Cross-Talk among Estrogen Receptor, Epidermal Growth Factor, and Insulin-like Growth Factor Signaling in Breast Cancer

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Abstract

Since the cloning of the estrogen receptor α (ERα) and subsequent identification of a second distinct form of ER, termed ERβ, a large volume of research has begun to define the molecular mechanisms of ER action. However, although great progress has been made, ER action is still poorly understood. It is expected that a better understanding of ER action may lead to novel strategies and targets for breast cancer prevention and treatment. One of the early-realized functions of the ER was regulation of growth factor signaling, but the degree of interaction between these two mitogenic signaling pathways could not have been anticipated. Recent evidence suggests that the ER and the growth-factor-signaling pathways intersect and directly interact at every level of signal transduction. The resulting synergism between ER and growth factors has been documented both in normal breast development and, importantly, in breast cancer progression and antiestrogen resistance. In this review, we will highlight our current understanding of the molecular mechanisms of cross-talk between ER and growth-factor-signaling pathways.

ER Structure and Function

The first indication that estrogen effects were mediated via a receptor came from the observation by Jensen (1) that rat uterine tissue could bind radiolabeled estradiol, and the solubilized nuclear receptor was subsequently isolated by centrifugation of homogenized uterine tissue (2). The ER is a member of a large family of nuclear transcriptional regulators (3), which include receptors for androgens, progestins, thyroid hormone, retinoids, and a number of members, termed orphan receptors, the ligands of which have yet to be isolated (4).

The cloning of ERs from a breast cancer cell line, MCF-7, led to a greater understanding of its role in mediating the diverse effects of estrogen (5). ERα mRNA is 6322 nucleotides long, encoding a protein of 595 amino acids that has a relative molecular mass of 66 kDa. The ERs are highly homologous between species, and these similarities led to the identification of seven regions within ERα (A-F) that perform the function of ligand binding (E), DNA binding (C), and transcriptional activation (A and E). The DNA-binding domain (DBD), domain C, is 66 amino acids in length and appears to form a putative zinc finger DNA-binding domain (6). Domain D contains a putative nuclear localization sequence (nls), which, when transferred to another protein, may confer nuclear localization activity (7), and the transcriptional activating function of ERα is contained within domains A and E. The first element (activating function 1, AF-1) contained in domain A is constitutively active and hormone independent, whereas AF-2 found in the E domain is responsive to hormone. A second distinct form of ER was recently cloned and termed ERβ (8). ERβ has significant homology to ERα and is able to bind estradiol and to increase ERE-driven reporter constructs similar to ERα. However, significant differences between ERα and ERβ signaling have been noted (9), and more research is needed to better understand ERβ action, which may define a role for ERβ in normal breast development or breast cancer (10).

ER binds to DNA at a region called the ERE, which is a palindromic sequence consisting of two hexanucleotide repeats (11). A number of EREs have been functionally identified in the promoter regions of estrogen-responsive genes, and, from these, a consensus ERE has been defined that is identical to the ERE of Xenopus laevis vitellogenin gene, i.e., 5′-GGTCA NNN TGACC-3′ (12). Many naturally occurring estrogen-responsive genes contain multiple copies of consensus or variant forms ("imperfect ERE") of the inverted repeat or contain only one-half of the palindrome. However, these variants of the consensus ERE can still bind ER and confer estrogen responsiveness. A large number of studies have characterized EREs in various genes, and different methods are described for identifying EREs (13). However, to date, only a very limited number of estrogen-responsive genes have been described. This is somewhat surprising because estrogen stimulation of ER-positive cells leads to a cascade of phenotypic changes and different gene expression profiles (14) that suggests a more complicated mechanism of ER action, such as direct interaction with AP-1 (15), which will be discussed later (see "Direct Interaction and Activation of Growth-Factor-Signaling Pathways by EROα below.

In addition to ER acting as a nuclear transcription factor, growing evidence showing that estrogen can have effects within minutes and, thus, before gene transcription can occur, suggests...
ER and Growth-Factor-Signaling Pathways Cross-Talk

Estrogen regulation of growth-factor-signaling pathways

ER Increases Expression of Stimulatory Growth-Factor-Signaling Pathways. The proliferative effect of estrogen on breast cancer cells can in part be accounted for by the ability of the ER to regulate expression of stimulatory growth-factor-signaling pathways (18). The interaction of ER with growth factor pathways is exquisitely intimate, with ER affecting every level of the signaling pathways (Fig. 1). The net effect of estrogen is to sensitize breast cancer cells to external growth factor simulation.

