

Cross-Talk between Estrogen Receptor and Growth Factor Pathways as a Molecular Target for Overcoming Endocrine Resistance

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Abstract

Introduced more than 100 years ago, endocrine therapy is still the most important systemic therapy for all stages of estrogen receptor (ER)-positive breast tumors. A major clinical problem limiting the usefulness of this therapy is tumor resistance, either *de novo* or acquired during the course of the treatment. Relatively new discoveries emphasize the complexity of ER signaling and its multiple regulatory interactions with growth factor and other kinase signaling pathways. Both genomic (nuclear) and nongenomic (membrane and cytoplasmic) ER activities contribute to this intimate cross-talk, which is probably a fundamental factor in endocrine resistance. New targeted therapies, especially against the epidermal growth factor receptor/HER-2 pathway, should be carefully evaluated in more (bio)logical strategies to enable them to be exploited appropriately. A strategy of combining endocrine therapy (particularly tamoxifen) with these inhibitors, to circumvent *de novo* and acquired resistance, will be discussed. We will also emphasize open questions and future challenges in the dynamic research field of molecular ER biology from the endocrine therapy perspective.

Introduction

Despite the significant achievements of endocrine therapy in the treatment of breast cancer, its application is greatly limited by both *de novo* and acquired resistance. Only 50% of all estrogen receptor (ER)-positive tumors are responsive at first presentation to antiestrogens such as tamoxifen; furthermore, at least in the metastatic setting, initially responsive tumors eventually become resistant to endocrine treatment, leading to tumor progression and death (1). Thus, it is imperative to better un-

derstand the mechanisms responsible for resistance and to explore new therapeutic strategies that will improve and prolong duration of response and circumvent endocrine-resistant tumor growth.

ER Activity and Cross-Talk with Other Growth Factor Signal Transduction Pathways. With new powerful molecular and imaging research tools, it is becoming clear that the mechanisms of action of ER are much more diverse and complicated than initially thought. Part of the pleiotropic effects of the ER pathway can now be explained by new discoveries regarding the intense cross-talk of ER with growth factor and other signaling pathways. This cross-talk, occurring at multiple levels, is bidirectional so that modulation of the ER pathway influences growth factor pathways and *vice versa* (2). Many recent studies emphasize the importance of this cross-talk in breast cancer etiology and progression (3). Furthermore, increasing evidence suggests that endocrine resistance is also intimately associated with certain growth factor and cellular kinase pathways (2, 4). These specific associations and cross-talk will be discussed here as potential therapeutic targets to circumvent endocrine resistance.

ER Genomic Activity and Cross-Talk with Growth Factor Signal Transduction. Estrogens and related ligands mediate most of their effects in breast cancer by binding to ER α , which originally was believed to exert most of its effects through direct activation of gene expression (genomic action). Many of these estrogen-responsive genes are key components of growth factor pathways, and their expression can be antagonized by antiestrogens like tamoxifen. These include genes coding for peptide growth factors, membrane tyrosine kinase receptors (TKRs), and a variety of principal cellular signaling molecules (2, 4 and references therein). These, in turn, not only play an important role in breast cancer proliferation and survival pathways, but can also directly reactivate the ER pathway. Importantly, however, an inhibitory relationship has also been documented. Depending on cell and ER subtype, the expression and/or activity of specific growth factor receptors is inversely related to ER expression and activity. Particular examples, probably highly significant to endocrine resistance, are the epidermal growth factor receptors (EGFRs) 1 and 2 (EGFR/HER-1 and HER-2/*neu*). Although the regulation of these genes by estrogen is still somewhat controversial, strong evidence suggests that the antiestrogens activate the transcription of these genes (5–7). Reciprocally, levels of EGFR, HER-2, and the third family member HER-3 are often inversely correlated with ER levels (8, 9).

