane-bound ER may explain the short-term effects of estrogen (occurring within minutes) identified in cultured cells more than two decades ago (19, 20). The membrane ER can directly interact with and/or activate IGF-IR, the p85 subunit of PI3K, Src, EGFR, and HER2 (2, 21–26). Working as a G-protein-coupled receptor, estrogen-bound membrane ER has been reported to activate Src, which in turn activates matrix metalloproteinase 2 to cleave heparin-binding EGF from the cell surface (26). This transmembrane form of EGF can then interact by autocrine or paracrine mechanisms with adjacent EGF receptors to initiate growth factor signaling. As shown below, selective estrogen receptor modulators like tamoxifen behave as estrogen agonists on the rapid ER effects in the membrane (2). However, in breast cancer cells with low levels of EGFR or HER2, these membrane functions of ER may be modest (2). In tumor cells with abundant EGFR, HER2, or the cytoplasmic proteins MTA1-S or MNAR (PELP-1), which can sequester ER outside the nucleus, this membrane-initiated steroid signaling may contribute more substantially to tumor growth and to resistance to endocrine therapies, particularly selective

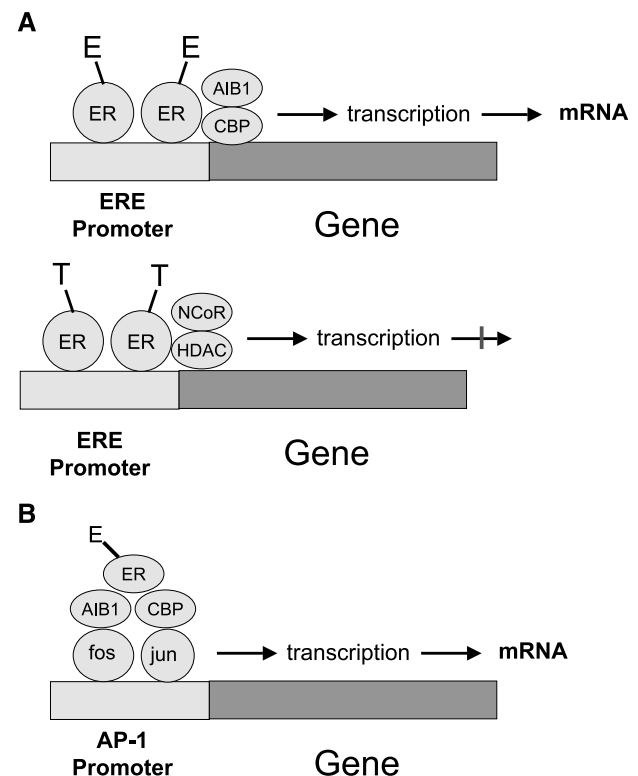


Fig. 2 Nuclear effects of estrogen (E) or tamoxifen (T) bound ER on estrogen-responsive element (ERE, A) or other transcription factor-dependent promoter sites (B). AIB1 and CBP are coactivators, whereas NCoR is a corepressor. Fos and jun are transcription factors in the AP1 family. HDAC, histone deacetylase.

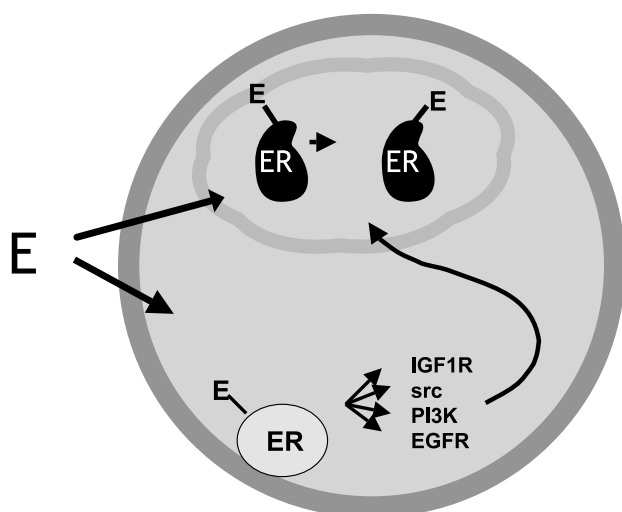


Fig. 3 Nuclear and membrane activities of ER. Membrane ER is activated by estrogen and tamoxifen, and can in turn activate several growth factor pathways. These pathways can then activate and augment nuclear ER functions.

