

Biology of Progesterone Receptor Loss in Breast Cancer and Its Implications for Endocrine Therapy

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A B S T R A C T

The response to endocrine therapy in breast cancer correlates with estrogen receptor (ER) and progesterone receptor (PR) status. ER-positive/PR-negative breast cancers respond less well to selective ER modulator (SERM) therapy than ER-positive/PR-positive tumors. The predictive value of PR has long been attributed to the dependence of PR expression on ER activity, with the absence of PR reflecting a nonfunctional ER and resistance to hormonal therapy. However, recent clinical and laboratory evidence suggests that ER-positive/PR-negative breast cancers may be specifically resistant to SERMs, whereas they may be less resistant to estrogen withdrawal therapy with aromatase inhibitors, which is a result inconsistent with the nonfunctional ER theory. Novel alternative molecular mechanisms potentially explaining SERM resistance in ER-positive/PR-negative tumors have been suggested by recent experimental indications that growth factors may downregulate PR levels. Thus, the absence of PR may not simply indicate a lack of ER activity, but rather may reflect hyperactive cross talk between ER and growth factor signaling pathways that downregulate PR even as they activate other ER functions. Therefore, ER-positive/PR-negative breast tumors might best be treated by completely blocking ER action via estrogen withdrawal with aromatase inhibitors, by targeted ER degradation, or by combined therapy targeting both ER and growth factor signaling pathways. In this review, we will discuss the biology and etiology of ER-positive/PR-negative breast cancer, highlighting recent data on molecular cross talk between ER and growth factor signaling pathways and demonstrating how PR might be a useful marker of these activities. Finally, we will consider the clinical implications of these observations.

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INTRODUCTION

Estrogen and the estrogen receptor (ER) play key roles in both normal breast development and breast cancer progression. Therapeutic strategies directed at inhibiting the action of ER using selective ER modulators (SERMs), targeting ER for degradation with selective ER downregulators (SERDs), or withdrawing estrogen by surgical (oophorectomy) or medical (luteinizing hormone agonists and antagonists) ovarian ablation or by aromatase inhibitors represent highly successful examples of targeted therapy for clinical breast cancer.¹⁻⁴

In this review, we will highlight the predictive value of ER and progesterone receptor (PR) in breast cancer and provide hypotheses to explain why a significant subset of breast cancers (ER-positive/PR-

negative) may be selectively more resistant to SERM therapy. The difference in clinical benefit from SERMs versus aromatase inhibitors in ER-positive/PR-negative tumors may be explained by new insights into the regulatory mechanisms of ER and PR that could simultaneously cause the ER-positive/PR-negative phenotype and the resistance to SERM therapy. These mechanisms invoke molecular cross talk between ER, PR, and growth factor-receptor signaling pathways and the relationship between the classical and nonclassical effects of ER in breast cancer cells. Here, we summarize the molecular signals regulating PR levels in breast cancer and highlight recent clinical data that support a strong rationale for the design and implementation of clinical studies using alternative endocrine therapies in ER-positive/PR-negative tumors.

ER AND PR IN BREAST CANCER

Expression and Prognostic Value of ER and PR

The importance of steroid hormones in breast cancer has been known for decades, and many studies have described the ontogeny of steroid receptor expression in both rodent model systems and the human mammary gland.^{5,6} The ER exists as two isoforms, ER α and ER β , which are encoded by two different genes (Fig 1). Like other steroid receptors, the ER has a structure consisting of a DNA-binding domain (DBD) flanked by two transcriptional activation domains (AF-1 and AF-2). The receptor binds its ligand estradiol in the ligand-binding domain (LBD). Studies in rodents have shown that ER α and ER β are expressed in the normal mammary gland⁷ and that expression of ER α , but not ER β , is critical for normal mammary gland ductal development.⁸ In humans, ER is also expressed in the normal breast, and a dramatic increase in ER α expression is seen in early hyperproliferative premalignant lesions.⁶