Nuclear ERs exert their genomic action as ligand-dependent transcription factors via two major transcriptional activation functions, the ligand-independent AF-1 and ligand-dependent AF-2 (10). Ligand binding to the ER results in receptor phosphorylation, dimerization, and the recruitment of specific

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coregulator proteins to responsive sequences in promoters of target genes. Depending on the nature of the ligand, tissue, cell, and gene, these coregulators can either activate (coactivators) or repress (corepressors) ER transcriptional activity (11). The absolute and relative levels of these coregulatory proteins in a given tumor cell therefore comprise an important factor in adjusting the agonist *versus* antagonist activity of mixed antiestrogens (SERMs) like tamoxifen on the ER transcriptional activities (4).

ER genomic activity is also up-regulated by various growth factor signaling pathways, such as epidermal growth factor and insulin-like growth factor type I (2). Many studies have shown that ER is activated by phosphorylation by kinases of diverse growth factor and other signaling pathways (3). Phosphorylation on specific sites residing in the AF-1 potentiates AF-1 autonomous activity, even in the presence of antiestrogens like tamoxifen, and may, therefore, play an important role in endocrine resistance (3, 12, 13). In addition, phosphorylation of ER coregulatory proteins by many growth factor, stress-response, and cytokine-related pathway kinases (4) has also been recognized recently as a regulatory mechanism of ER signaling. Thus, ER coregulators should also be considered as key mediators of growth factor signals to the ER pathway (14).

ER Nongenomic Activities and Cross-Talk with Growth Factor Signal Transduction. Important studies from recent years have now confirmed early and somewhat undervalued observations showing that in response to their ligands, ERs, as well as other nuclear receptors, can rapidly and transiently initiate signaling cascades originating from the membrane or the cytoplasm via direct association with and activation of many signal transduction components (15, 16). Through these interactions, estrogens and many other steroids rapidly regulate various cellular responses and processes. This nongenomic action (also called rapid, non-nuclear, or nonclassical action, or membrane-initiated steroid signaling), in contrast with the genomic action of ER, occurs outside the nucleus, is independent of gene transcription (though eventually it may also influence gene transcription), and is manifested in seconds to a very few minutes. Nongenomic ER signaling has been documented in many tissues (17). In some of these tissues, which are predominantly nonreproductive in function, most, if not all, of the estrogen responses are mediated by this rapid nongenomic action of the ER (18), a finding with profound implications for the future of SERM development.

The identity of the steroid receptors that execute the nongenomic signaling, their cellular localization, and the means by which they operate still await clarification. However, new evidence suggests that in the case of estrogens, these receptors are a small subset of the classic ERs, or perhaps of a short-form splicing/translational variant of ER α (19, 20), which reside in both the cytoplasm and the plasma membrane, predominantly at the caveolar domains (21). Membrane receptors genetically unrelated to the classic ERs may also be involved (22). Interestingly, the short-form variant of ER α , which lacks most of the AF-1 domain, has been detected in various estrogen target cells, including breast cancer cells (23). Furthermore, the role of nongenomic ER activity in mediating estrogen-induced growth and survival effects in breast tumor cells has now been well documented, and the physical existence and function of both

membrane and cytoplasmic ERs has been established by biochemical, immunohistological, and genetic methods (24, 25).

Membrane ER α can directly or indirectly activate membrane TKRs in many cells including breast cancer cells. A direct physical association between ER α and insulin-like growth factor receptor after estrogen treatment leads to activation of insulin-like growth factor receptor and the downstream extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK). This interaction is completely blocked both by the potent antiestrogen fulvestrant and by inhibitors of MAPK kinase (26). ER α also interacts directly with HER2 (27), and it is this interaction that protects HER-2-overexpressing breast tumor cells from tamoxifen-induced apoptosis (27). Estrogen-activated membrane ER also phosphorylates and activates EGFR in a process that involves activation of G-proteins, c-Src, and matrix metalloproteinases (28). In addition, ER also directly associates with many other key signaling molecules such as c-Src (29, 30), Shc (31), and the p85 α regulatory subunit of phosphatidylinositol-3-OH kinase (29, 32). Many of the above interactions lead to activation of key secondary signaling messengers and downstream kinase pathways such as the p21Ras/ERK 1/2 MAPK and the PDK1/AKT. These kinase signals, in turn, activate the nuclear ER transcriptional activity as well as other components in the transcriptional machinery, thus promoting ER-dependent transcription (32–34). Therefore, the genomic and the nongenomic actions of ERs seem to be complementary and even synergistic via cross-regulatory interactions mediated through cross-talk with other growth factor and cellular signal transduction pathways.