estrogen receptor modulators (2, 27–32). In such tumors bidirectional crosstalk between ER and growth factor pathways results in a positive feedback cycle of cell survival and cell proliferative stimuli. Clinically, it may be crucial to block this crosstalk by inhibiting both signaling networks to achieve optimal therapeutic activity. Finally, the role of an ER splice variant (46 kDa) missing exon 1, which may mediate rapid estrogenic effects in some tissues (33, 34), needs to be more thoroughly investigated in breast cancer. Importantly, this short form of ER has been documented in breast cancer cell lines (35).

ER/HER2 CROSSTALK IN BREAST CANCER CELLS

To study this crosstalk between ER and growth factor receptor pathways and its clinical implications in more detail, we have used two cell lines, the ER-positive MCF-7 cell line, which expresses very low levels of EGFR and HER2, and a derivative cell line, MCF-7/HER2-18, which has been engineered to overexpress the HER2 oncogene at levels similar to those in patients' tumors amplified for the gene. These cell lines express similar levels of ER and also are naturally amplified for the ER coactivator AIB1, which may be a crucial downstream target for the EGFR/HER2 kinase signaling cascade (3, 7). We previously reported that growth of the MCF-7 xenografts in athymic mice is stimulated by estrogen and inhibited by estrogen deprivation either alone or in the presence of tamoxifen (36). The antitumor effects of these endocrine therapies in this model are temporary because acquired resistance develops to both therapies after several months of treatment. This acquired resistance to tamoxifen is due to a change in tumor biology such that tamoxifen stimulates rather than inhibits growth.

We have recently reported that growth of the MCF-7/HER2-18 tumors is stimulated from the outset by both estrogen and tamoxifen as a mechanism of *de novo* resistance

when these cells are grown as xenografts in athymic mice (2). Both MCF-7 and MCF-7/HER2-18 tumors are, however, strikingly inhibited by estrogen deprivation alone. Therefore, in tumors with abundant ER, AIB1, and HER2, tamoxifen behaves as an estrogen agonist and stimulates tumor growth, whereas estrogen deprivation therapy remains effective and inhibits growth. These results suggest the possibility that aromatase inhibitors (estrogen deprivation) might be more effective than tamoxifen in patients with ER-positive tumors that overexpress HER2, an idea that is supported by results from recent clinical studies (37, 38).

Thus, ER-positive/HER2-positive tumors are not necessarily estrogen independent, but, on the contrary, can be strikingly sensitive initially to therapies designed to lower the levels of estrogen, which based on laboratory studies inhibits both the nuclear and membrane activities of ER. In fact, phosphorylated Erk1/2 MAPK, a signaling molecule in the EGFR/HER2 pathway, was significantly reduced by estrogen deprivation but was increased by both estrogen and tamoxifen in the MCF7/HER2-18 xenograft tumors, confirming the crosstalk between ER and the growth factor signaling pathway (2).

This crosstalk was studied in more detail using these two cell lines growing in tissue culture (2). In MCF-7 cells, short-term treatment with either estrogen or tamoxifen induced phosphorylation of ER on Ser¹¹⁸. ER phosphorylation at this residue, therefore, does not cause tamoxifen resistance because the drug is a potent antagonist in these cells. Neither estrogen nor tamoxifen induced detectable phosphorylation of EGFR, HER2, Erk1/2 MAPK, or Akt, although, as expected, heregulin and EGF did activate the growth factor signaling pathway. Thus, there was little receptor crosstalk in these cells via the membrane ER activity. However, in the MCF-7/HER2-18 cells and in the BT474 cells (which also express ER, high levels of AIB1, and are amplified for HER2), both estrogen and tamoxifen, like EGF and heregulin, caused rapid phosphorylation of all of these growth factor signaling intermediates within minutes of adding them to the culture media (2). These effects were all inhibited by the EGF receptor tyrosine kinase inhibitor gefitinib, suggesting that this receptor mediates, at least in part, the rapid effects of estrogen and tamoxifen in these tumors.