One of the most studied ER-regulated genes is PR, which mediates progesterone's effects in the development of the mammary gland and breast cancer.⁹ PR is expressed as two isoforms (PR-A and PR-B) from a single gene (Fig 1).¹⁰ Like ER, PR contains a DBD, LBD, and multiple AFs.¹¹ Studies in the rodent mammary gland have shown that PR is critical for lobuloalveolar development of the gland¹² and that the ratio of PR-A to PR-B is critical for proper mammary gland development.¹³ Interestingly, an overabundance of PR-A in human breast cancers has recently been reported to be associated with resistance to tamoxifen,¹⁴ whereas a functional promoter polymorphism that results in increased production of PR-B is associated with an increased risk of breast cancer.¹⁵ The dramatic increase in breast cancer incidence in women taking both estrogen and progesterone for hormone replacement therapy, compared with estrogen alone, emphasizes the importance of progesterone and the PR in breast cancer.¹⁶

Approximately 75% of primary breast cancers express ER, and more than half of these cancers also express PR.¹⁷

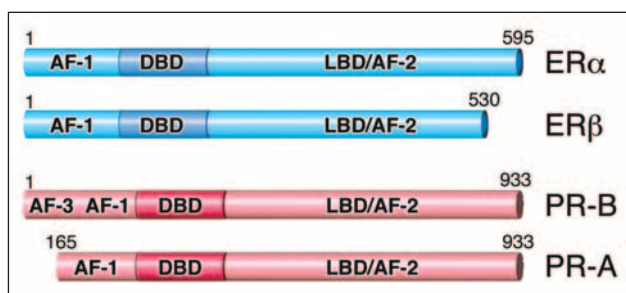


Fig 1. Structure of estrogen receptor (ER) and progesterone receptor (PR). ER consists of two isoforms (ER α and ER β) that are transcribed from two genes. PR also consists of two isoforms (PR-A and PR-B) that are transcribed from a single gene using an alternative promoter and translation start site. DBD, DNA-binding domain; LBD, ligand-binding domain.

Both ER and PR are prognostic factors, although both are weak and lose their prognostic value after long-term follow-up.¹⁸ PR is an estrogen-regulated gene, and its synthesis in normal and cancer cells requires estrogen and ER. Therefore, it is not surprising that ER-positive/PR-positive tumors are more common than ER-positive/PR-negative tumors. The etiology of ER-positive/PR-negative tumors is currently unclear. Some studies have shown that ER and PR status can change over the natural history of the disease or during treatment.¹⁹ For instance, sequential breast cancer biopsies have shown that ER levels are reduced slightly with intervening endocrine therapy, although complete loss is uncommon. In contrast, PR levels decrease more dramatically during tamoxifen therapy, with up to half of tumors completely losing PR expression when resistance develops.²⁰ These ER-positive/PR-negative metastatic tumors then display a much more aggressive course after loss of PR compared with tumors retaining PR, and patients then have a worse overall survival, indicating a change in tumor cell-regulatory mechanisms.^{20,21} Whether and how the loss of PR affects the poor clinical course of these tumors is at present unclear.

Although ER-positive/PR-negative primary untreated tumors may simply evolve by loss of PR from subclinical ER-positive/PR-negative tumors, the differences in the biology and outcome of ER-positive/PR-negative tumors suggest that some of these tumors may initially evolve separately as ER-positive/PR-negative tumors, representing their own individual stable phenotype from the outset. Indeed, recent prospective studies have shown that ER-positive/PR-negative tumors have their own unique epidemiologic risk factors.²² For instance, the incidence of each of the four receptor tumor subtypes (ER-positive/PR-positive, ER-positive/PR-negative, ER-negative/PR-positive, and ER-negative/PR-negative) differs with age,²³ pregnancy history, postmenopausal hormone use, and body mass index after menopause.²⁴ We have also found similar associations^{24A} between ER-positive/PR-negative tumors and older age compared with ER-positive/PR-positive tumors. Importantly, many of these associations remain for PR by itself, even when corrected for quantitative ER levels, highlighting the significance of both receptors not only in breast cancer treatment, but also in their etiology.