ER nongenomic activity, like the genomic activity, is highly dependent on the ligands, coregulatory proteins, and the growth factor signaling milieu. In contrast to ER genomic activity, the nongenomic action, perhaps in part due to its transient nature, can in fact be stimulated by SERMs like tamoxifen while being mostly blocked by the potent antiestrogen fulvestrant (16, 34, 35). This ability of tamoxifen to activate at least some nongenomic ER action may be an important factor in its agonistic actions. Tamoxifen can also activate the expression of some membrane TKRs (*i.e.*, members of the HER family). Of note, activation or overexpression of growth factor signaling (*e.g.*, HER-2) alters ER subcellular distribution and augments nongenomic activities in breast cancer experimental systems in response to both estrogen and tamoxifen (27, 33, 34, 36). Together these observations may explain the poor outcomes seen when tamoxifen is combined with chemotherapy agents.

The contribution of nongenomic ER action in endocrine resistance needs to be investigated. How do coregulators of the genomic ER action, as well as specific coregulators of nongenomic ER action [*e.g.*, MTA1s (36) and MNAR/PELP1 (30)], modulate this ER nongenomic action in response to different endocrine therapies? How do other growth factor signaling pathways affect ER nongenomic activity in both sensitive and resistant tumors? These and many other related key questions are still open and certainly need much more basic and clinical research to be fully answered. With the current intense research interest in the importance of the nongenomic actions of ER in estrogen signaling in general, and especially in breast cancer, some of these answers may soon mature and may advance our

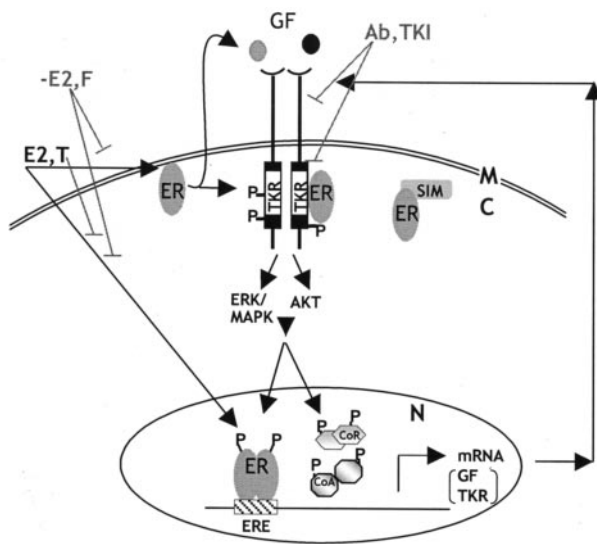


Fig. 1 Estrogen receptor (ER) and growth factor (GF) pathway cross-talk in endocrine resistance: a working model. The ligand estrogen (E) induces genomic-nuclear (N) ER activity, which results in increased gene transcription, including important genes in the growth factor pathways. Estrogen deprivation ($-E_2$), the antiestrogen tamoxifen (T), and the potent antiestrogen fulvestrant (F) antagonize this activity. However, both estrogen and tamoxifen can turn on nongenomic membrane (M) and/or cytoplasmic (C) ER, which, in turn, through multiple interactions with signaling intermediate molecules (SIM; see text), can activate tyrosine kinase receptors (TKR) and cellular kinase cascades. Subsequent phosphorylation (P) of the ER and its coactivators (CoA) and corepressors (CoR) by these kinases (e.g., ERK/MAPK and AKT) then potentiates the genomic-nuclear ER activity. Increased GF signaling (by overexpression of GFs or TKRs) augments both activities of the ER and can result in endocrine resistance to antiestrogens like tamoxifen. This cycle can possibly be broken either by inhibitors of the GF/TKR pathways [TKIs or antibody (Ab)-based], which restore sensitivity to tamoxifen, or by abolition of the ER pathway by ligand deprivation or by ER degradation using fulvestrant (ERE, promoters containing diverse ER-responsive elements.)

understanding of how to improve endocrine therapy and prevent resistance.