It has been previously shown that the MAPK pathway can phosphorylate the ER coactivator AIB1, and we reasoned that increased tamoxifen estrogen agonist activity and tamoxifen-stimulated growth in the presence of high HER2 might be a consequence of the functional activation of AIB1 via EGFR/HER2 signaling (7). Heregulin treatment phosphorylated AIB1 in MCF-7 cells, but estrogen and tamoxifen did not (2). In contrast, in MCF-7/HER2-18 cells, AIB1 phosphorylation was observed not only in cells treated with heregulin but also in cells treated with estrogen and tamoxifen. This phosphorylation was blocked by pretreatment with gefitinib, again suggesting that many of the effects of estrogen and tamoxifen in the MCF-7/HER2-18 cells are due to ER-mediated activation of EGFR and/or HER2.

We also examined the effects of tamoxifen on a panel of estrogen-regulated genes, hypothesizing that it might act similarly to estrogen in stimulating gene expression in the MCF-7/HER2-18 cells (2). Indeed, we found that whereas tamoxifen, as expected, had no agonist activity on gene expression in the MCF-7 cells, it increased the expression of

several estrogen-regulated genes in the MCF-7/HER2-18 cells nearly as well as estrogen itself. Furthermore, these effects were totally abolished by gefitinib. Thus, in these cultured cells, tamoxifen not only stimulates cell proliferation but also behaves as an estrogen agonist on the nuclear-initiated steroid signaling activity of ER to regulate estrogen target genes. Several of these genes, including those encoding IRS1 and cyclin D1, are potentially important for tumor growth.

To examine the mechanism by which tamoxifen exerts estrogenic activity on gene expression in the MCF-7/HER2-18 cells, we examined the ER transcription complex components binding to the promoter region of the well-known estrogen target gene, PS2 (2). In MCF-7 cells estrogen treatment induced occupancy of the promoter by ER and by coactivator complexes including AIB1, P300, and CBP, leading to acetylated histones. Tamoxifen, in contrast, induced occupancy by ER complexed with the corepressor NCoR and histone deacetylase-3. However, in the MCF-7/HER2-18 cells, both estrogen and tamoxifen induced the formation of coactivator complexes, thereby explaining agonist effects of tamoxifen on endogenous estrogen-regulated gene expression. Interestingly, the ability of tamoxifen-bound ER to recruit coactivator complexes to the PS2 promoter was also completely reversed by the addition of the EGFR tyrosine kinase inhibitor gefitinib. Suppression of the growth factor receptor pathway led to the replacement of coactivator complexes with corepressor complexes in the presence of tamoxifen-bound ER, indicating that the crosstalk between EGFR/HER2 and ER signaling was totally responsible for the agonist activity of tamoxifen.

Because gefitinib blocked EGFR and HER2 crosstalk, dissociated coactivator complexes from tamoxifen-bound ER, and restored antagonist effects of tamoxifen on gene expression, we also examined its effects on tamoxifen-stimulated tumor cell proliferation using a variety of different *in vitro* and *in vivo* measures (2). Simultaneous treatment of MCF-7/HER2-18 cells with tamoxifen and gefitinib reduced growth factor signaling, inhibited AIB1 phosphorylation, and most important, blocked growth stimulation by tamoxifen by reestablishing its potent antagonist qualities (Fig. 4; ref. 2). Of note, gefitinib treatment only modestly inhibited estrogen-induced tumor growth. These data suggest that the effects of estrogen on tumor growth are only partially dependent on EGFR/HER2 activation, whereas tumor growth induced by tamoxifen is totally dependent on this pathway.