ER and PR Predict Response to Endocrine Therapy

ER status is a strong predictor of response to endocrine therapy. In the adjuvant setting, a meta-analysis showed that tamoxifen significantly reduces recurrence and death only in patients with ER-positive tumors.²⁵ Similar results have been shown in two trials prospectively designed to test the value of tamoxifen in ER-negative tumors^{26,27} and also in retrospective analyses of several clinical trials.²⁸ In several

studies, it has been shown that response to tamoxifen is directly related to ER levels,²⁹ but there are responses in tumors that have as little as 4 to 10 fmol/mg of ER protein or as few as 1% to 10% of cells positive for ER by immunohistochemistry.³⁰

Although ER is an accepted predictor of response to endocrine therapy, the role of PR has been more controversial. For instance, the Oxford overview of all trials of tamoxifen therapy in early breast cancer found that PR status did not predict benefit.²⁵ However, variability in assay methodology and lack of quality control in many laboratories raise questions about this data. In contrast to this, we have recently published a retrospective analysis of two of the largest data sets of patients (n = 15,871) with early breast cancer treated with endocrine therapy (nearly all received tamoxifen), with ER and PR levels measured in two standardized quality-controlled clinical laboratories.¹⁸ In this study, we found that patients with ER-positive/PR-positive tumors benefited much more from adjuvant tamoxifen therapy than patients with ER-positive/PR-negative tumors (Fig 2). Multivariate analyses showed that both ER and PR were independent predictors of overall survival, with the reduction in relative risk of death being significantly greater in ER-positive/PR-positive compared with ER-positive/PR-negative tumors. Importantly, PR still had predictive value even when ER was considered as a continuous variable, indicating that the predictive information is independent of quantitative ER levels¹⁸ and that PR adds predictive information to ER. The predictive value of PR in the adjuvant tamoxifen setting has also been shown in two smaller studies.^{31,32}

Consistent with the predictive power of PR in the adjuvant setting, several studies have shown that elevated PR levels significantly and independently correlate with increased probability of response to tamoxifen, longer time to treatment failure, and longer overall survival in patients

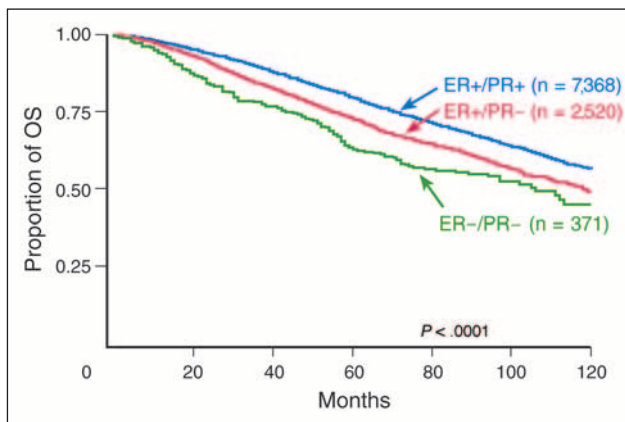


Fig 2. Overall survival (OS) according to tumor receptor status in women treated with endocrine therapy. All patients were treated with systemic endocrine therapy (tamoxifen in > 90%). ER+, estrogen receptor positive; ER-, estrogen receptor negative; PR+, progesterone receptor positive; PR-, progesterone receptor negative. Data adapted.¹⁸

with metastatic disease.³³ As with ER, response is directly and positively related to PR levels.²⁸ Similarly, two recent neoadjuvant studies showed a better response (clinical outcome and proliferation rate) to endocrine therapy in PR-positive tumors compared with PR-negative tumors.^{34,35}

ER-positive/PR-negative Breast Tumors Are Selectively Resistant to SERMs

Supporting the studies indicating a role for PR in predicting response to adjuvant antihormone therapy are recent data from the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial, which randomly assigned postmenopausal women with early breast cancer to 5 years of treatment with the aromatase inhibitor anastrozole, tamoxifen, or the combination.³⁶ A recent preliminary analysis of this trial with respect to hormone receptor status showed that, when all treatments are combined together (termed endocrine therapy), 7.6% of patients with ER-positive/PR-positive tumors had a recurrence (breast cancer event) over a 47-month follow-up period, whereas 14.8% of patients with ER-positive/PR-negative tumors experienced recurrence (Fig 3A). Importantly, however, a careful analysis of the individual treatments (anastrozole v tamoxifen v the combination) reveals tantalizing hints as to the biology of this reduced efficacy. The inferior outcome in the ER-positive/PR-negative subgroup was restricted largely to the tamoxifen and combination treatment arms (Fig 3B). In contrast, the anastrozole only arm showed only a trend for anastrozole to be superior to tamoxifen in the ER-positive/PR-positive subset. Indeed, this preliminary hormone receptor data suggest that the overall benefit of anastrozole