Estrogen can increase expression of IGF-II (19, 20) and TGFα (21), two autocrine stimulators of breast cell proliferation. Estrogen can also increase PDGF expression (22), and this may act in a paracrine manner to stimulate fibroblast to secrete growth factors (e.g., IGF-I) that in turn increase breast cancer cell growth. In addition to increasing actual growth factor expression, estrogen can sensitize breast cancer cells to growth factors by increasing expression of growth factor receptors such as IGF-IR (23, 24) and EGFR (25). Increased receptor numbers allow cells to respond to lower concentrations of EGF and IGF and confers synergism when cells are treated with a combination of estrogen and growth factors (26).

Recent evidence has shown that estrogen can also increase expression of intracellular signaling intermediates such as IRS-1 and -2 (24, 27). IRSs are a family of intracellular proteins that integrate and coordinate extracellular signals within the cell (28). IRSs can be activated by receptors for a number of ligands, including insulin, IGFs, growth hormone (GH), prolactin, oncostatin, interleukins, and IFNs; and, in turn, IRSs can signal to proliferation, survival, and migration (28). Estrogen regulation of critical signal integrators such as IRSs could in part explain the pleiotropic effects elicited by estrogen. Estrogen-increased IRS levels can enhance the ability of IGF-IR to signal, and, thus, may be responsible for the observed synergism between IGF and estrogen.

Although estrogen can clearly regulate expression of multiple-growth-factor signaling elements, ER is also able to regulate expression of nuclear transcription factors that are critical for growth factor signaling. Estrogen causes rapid induction of c-myc (29), c-fos (30), and c-jun (30). In addition to ER regulation of transcription factors, ER can also regulate expression of cell cycle components such as cyclin D1 and p21 (31). It has recently been shown that synergism between ER and IGF may occur directly at the level of the cell cycle, with ER decreasing the levels of p21 (a cdk inhibitor) and thereby allowing IGF to potently activate cdk complexes in the absence of cdk inhibitors.

ER-mediated regulation of transcription factors and cell cycle expression occurs very fast (within hours), before changes in growth factor pathways are observed (hours to days), and this has led some investigators to propose that growth factors are not important for estrogen-mediated proliferation (32). In fact, it is probably a combination of estrogen directly affecting the cell cycle and interaction with growth factor pathways that allows estrogen to be such a potent mitogen.

Whereas estrogen can clearly increase expression of positive growth-factor-signaling pathways, antiestrogens confer the opposite effect, down-regulating the same components. Thus the net effect of antiestrogen treatment is to block the cell cycle and render breast cancer cells insensitive to growth factor stimulation. In addition, antiestrogens have also been shown to increase expression of two tyrosine phosphatases, LAR and FAP-1, that can specifically dephosphorylate and deactivate IGF-IR and EGFR (33, 34).

ER Decreases Expression of Inhibitory Growth-Factor-Signaling Pathways. Whereas estrogen can increase positive growth-factor-signaling elements, it also down-regulates negative signaling elements. In particular, estrogen decreases expression of TGFβ (35), a potent growth inhibitor and activator of cell death in breast cancer cells. In the IGF signaling pathway, estrogen decreases IGFBP-3 levels (36). IGFBP-3 can inhibit breast cancer cell growth by binding and sequestering IGF ligand and by activating cell death via a specific IGFBP-3 receptor (37). In addition, estrogen can down-regulate IGF-IR expression (38), another inhibitor of breast cancer cell proliferation and a putative tumor suppressor gene (39). However, estrogen reduction of IGF-IR expression may be an indirect result of estrogen stimulation of IGF-II expression, which is bound by IGF-IR and results in IGF-IR internalization and reduced cell surface levels.

Direct Interaction and Activation of Growth-Factor-Signaling Pathways by ERα

Estrogen regulation of growth-factor-signaling pathways are long-term effects (days) that can, in most part, be accounted for by the ability of a nuclear ER to alter gene transcription. However, there have been a number of reports showing that estrogen can have effects that cannot be accounted for by the classical model of nuclear ER activating gene transcription (16).
Several lines of evidence suggest that ERα can actually exist, and bind estrogen, in the membrane or cytoplasm (Fig. 2). This has been shown by biochemical fractionation of the membrane in cells that overexpress ERα (40) and by direct visualization of membrane ERα, using immunocytochemistry (41). A plausible hypothesis is that ERα is either transiently in the cytoplasm when it is first translated, or that ERα shuttles between the nucleus and cytoplasm (ERα contains both nuclear localization sequences and nuclear export sequences).