CLINICAL IMPLICATIONS

These laboratory data provide a possible mechanism by which some HER2-overexpressing ER-positive tumors become resistant to selective estrogen receptor modulators like tamoxifen. We recently reported in a study of patients treated with tamoxifen adjuvant therapy that tumors expressing high levels of both HER2 and AIB1 are relatively resistant to tamoxifen therapy (3), an observation that is similar to the results using our experimental models. The clinical trial also showed that if either AIB1 or HER2 is expressed at low levels, then the tamoxifen-resistant phenotype does not occur. It is interesting to note, then, that in the wild-type MCF-7 cells, which overexpress AIB1 but not HER2, tamoxifen behaves as an estrogen antagonist, whereas

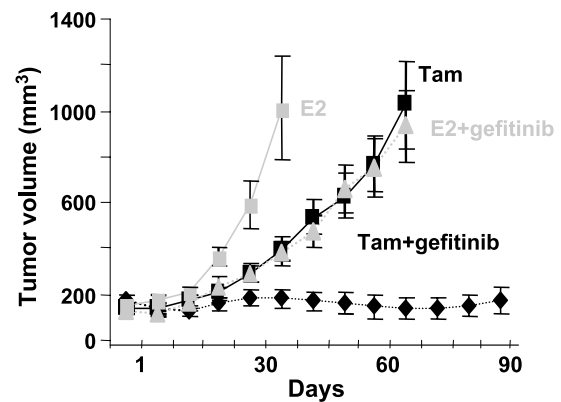


Fig. 4 Athymic nude mice bearing MCF-7/HER2-18 tumors were treated with estrogen (E2) or tamoxifen (Tam), either alone or in combination with the tyrosine kinase inhibitor gefitinib, and tumor growth was followed. Mean tumor volume in each group ($n = 8$); bars, 95% confidence intervals. Reprinted with permission from ref. 2.

in the MCF-7/HER2-18 cells it has lost its antagonist activity, resulting in *de novo* resistance to the drug.

Our data also provide a possible explanation for the relative superiority of estrogen deprivation therapy with aromatase inhibitors compared with tamoxifen in HER2-overexpressing breast cancers observed in two clinical trials (37, 38). Although these neoadjuvant studies were small, patients were treatment naive, allowing accurate correlations between biological markers and tumor response. Both studies suggested that aromatase inhibitors were more effective than tamoxifen in tumors that are amplified for HER2. We are currently measuring AIB1 in these tumors. Aromatase inhibitors lower the amount of estrogen available to bind the ER and so would be expected to shut off both the nuclear-initiated and membrane-initiated steroid signaling activities of the receptor in HER2-positive tumors. Tamoxifen, on the other hand, although it might still antagonize ER nuclear-initiated steroid signaling activity, at least on some ER-dependent genes, behaves as an estrogen agonist on its membrane-initiated steroid signaling activity, which could lead to loss of its antagonist profile and less benefit to the patient. Although these studies need confirmation, it is interesting that they support the biological principles arising from the laboratory models.

Aromatase inhibitors, at least in one large recently reported adjuvant trial, were also impressively more effective than tamoxifen in tumors that are ER positive/PR negative (38). Reduced benefit with tamoxifen adjuvant therapy in ER-positive/PR-negative tumors was also recently reported in a very large data set in which the receptors were measured in a central reference laboratory (39). These observations are difficult to explain if one imagines that PR loss in a tumor simply reflects a nonfunctional ER pathway (40). In that situation, neither tamoxifen nor an aromatase inhibitor would be beneficial because the tumor would be ER independent. Recent laboratory and clinical studies shed light on this observation. It has recently been reported that growth factor signaling through IGF-1R or EGFR/HER2 results in down-regulation of transcription of the PR gene (41). This may be due to ER complexed with the transcription factors fos and jun at an activator protein recognition site in the promoter of the PR gene (42). Although PR loss has several possible explanations, in some

tumors it may reflect active growth factor signaling. In fact, it has been previously reported that ER-positive/PR-negative tumors more frequently express higher levels of HER2 than ER-positive/PR-positive tumors (43). Thus, estrogen deprivation therapy might be more beneficial than tamoxifen in ER-positive/PR-negative tumors for reasons similar to those in HER2-positive tumors, in which the growth factor signaling cascade reduces the antagonist qualities of tamoxifen (42).