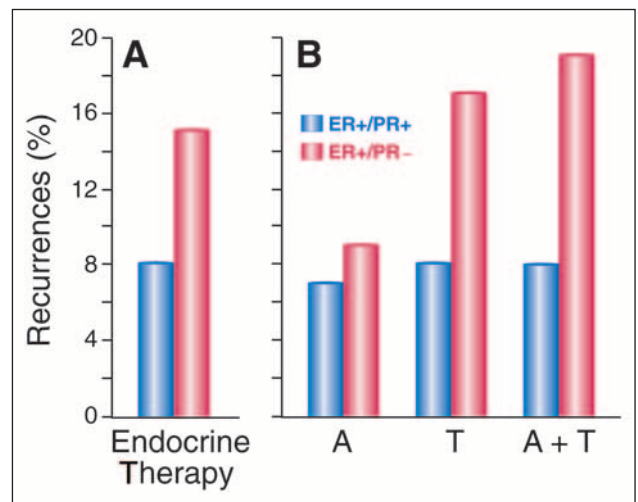


Fig 3. Breast tumor recurrences in the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial according to hormone receptor status. (A) Breast tumor recurrence according to hormone receptor status including data combined from the three treatment arms of the ATAC trial (termed endocrine therapy). (B) The three individual arms of the ATAC trial. A, anastrozole; T, tamoxifen. ER, estrogen receptor; PR, progesterone receptor. Data adapted.³⁷

over tamoxifen in the ATAC trial is largely a result of the reduced efficacy of tamoxifen in the patients with ER-positive/PR-negative tumors.

Collectively, these data indicate that ER-positive/PR-negative breast tumors are less responsive to SERM therapy compared with ER-positive/PR-positive tumors. It is noteworthy that in the 1970s it was hypothesized that PR might provide additional information to more accurately predict which patients will respond to hormonal therapy.³⁸ This theory was based on the rationale that PR is induced by estrogen in ER-positive breast cancer cell lines and, therefore, that PR would serve as an indicator of a functionally intact ER pathway. However, this simple theory fails to fully explain differences such as those seen between anastrozole and tamoxifen in the ATAC trial, which presumably are mediated via the ER.

Increased Growth Factor Signaling Is Associated Both With the ER-Positive/PR-Negative Phenotype and With SERM Resistance

Several reports suggest that high growth factor activity in breast cancers may be associated with decreased PR levels. Konecny et al³⁹ recently reported that patients with higher levels of human epidermal growth factor receptor 2 (HER2) in their tumors had statistically significantly lower levels of ER/PR than patients with lower levels of HER2. Intriguingly, their study also revealed that relatively low levels of HER2 amplification/overexpression were associated with marked decreases of PR but not ER, thus causing tumors to be ER-positive/PR-negative. Recently, Dowsett et al³⁵ found that 25% of ER-positive/PR-negative tumors overexpressed HER2 compared with 10% of ER-positive/PR-positive tumors. Several other studies also suggest that ER-positive/PR-negative tumors are more likely to be higher grade and to be amplified for HER2 than ER-positive/PR-positive tumors.^{21,40} We have also recently found that ER-positive/PR-negative tumors have higher levels of epidermal growth factor receptor (EGFR) and HER2 compared with ER-positive/PR-positive tumors (Arpino et al^{24A}).