When ERα is in the cytoplasm or membrane, it may interact with IGF-IR (42). This interaction occurs only in the presence of estrogen and results in activation of IGF-IR and downstream signaling through MAPK. Interestingly, this interaction occurs only with ERα and not with ERβ. ERα is also able to directly interact with the p85 subunit of phosphatidylinositol 3'-kinase (43). This again is an interaction that occurs only in the presence of estrogen and is only performed by ERα and not ERβ. Whereas the ER/IGF-IR interaction was shown by coimmunoprecipitation, and thus may not be a direct interaction, ERα binds p85 in vitro binding assays, which indicates that this is direct binding. It is interesting to note that IGF-IR may bind p85, setting up the possible ternary complex IGF-IR/p85/ERα that can be activated by estrogen. Because IGF-IR and p85 are upstream of two of the most potent cell proliferation (MAPK) and cell survival (e.g., Akt) signaling pathways, understanding the significance and relevance of this potential complex is needed. Evidence also suggests that ER can bind, and be phosphorylated by, c-src (44).

Although the former effects rely on cytoplasmic or membrane-bound ER, nuclear ERα can also bind other nuclear proteins that are involved in growth-factor signal-transduction pathways. For instance, ER can directly interact with AP-1 (15) and SP-1 (15). Binding of ER to AP-1 allows transactivation at AP-1 sites, allowing estrogen induction of genes (e.g., IGF-1) in the absence of ER binding to DNA (15). In contrast, ER binding to SP-1 allows synergistic transactivation potential at ER and SP-1 sites that are in close proximity. It is unknown at present how many genes are regulated by ER in this fashion, but direct interaction of ER with other nuclear transcription factors may account for the large number of genes that are regulated by estrogen.

ER is able to directly interact with, and be activated by, cyclin D1 (45, 46). Cyclin D1 activation of ER is separable from its function as a cdk regulator. Two recent reports have given insight into how cyclin D1 may activate the ER. Cyclin D1 can interact with two coactivators of the ER, namely SRC-1 (47) and p300 (48). Cyclin D1 can interact with SRC-1 through the classical leucine-rich coactivator binding motif used by the ER to bind SRC-1 (47). Thus, cyclin D1 could act as a bridge between ER and coactivators and allow transcription activation. Furthermore, in the presence of estrogen, ER presumably interacts with coactivators directly and through cyclin D1. Importantly, growth factors are known to increase cyclin D1 expression (49), and, therefore, it is possible that in ER-positive breast cancer cells, this would result in ligand-independent activation of the ER.

**Growth Factor-mediated Activation of the ER**

As stated earlier, several growth factor pathways can activate the ER (50). Deletion of functional domains of the ER first indicated that ER lacking the AF-1 domain was activated by estradiol, but not by EGF or IGF-I (51–53), implicating AF-1 in ligand-independent activation of ER. Furthermore, ER lacking AF-2 was activated by EGF and IGF-I, but not by estrogen. Estrogen activation of the ER is associated with an increase in phosphorylation of the receptor (54–56). Growth factors have also been shown to cause an increase in phosphorylation in the ER (57–60). Confirming earlier data implicating AF-1 in ligand-independent action, it was shown that MAPK, which is downstream of a number of growth factor receptors, could phosphorylate Ser118 in the AF-1 domain of the ER (57, 58). Overexpression of a dominant active ras mutant, c-Ki-RasV12, or constitutively active MAPK, increased the phosphorylation and transcriptional activity of AF-1 (57) but not AF-2 and a mutant AF-1 with Ala118. Additionally, dominant-negative Ras (RasN-17), or dominant negative MAPK, inhibited EGF-induced transcriptional activation of the ER (58). Thus, the role for MAPK phosphorylation of AF-1 in growth factor-induced activation of ER is firmly established.

Recent data suggest a mechanism for ligand-independent activation of the ER by MAPK. Mouse ERβ can be activated by MAPK phosphorylation of Ser124 (61), resulting in increased association between ERβ and an enhancer of ER-mediated transcriptional activity, SRC-1. This would potentially allow ER to interact with the basal transcription machinery and enhance transcription. Interestingly, Ser124, which is phosphorylated by MAPK in mouse ERβ and is involved in enhanced association of SRC-1, corresponds to Ser118 in human ERα. However, in contrast, it has also recently been shown that GRIP-1 (SRC-2) binding to the AF-1 domain of ERα is independent of Ser118 (62).