Targeting ER-EGFR/HER-2 Cross-Talk: Is a Combination of Endocrine Treatment and EGFR/HER-2 Targeted Therapy a More (Bio)logical Strategy? The extensive bidirectional cross-talk between the ER pathway and growth factor and cellular signaling pathways appears to be important to the operation of these pathways and to play a critical role in breast cancer pathology, especially in endocrine resistance. It has been suggested that an increase in growth factor and cellular kinase signaling in breast tumors potentiates the ER pathway, which in turn reactivates growth factor signaling via both genomic and nongenomic activities, resulting in a stimulatory cycle that intensifies activity in the ER and EGFR pathways (32–34). This increased cross-talk might make the pathways more dependent on each other, thereby rendering the tumors more sensitive to specific inhibitors of these pathways (Fig. 1). However, it may also neutralize the efficacy of SERMs like tamoxifen, because tumor resistance to these SERMs is associated with an altered balance in their agonist *versus* antagonist activities.

Substantial clinical and experimental evidence suggest that

in fact breast tumors with increased growth factor signaling, especially of EGFR/HER-2, are associated with reduced response to tamoxifen, which in experimental systems can actually stimulate their growth (37–39). Similarly, acquired resistance to estrogen deprivation is associated frequently with an estrogen-hypersensitivity phenotype and increased levels of ERK 1/2 MAPK activities (40). Results from our recent clinical trial, finding that high levels of HER-2 together with the ER coactivator AIB1 are associated with a poor disease-free survival in patients receiving tamoxifen adjuvant therapy (41), further emphasize the significance of this cross-talk and the role of coactivators in clinical tamoxifen resistance. These tumors, wherein both estrogen and tamoxifen can act as potent growth factors in turning on the EGFR/HER-2 pathway, may still be highly sensitive to a therapy that withdraws or lowers the estrogen levels (38). A recent neoadjuvant study in ER-positive primary breast cancers, demonstrating the superiority of aromatase inhibitor therapy in patients with EGFR/HER-2-positive tumors (42), provides additional strong clinical support to this scenario.

Potent, promising new targeted therapies, either antibody-based or small molecule kinase inhibitors, have been introduced recently to the clinic. Unfortunately, most of these drugs, especially against the EGFR/HER-2 pathway, have failed to elicit a significant response in many solid tumors including breast cancer (43). An exception is the HER-2-targeting monoclonal antibody trastuzumab (Herceptin), which shows a significant response in HER-2-overexpressing tumors, although even then the activity is transient and the rate of response is unsatisfying (44). Given the importance of cross-talk in modulating EGFR/HER-2 activity, a more biological approach in ER-positive breast tumors is to use these drugs in combination with antiestrogens such as tamoxifen rather than as single agents. Taking into account that acquired resistance to tamoxifen is associated with up-regulation of growth factor signaling, especially of the EGFR/HER-2 (45, 46), a combination strategy may have important therapeutic potential in many ER-positive tumors besides those that overexpress HER-2. Our studies and others indeed suggest that in experimental systems, EGFR/HER-2-targeted therapy, either with trastuzumab for HER-2-overexpressing tumors (47) or with specific tyrosine kinase inhibitors such as the EGFR-selective inhibitor gefitinib (Iressa; Refs. 45, 46) and the dual EGFR/HER-2 inhibitor GW572016 (48) for ER-positive tumors with normal/low levels of EGFR/HER-2, can improve tamoxifen performance and reverse or delay tamoxifen-resistant tumor growth. Of note, these drugs have only minimal or no growth inhibitory effects on estrogen-stimulated tumor growth when used as single agents. A preliminary report (49) suggests a considerably higher response rate to gefitinib in breast cancer patients with tamoxifen-resistant tumors, in contrast with other reports from trials with no criteria for patient selection (50, 51). These results additionally suggest the potential of this strategy in the clinic. Larger clinical trials are now starting to evaluate the promise of combining endocrine therapy (*i.e.*, tamoxifen) with an EGFR tyrosine kinase inhibitor (gefitinib) to overcome *de novo* resistance and delay acquired resistance. Parallel trials using trastuzumab are also currently ongoing.