The cumulative preclinical and clinical data suggest that the optimal initial treatment for ER-positive/HER2-positive or ER-positive/PR-negative breast cancer might be an aromatase inhibitor rather than a selective estrogen receptor modulator, a hypothesis that should be investigated in future studies. Alternatively, an ER down-regulator like fulvestrant might also be effective in such tumors by inducing ER degradation, and, thereby, blocking both the nuclear-initiated and membrane-initiated steroid signaling ER activities similar to aromatase inhibition. Finally, such tumors might also be treated effectively by combining tamoxifen with a growth factor inhibitor such as gefitinib, trastuzumab, or other drugs that inhibit these kinases or downstream intermediates in the PI3K/Akt or Erk1/2 MAPK pathways. It could be argued that this biology is no longer relevant and that aromatase inhibitors should now be used as adjuvant therapy in all postmenopausal patients with ER-positive tumors. But if tamoxifen could be made more effective without compromising its bone-sparing effect, it would remain an attractive alternative. Furthermore, it is possible that in some patients, such as those with ER-positive and PR-positive tumors, the sequence of initial tamoxifen for 3 to 5 years followed by an aromatase inhibitor might be superior to initial therapy with an aromatase inhibitor, an idea that could be tested in ongoing trials. Recently reported large clinical trials suggest that this is a very effective strategy (44, 45). Although monotherapy with a growth factor inhibitor will likely be suboptimal in ER-positive tumors, double blockade using both ER-targeted therapies and therapies targeting the growth factor receptor cascade is an attractive strategy now being tested in clinical trials.

OPEN DISCUSSION

Dr. Steven Come: Are the coactivators equally important to the ER cell membrane functions as they are to genomic functions, or are these only active in the nucleus?

Dr. C. Kent Osborne: We don't know. There is a kinase-binding domain on AIB1, but when you stain for it, it is nuclear in every experiment we've done. Bert O'Malley has some data that some of the AIB1 is cytoplasmic or outside the nucleus, but we don't see it, perhaps because of less sophisticated microscopy [Wu et al. *Mol Cell Biol.* 2002;10:3549–61]. It is a very small pool of estrogen receptors, so it still could be there, but you wouldn't see it. This is an important issue to sort out. We and others are knocking down AIB1 with siRNA, then looking to see what its effects are on the membrane activity as opposed to the nuclear activity.

Dr. Come: Wouldn't it be possible that the whole idea of genomic and nongenomic isn't right, and the resistance mechanism is simply growth factors working through coactivators on classical genomic ER function?

Dr. Osborne: I think the estrogen receptor is important, but the roles of AIB1 are many. It's a promiscuous coactivator. It is a coactivator for many other transcription factors in addition to the estrogen receptor. Also, AIB1 is phosphorylated on about eight or nine different sites, and many of those are phosphorylated by different kinases in the growth factor signaling pathway. I think that AIB1 might well be important for some other function in the cell that is contributing to resistance other than its classical effect on estrogen-responsive element-mediated transcription. Also, progesterone receptor is frequently lost when resistance develops, which you would not expect if genomic activity was high.

Dr. Richard Santen: Rakesh Kumar has shown that MNAR, also called PELP-1, is another coactivator [*J Biol Chem* 2001;276: 38272–79]. It binds and activates Src, and Src then is involved in the phosphorylations that are necessary for the nongenomic effects to take place. I think it is probably the first example of a protein which is both a coactivator on transcription and also is involved in the nongenomic pathways.

Dr. Osborne: Dr. Kumar also thinks that it sequesters ER out of the nucleus and into the cytoplasm and that it is responsible for augmenting the membrane pool when there is a lot of growth factor activity. Another example is MTA1 short form, a corepressor of ER that seems to sequester ER out of the nucleus as well.

Dr. Stephen Johnston: So you are saying that in the HER2-positive, estrogen-deprived resistant cells ER is not a big player because it is lost? That is very different from tamoxifen resistance, where you are saying that the agonist response to the nongenomic component is through ER. There may be growth factor signaling, but the ER in terms of its nongenomic involvement or indeed its genomic involvement is not an issue in resistance in estradiol-deprived cells.

Dr. Osborne: Exactly. Remember, normally, there is an interplay between HER2 activity and loss of ER transcription, so you would think that both tamoxifen as an antiestrogen and estrogen deprivation would up-regulate that in the same way and cause ER to be shut off, but it doesn't do so. In fact, ER remains quite high in the tamoxifen-resistant HER2-overexpressing tumors. So there is clearly a big difference between resistance to estrogen deprivation and resistance to tamoxifen.

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