Interestingly, although analyses in COS-1 and HeLa cells showed that phosphorylation of AF-1 may be responsible for EGF and IGF-I activation of ER, studies in SK-N-BE neuroblastoma cells transfected with ER have shown that insulin activation of the ER also requires Ras (63, 64) but occurs through the AF-2 domain (65). Supporting this hypothesis, Ser118 is actually indispensable for insulin activation of the ER (60). This observation is supported by the fact that insulin causes different ER electrophoretic migration pattern in COS-1 cells.
cells versus SK-N-BE, which suggests that in SK-N-BE cells, ER becomes phosphorylated on different residues and has a different mechanism of activation.

The phosphorylation of ER by MAPK has been most studied and in part may explain how growth factors activate the ER. However, increasing evidence suggests that other growth factor-activated kinases can also phosphorylate and/or activate the ER (Fig. 3). These include Akt (15), JNK (66), pp90rsk (67), and p38 MAPK (68). In addition, ER coregulators, which serve as a bridge between ER and the basal transcription machinery, are also subject to phosphorylation by the same kinases and may also be a potential mechanism for ligand-independent activation of the ER (69). It is clear, therefore, that many kinases can phosphorylate the ER and its cofactors, and that more studies will be needed to define the role of these kinases in growth factor-mediated activation of ER and antiestrogen resistance.

Role of Growth Factors in Antiestrogen Resistance

The tight regulation of growth factor signaling by estrogen and antiestrogens has led a number of groups to study dysregulation of growth factor signaling in antiestrogen resistance (70). This has been performed by examining growth factor signaling in antiestrogen-resistant breast cancer cells in vitro, and by overexpressing individual growth-factor-signaling elements and then assessing antiestrogen sensitivity. It has been found that overexpression of many growth-factor-signaling elements, such as IGF-II, EGFR, ErbB2, IRS-1, cyclin D1, AP-1, results in estrogen-independent growth and/or antiestrogen resistance (71). This suggests that there may be multiple pathways for acquiring antiestrogen resistance, and this hypothesis has been borne out by the examination of growth factor signaling in antiestrogen-resistant breast cancer cells lines. In each of these models different growth-factor-signaling pathways seem to have been dysregulated, and there is not a single pathway that is clearly responsible. An excellent example of this is the EGFR/MAPK-signaling pathway in MCF-7 models of estrogen resistance. Several groups have selected MCF-7 variants that are either estrogen-independent or antiestrogen-resistant by long-term culture of MCF-7 cells in the absence of estrogen or the presence of antiestrogen. Several studies have shown that this results in the generation of MCF-7 cells that have increased EGFR and MAPK activity (72–74). However, studies have also found no perturbation in EGFR expression and MAPK activity using similar methods (75). Similarly, we have recently shown that MCF-7 cells that have been selected for estrogen independence by long-term estrogen withdrawal do not have increased MAPK activity (76). Although differences may be attributable to experimental variation in technique and parental cells, these data again suggest that multiple pathways, both ER-dependent and ER-independent, may contribute to antiestrogen resistance (71).

Clinical Significance of ER and Growth Factor Cross-Talk

Several observations from breast cancer cells in vitro have been replicated in primary breast cancer specimens. The most obvious of these is that genes found to be estrogen regulated in vitro have expression levels that correlate with the ER status of breast cancer specimens. For instance, in the IGF signaling family, ER status correlates with expression of IGF-IR (77, 78), IGF-binding proteins (79), and IRS-1 (80). Furthermore, these same components have prognostic significance in ER-positive breast cancer patients (24, 81). What is not clear, however, is the role of these same components in clinical antiestrogen resistance. Despite this uncertainty, several small studies have given preliminary evidence that growth-factor-signaling pathways may be overexpressed in antiestrogen-resistant breast cancer (70).

Summary

In this review, we have highlighted the exquisite and complex molecular interactions between ER and growth factor signaling. It is clear that, with the observations that have been made, we have thus far only scratched the surface and that more intricate cross-talk will continue to be discovered. Whereas we have concentrated on cross-talk between ER and growth factors in this review, there is new and growing evidence that ER can cross-talk with many other signaling pathways and affect other biological processes, such as cell movement, angiogenesis, and cell survival. We and others hope that through additional molecular studies of ER action, we can define new targets of ER cross-talk that may prove useful in the prevention or treatment of breast cancer.

Open Discussion

Dr. Kent Osborne: There have been discussions about how to begin to integrate tyrosine kinase inhibitors and other newer agents in breast cancer. People are saying you’ve got to show that they inhibit breast cancer growth by themselves before you can start combining them. I think that’s nonsense. Based on the molecular data that you and others have shown, it makes total sense to do studies with combinations of these various endocrine agents, once you know the safety and the dose that seems to have a biological effect on the target.