High HER2 signaling has been associated with reduced tamoxifen efficacy in many, although not all, clinical studies.⁴¹ However, this may be in part a result of the fact that tumors that overexpress HER2 are more likely to be ER negative and PR negative. In fact, when analyzed as a continuous variable, HER2 is inversely related to ER and PR even in the subset of ER-positive patients.³⁹ Despite this, Dowsett et al³⁵ showed that tamoxifen resistance in ER-positive/HER2-positive tumors was unrelated to ER levels, and Arpino et al^{24A} found that EGFR levels predicted resistance to tamoxifen in multivariate analysis considering ER levels as a continuous variable.⁴² Therefore these studies suggest that reduced ER expression in EGFR- or HER2-positive tumors may not fully account for the tamoxifen

resistance. Other studies suggest that HER2 status alone is not a strong predictor of tamoxifen resistance in ER-positive tumors, although studies like this in metastatic breast cancer rely on the hormone receptor status of the primary tumor and not the metastasis.⁴³ Levels of ER, PR, or HER2 can change over time or with treatment as tumors progress to overt metastasis.^{44,45}

Two recent neoadjuvant studies confirmed the observation that PR-negative tumors respond less well to hormonal therapy than PR-positive tumors.^{34,35} Furthermore, Ellis et al³⁴ went on to show that tamoxifen was less effective than an aromatase inhibitor in patients whose tumors were EGFR-positive and/or HER2-positive, again reiterating the potent cross talk that can occur between this pathway and ER, perhaps contributing to relative resistance. A recent retrospective study demonstrating a poor disease-free survival for patients receiving adjuvant tamoxifen whose tumors expressed high levels of both HER2 and the ER coactivator AIB1⁴⁶ further implicates growth factor activity interacting with the ER pathway in resistance to SERMs.

The HER family of receptors is upstream to the PI3K/Akt/mTOR signaling cascade.⁴¹ Phosphatase and tensin homolog (PTEN) is a negative regulator of this pathway, and loss of PTEN, which is associated with activation of this survival pathway, has recently been correlated with loss of PR in clinical breast cancer specimens.⁴⁷ In another smaller study, loss of PTEN correlated with loss of both ER and PR; however, this study did not specifically examine ER-positive/PR-negative tumors.⁴⁸ Importantly, it has been reported that loss of heterozygosity (LOH) at chromosome 10q23, which harbors the *PTEN* gene, occurs in approximately 30% to 40% of sporadic breast cancers and that this LOH is associated with higher histologic grade and specific loss of PR but not ER expression.⁴⁹

Finally, Akt, a key downstream kinase, not only down-regulates PR levels, but it also induces ligand-independent activation of ER.⁵⁰ ER signaling might then activate other cell survival pathways, thereby protecting breast cancer cells from tamoxifen-induced apoptosis⁵¹⁻⁵³ or reducing tamoxifen's antagonist properties, resulting in tamoxifen-stimulated growth and treatment resistance. Clinical studies have reported that high levels of phosphorylated Akt predict worse outcome among endocrine-treated patients.⁵⁴

MOLECULAR BASIS FOR ER-POSITIVE/PR-NEGATIVE BREAST CANCERS AND RESISTANCE TO SERMS

ER Structure and Function

Overview. The ER is a member of a large family of nuclear transcriptional regulators.⁵⁵ Most research on the biology of ER has focused on its function as a nuclear transcription factor; however, ER can also bind to other transcription factors and proteins and function as a coregulator to enhance

genes not traditionally thought of as estrogen target genes. Finally, recent evidence also suggests that estrogen can bind ER located in or near the plasma membrane and rapidly activate other signaling pathways. To better define these mechanisms of action, a recent consensus nomenclature was proposed⁵⁶ whereby estrogen action in the nucleus is termed nuclear-initiated steroid signaling (NISS) and estrogen action at the plasma membrane is termed membrane-initiated steroid signaling (MISS). Both of these modes of ER action will now be briefly discussed.

NISS. ER is a hormone-regulated nuclear transcription factor that can induce expression of a number of genes (eg, PR).⁵⁷ On ligand activation, ER binds to estrogen response elements in target genes, recruits a coregulator complex, and regulates transcription of specific genes (Fig 4, pathway 1).^{58,59} ER has also been shown to act in a nonclassical manner without a need for DNA binding by modulating gene expression at alternative regulatory sequences such as AP-1,⁶⁰ SP-1,⁶¹ and USF sites⁶² (Fig 4, pathway 2).