Future Challenges. As the complexity of ER mechanisms of action is coming to light, so is the complexity of the mechanisms responsible for endocrine sensitivity and resistance in breast cancer. Both the genomic and the nongenomic activities of ER, as well as their complex interplay with many other growth factor and cellular signaling pathways, are now recognized as major factors influencing tumor sensitivity to various types of endocrine therapies. Therefore, we can no longer view ER for diagnostic and therapeutic purposes as a simple nuclear transcription factor. The standard for assessing tumor ER status is a simple immunohistochemistry assay detecting nuclear ER. This may need to be challenged. Other antibodies, assays, conditions, and perhaps even additional microscope modalities should be explored to allow the detection of membrane and cytoplasmic ER. Two related questions arise: (a) can we identify a set of markers that will distinguish tumors with increased membrane ER activity; and (b) is increased or dominant tumor membrane ER (nongenomic) activity related to the ER+/progesterone receptor negative phenotype of tumors, and is it responsible for the relatively poor response of these tumors to tamoxifen (52)? In addition, ER signaling should no longer be evaluated separately from the rest of the cellular and tumor signaling systems. Breast cancer is a heterogeneous malignancy, and signaling intermediates and pathways may vary between patients, thus leading to different patient responses. But what is the true predictive value of the various signaling components of the multiple pathways that modulate ER activity? The “predictive value” itself should now be considered in a broader sense, *i.e.*, the value related to different classes of endocrine therapies, other targeted therapies, and the appropriate combinations. With new diagnostic tools, some of these questions should be testable in recent or ongoing large clinical trials, such as the IMPACT and the ATAC trials, which already have the important “built-in” histopathology component. Of course, many more clinical trials would be needed to fully establish the predictive and therapeutic potentials of these signaling components and the novel targeted drugs before they will become an integral part of breast cancer management. Careful design of future trials, with more consideration for the biological interactions of the variety of targets and drugs, may accelerate, via smaller trials, the availability of these promising therapies to the welfare of breast cancer patients.

Open Discussion

Dr. Robert Nicholson: *In vitro* it is easy enough to get a range of growth factors into breast cancer cells and then look at the ability of gefitinib to inhibit those various growth factors. At the beginning of our studies we selected doses which we could demonstrate would block EGFR signaling but wouldn't necessarily block signals of IGF-1 receptor or other pathways. But how can you be confident with *in vivo* experiments that you are looking at something which is selective when, if you go up a dose level, these tyrosine kinase inhibitors will then start to affect many growth factor signal pathways?

Dr. Schiff: We know that what we are blocking in the beginning is the EGFR/HER-2 dimer. But when we select for even higher HER-2, the signaling becomes mainly HER-2/HER-2 and gefitinib doesn't work anymore, though trastuzumab

does, at least partially. We have also presented our data on the inhibition of the IGFR by gefitinib; what we are finding there is that a subset of EGF receptors in the membrane associate with IGFR once we induce cells with IGF. The resulting signaling from this interaction can be blocked by gefitinib, but not the rest of the IGF-specific signaling. So I think that we are working both *in vivo* and *in vitro* in the most specific dosages.

Dr. Matthew Ellis: In clinical samples there is a huge range of HER-2 and HER-1 expression, and certainly within the context of HER-2 there can be expression without gene amplification and then expression with gene amplification, which can lead to extreme levels of presumably HER-2-driven signaling. You focus on one clone to produce this model, which would probably reflect modest levels of HER-2 expression. Have you tried to generate an inducible clone where you can give different levels of an inducing factor to produce different levels of signaling, or have you perhaps looked at different clones?