Dr. Carlos Arteaga: Yes, I agree. Cancer cells are very smart, and they probably program themselves in such a way that they can anticipate or eventually bypass every single drug or stress that they encounter. Single cells persist in the bloodstream for months to years trying to extravasate and colonize. So I think that the idea of multiple anti-signaling strategies is very rational.

Dr. Per Lonning: Your in vitro data are beautiful, but
there is always an alternative explanation because of the redundant mechanisms. I'm concerned about extrapolating from in vitro to in vivo without knowing what each individual drug does in vivo in relation to that therapeutic outcome. I think we should use these opportunities we have now with the new biology to explore what each individual drug is doing in vivo.

Dr. Osborne: I think you're right, but just because we don't see significant tumor shrinkage from one of these factors doesn't mean we shouldn't also do studies combined with endocrine therapy to block potential mechanisms of resistance.

Dr. Steven Come: Nobody says that the paradigm of chemotherapy where each agent in a combination has to have independent activity has anything to do with the biological strategy for treating cancer. As Dr. Wakeling was showing yesterday with Iressa and tamoxifen resistance, an agent might be used to inhibit resistance rather than for a positive effect.

Dr. Arteaga: The impact of these interventions is going to be dependent on the biological window in which you intervene. The oncogenic events that are being discussed here occur early in the carcinogenic process. They are mechanisms by which these tumors destabilize their genome and then accumulate all kinds of mutations which, in turn, become the drivers of the tumor phenotype. So I think a rational idea would be to move these treatments early, before the initial oncogenic events become dispensable.

Dr. Stephen Johnston: Going back to the wild-type MCF-7 cells, if you do an immunoprecipitation, does ER bind to p85/p13K in the wild-type cells to the same extent? Secondly, what does tamoxifen do to insulin receptor substrate-1 (IRS-1) levels in both wild-type and in the ER-transfected C4-12 cells?

Dr. Lee: Yes, ER binds p85 in MCF-7 cells. Tamoxifen decreases IRS-1 levels in wild-type MCF-7 cells. But in the ER-negative cells they have no IRS-1 left. If you then put ER into those cells, then tamoxifen decreases it, because it's a transcriptional effect. So in that model, the ER-transfected C4-12 cells act just like MCF-7 cells. That's what's different in that model from most other models. When C4-12 became ER negative, they have an increase in cell numbers, but that increased growth is not associated with any increase in S-phase. Basically, it looks like there is a decrease in their apoptotic rate, and that's how they're increasing their cell number. So maybe that's the key that allowed us to put in ER and then switch on that proliferation again, because those ER-negative cells don't have unregulated proliferation. I think their cell cycle clock is probably normal.

Dr. Anthony Howell: What's happening in the ER-negative cells in those other models that are transfected with ER—do they stop proliferating? ER is basically antiproliferative in normal cells. So it's doing the right thing when you transflect it into other cell lines, but not in yours.

Dr. Lee: I think that's wrong, because in the original breast cancer cells, ER was proliferative. The breast cancer cell is clearly different from the normal breast cell. In the normal breast, it's really unclear what ER is doing in terms of proliferation. It may be a growth inhibitor or may be stimulating neighboring ER-negative cells to grow. But in the breast cancer cell, it's clear that the ER is causing proliferation. So when they took those ER-negative cells and then put them back in ER, that's an unusual finding for proliferation to be inhibited.

Dr. Osborne: The ER-negative cell line conceivably lacks other things aside from the receptor itself. One possibility would be that when you add ER to ER-negative cells, the ER squelches other pathways. It's going to take SRC-1 coactivators away from whatever else they do. After all, ER regulates growth factor pathways.

Dr. Lee: Yes, ER inhibition of growth in ER-negative cells could be a nonspecific effect.

Dr. Arteaga: Are there any genes that are differentially induced by the combination versus estrogen alone? There was a significant difference in expression whether you use each alone or both combined. That implies there are some genetic programs that require IGF-I that would not occur if there was not enough estrogen-induced up-regulation of the IGF-I system to recruit IGF-I signals at the promoter level.

Dr. Osborne: Maybe we should do some expression arrays, each hormone alone and then together, and see if there are different sets of gene profiles.

Dr. Lee: That's what we intend to do. There's a whole different set of events with the combination of estrogen and IGF that don't occur with the single agents. That is the main question: is there a unique set of genes that may be critical for that process that we could target?

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*Clin Cancer Res* 2001;7:4429s-4435s.

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