ER action is controlled by a growing number of coregulatory proteins termed coactivators and corepressors that recruit enzymes that modulate chromatin structure to facilitate or repress gene transcription.^{63,64} It has been postulated that the ability of SERMs to either activate or repress ER action is in part controlled by the cellular environment of coregulators. Indeed, the first experiments to test this showed that altering coregulator levels could change SERMs from antagonists to agonists but that this was cell specific.⁶⁵ Further studies showed that there were cell type- and promoter-specific differences in coregulator recruitment that determine the cellular response to SERMs.⁶⁶

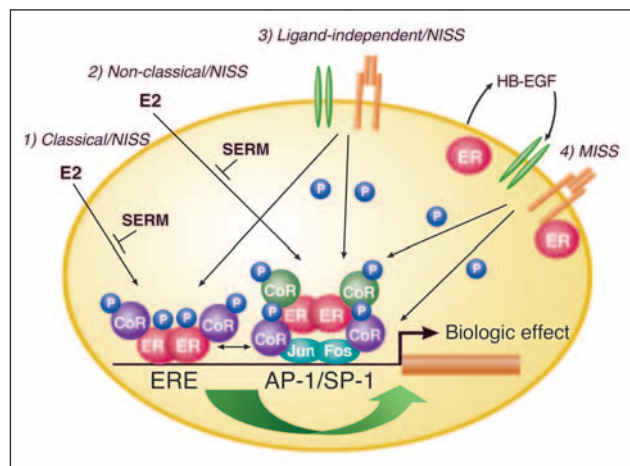


Fig 4. Estrogen receptor (ER) action. Schematic indicating the major pathways of ER action that have been elucidated using breast cancer cell lines. All of these pathways can act in a synergistic manner to orchestrate the overall change in gene expression when estrogen stimulates a breast cancer cell. NISS, nuclear-initiated steroid signaling; HB-EGF, heparin-binding epidermal growth factor; MISS, membrane-initiated steroid signaling; SERM, selective estrogen receptor modulator; P, progesterone; CoR, coregulator; E2, estradiol; ERE, estrogen response element.

Given these observations, studies have been performed that examined coregulator levels in breast cancer and associations with prognosis and hormone resistance,^{63,67} although more studies are needed, in particular concerning a fundamental understanding of the determinants of coregulator levels.

ER also interacts with several other nuclear proteins that themselves are critical in breast cancer. For instance, ER can bind BRCA1, and this interaction results in a reduction of ER activity.⁶⁸ Mutated BRCA1 protein derived from genetic carriers fails to repress ER, providing a hypothesis for the hormone-dependent cancer formation that is suppressed by ovarian ablation in these patients.⁶⁹ However, once breast tumors appear in BRCA1 carriers, the tumors are invariably ER negative, indicating that, at this stage, BRCA1 has no ER-dependent function. ER also directly interacts with and is activated by cyclin D1.^{70,71} Cyclin D1 can also interact with two coactivators of the ER, steroid receptor coactivator (SRC)-1⁷² and p300.⁷³ Thus, cyclin D1 could function as a bridge between ER and coactivators to augment transcriptional activation.

In addition to estrogen, several other stimuli can enhance NISS in a ligand-independent manner (Fig 4, pathway 3). Growth factors, such as insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF), heregulin, and transforming growth factor alpha,⁷⁴ neurotransmitters such as dopamine,⁷⁵ signaling molecules such as cyclic adenosine monophosphate,⁷⁶ and membrane-permeable phosphatase inhibitors⁷⁷ can all potentiate NISS. One of the mechanisms for ligand-independent activation may be phosphorylation of ER or its coregulators at specific sites induced by growth factors and stress-related kinases.⁷⁸ ER is phosphorylated at several sites by multiple kinases including the extracellular regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinases (MAPKs),^{79,80} cyclin-dependent kinase (CDK)-2,⁸¹ CDK-7,⁸² c-SRC,⁸³ protein kinase A,⁸⁴ pp90^{rsk1},⁸⁵ and AKT.⁵¹ Phosphorylation of ER α at serine residues clustered in its amino terminus (Ser 104/106, 118, and 167) enhances transcriptional transactivating activity arising from the ligand-independent AF-1 domain.^{84,86} ER dimerization and DNA binding are regulated by phosphorylation at serine 236 in the DBD⁸⁷ and by tyrosine phosphorylation at residue 537 within the LBD, with the latter modification also involved in estrogen binding.⁸⁸ Finally, phosphorylation on threonine 311, which is induced by estrogen activation of the p38 MAPK, promotes ER nuclear localization via nuclear export regulation and enhances ER interaction with coactivators.⁸⁹