Dr. Schiff: We have done up to now only these clones. I think that with less HER-2 there will be an even stronger interaction between the two pathways. To answer your question, we are going to do the inducible model.

Dr. Richard Santen: I would like you to comment on whether the HER-2-overexpressing MCF-7 cells are really hypersensitive to lower amounts of estrogen than would be necessary to stimulate the wild-type cells. With the very potent aromatase inhibitors and the ability to suppress estrogen quite effectively, I'd be interested to know if you have done formal dose response curves and looked at tumor growth in response to much lower concentrations of estrogens in your HER-2-expressing cells.

Dr. Schiff: No, we haven't done that. I showed you how sensitive these HER-2 tumors are to estrogen deprivation *in vivo*. *In vitro* when we try to take ligand away we can still see high basal activity, so I don't want to say that there is no ligand-independent activity. I'm not sure how much you can really deplete ligand *in vitro*.

Dr. Myles Brown: You gave your thoughts about why you need a nongenomic ER to be involved in the signaling. I saw that there was signaling, but what is the evidence that it is linked to the growth, that the ability of ER to alter MAP kinase signaling is linked to the ability of ER to stimulate growth of the MCF-7 or the MCF-7/HER-2?

Dr. Schiff: We don't have a direct answer for that right now. However, we are using the expression of some classical ER-dependent genes as readout for classical genomic ER activity. When tumors begin to advance on tamoxifen, we see a nice reduction of these classical genomic markers, and when we let them advance further, we completely lose this set of markers, and the nongenomic activity is now more important. We can now show more signaling in the membrane, which we can modulate by adding or removing estrogen or tamoxifen. Whether it's true membrane ER signaling or whether it's cytoplasmic ER signaling we don't know yet.

Dr. Ellis: Do you think that the concentration on the HER-1 and HER-2 pathways is really justified? If you look at ER-positive breast cancer in reasonable subsets, perhaps only 10 to 15% of those cells are ER positive, HER-1 or -2 positive, at baseline; the limited data available would suggest that there isn't huge upregulation of HER-2 during the acquisition of tamoxifen

resistance. I know that Mitch Dowsett presented some nice data at ASCO, but he was only talking about 1 or 2 cases. It was rather uncommon that during acquired tamoxifen resistance in the clinical situation you went from being HER-2 negative to HER-2 positive. So, clearly to me there are other potentially more dominant pathways to resistance than this. Have you any thoughts about what those pathways might be?

Dr. Schiff: I don't think EGFR/HER-2 will be the only answer. We think that a lot of the stress-related pathways, specifically the AP-1/JNK and the p38 MAP kinase pathways, may also be involved. Right now we have mainly indirect evidence for these, because we see that high levels are associated with resistance. Furthermore, using a dominant negative for API, we find that tamoxifen-stimulated tumors are more sensitive than estrogen-stimulated tumors, and this is with low levels of EGFR/HER-2, so it's not only the EGFR/HER-2.

Dr. Nicholson: We do have a model of fulvestrant resistance where we take endocrine-responsive MCF-7 cells, treat them with fulvestrant for long periods of time, and eventually the tumor cells will regrow. In that particular model there's certainly a downregulation of estrogen receptors within the cancer cells, and there's a normal increase in EGFR/HER-2 that you see within that particular model, but if you take fulvestrant out of the system, they will regenerate estrogen receptor signaling. So maybe there are going to be pathways whereby resistance can be acquired, and maybe that is taking place through adaptive changes in the cancer cells, some of which may be reversible.

Dr. Schiff: The key word is adaptation. I think that ER may be lost at the level of mRNA, but not at the level of the gene. Indeed, at the last ASCO meeting, it was shown that if you treat ER-negative tumors with trastuzumab, they can become ER-positive again. We have never seen that before.

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