However, phosphorylation of ER coregulators is probably as important as phosphorylation of ER itself in communicating growth factor and other signaling effects on the ER pathway.⁹⁰ Several key signaling kinases, including the mitogenic ERK1/2, the stress-related kinases I kappa B kinase,⁹¹ c-Jun NH2-terminal kinase (JNK), and the p38

MAPK,⁹² have all been suggested to phosphorylate members of the p160 SRC family of coactivators, and especially AIB1, which is often gene amplified or overexpressed in breast tumor cells. Phosphorylation of coactivators can augment their activity on ER-dependent transcription, even in the absence of ligand or in the presence of antiestrogens, by increasing their subcellular nuclear localization,⁹¹ their interaction with the ER, and their ability to recruit other transcriptional coregulators, such as the CBP/p300 coactivator, to the receptor-promoter complex.⁹⁰ In addition, phosphorylation may also directly activate intrinsic enzymatic activities of the coactivators such as acetyltransferase activity.⁹³ A recent identification of the phosphorylation sites on SRC-3⁹² has shown that multiple upstream signaling cascades phosphorylate different combinations of sites and that this may serve as a mechanism for SRC-3 to integrate and coordinate multiple kinase signaling cascades.

Like ER's coactivators, corepressor action on the ERs is also regulated and potentially negated by multiple signal transduction pathways.^{53,94} Direct phosphorylation of corepressors or of interacting components in the corepressor complex and consequent changes in their affinity for the cognate transcription factors and in their cellular distribution are mechanisms that might be responsible for the observed inhibition of corepressor action by growth factors and kinases.^{95,96}

MISS. In addition to ER acting as a nuclear transcription factor, growing evidence showing that estrogen can have rapid cellular effects that occur within minutes, long before its effects on gene transcription, suggests other mechanisms of action.⁹⁷ ER can be detected in or near the plasma membrane, where it can directly interact with and modulate several signaling pathways. This type of ER signaling is now referred to as MISS (Fig 4, pathway 4).

Several lines of evidence suggest that ER α does function outside the nucleus, perhaps in the membrane or cytoplasm, although the identity of the receptors mediating the MISS actions of steroid hormones still remains controversial. Non-nuclear localization of ER has been shown by biochemical fractionation of the membrane in cells that overexpress ER α ⁹⁸ and by direct visualization of membrane ER α using immunocytochemistry.⁹⁹ In addition, an alternative splicing/translational variant of ER α ^{100,101} that binds to the membrane may transduce the rapid estrogen signaling in some cells. Despite this evidence, membrane receptors distinct from the classical ERs may also be involved¹⁰² because rapid estrogen effects have been documented in several ER-negative cell lines,¹⁰³ although the relevance of this data to clinical breast cancer is suspect given the lack of effect of endocrine therapy in ER-negative tumors.

Cell culture studies have shown that ER α can interact at many levels of the IGF signaling pathway (Fig 5). It has previously been shown that estrogen can increase expression of IGF-I receptor (IGF-IR)¹⁰⁴ and insulin receptor substrate-1 (IRS-1)¹⁰⁵ and sensitize cells to IGFs and, conversely, that IGFs can phosphorylate and activate ER in a ligand-independent manner.¹⁰⁶ However, recent studies have shown numerous interactions between proximal IGF signaling intermediates and putative membrane or cytoplasmic ER (Fig 5). Estrogen stimulates association between ER and IGF-IR, and this results in activation of IGF-I signaling via ERK1/2.¹⁰⁷ Estrogen also stimulates association between ER α and the p85 subunit of phosphatidylinositol 3'-kinase (PI3K), resulting in subsequent PI3K activation.¹⁰⁸ Both of these interactions are inhibited by antiestrogens and do not occur with ER β . ER α has been shown to interact with the IGF-IR downstream signaling intermediates (IRS-1¹⁰⁹),

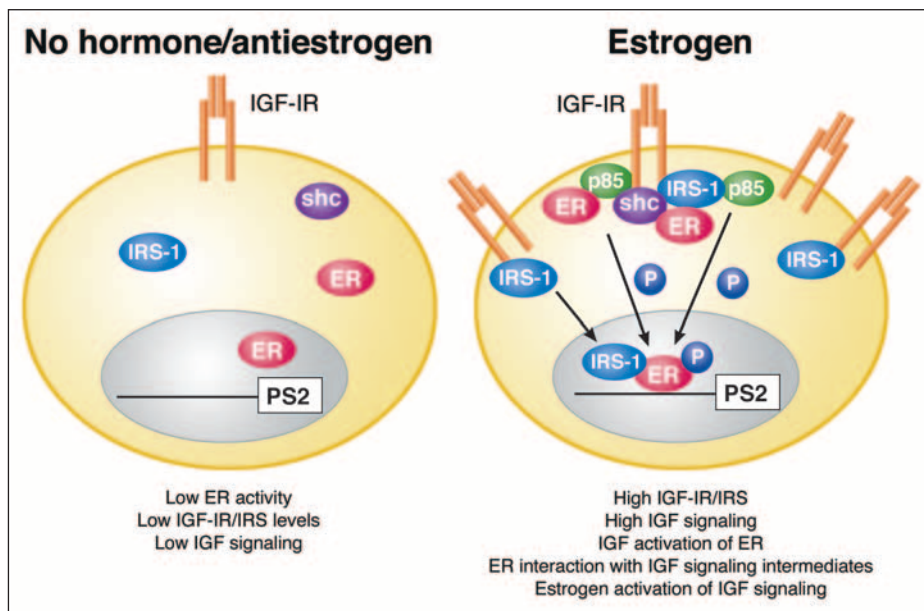


Fig 5. Interaction of estrogen receptor (ER) with the insulin-like growth factor (IGF) signaling pathway. Schematic showing the multiple levels of interaction between the ER and IGF signaling pathways. IGF-IR, insulin-like growth factor-I receptor; IRS-1, insulin receptor substrate-1; P, progesterone.

and on estrogen stimulation, IRS-1 translocates to the nucleus and can be found bound to ER on the pS2 promoter.¹¹⁰ ER is also able to interact with shc, which seems to be critical for the translocation of ER to the membrane. Importantly, reduction of shc expression inhibits formation of an ER/IGF-IR complex, and reduction of IGF-IR inhibits ER/shc association. Therefore, it is interesting to note that ER can bind all elements of the proximal IGF-IR signaling cascade, establishing a complex that may be activated by estrogen. The exact mechanism of action of this complex and the resulting functional significance are still to be deciphered.

Membrane ERs have also been shown to predominantly localize to discrete caveolar domains where they physically interact with the scaffold caveolin proteins¹¹¹ and act as G protein–coupled receptors.¹¹² G protein activation, via c-Src, leads to activation of matrix metalloproteinases, which in turn, cleave and liberate heparin-binding EGF. This free heparin-binding EGF, acting in an autocrine manner, is responsible for the activation and phosphorylation of EGFR and, subsequently, its downstream kinases ERK1/2 and PI3K. This molecular scenario is ER-dependent and is induced by estrogen and SERMs like tamoxifen, although it is blocked by the pure ER antagonist fulvestrant.¹¹² Interestingly, overexpression of the EGFR family member HER2/*neu* (HER2) has also been shown in experimental systems to potentiate MISS in response to both estrogen and tamoxifen.^{113,114} It is this ER activity that may be a critical element in blocking tamoxifen-induced apoptosis in HER2-overexpressing breast cancer cells,¹¹⁵ and it may contribute to increased estrogen activity of this SERM and then to tamoxifen-stimulated growth as a mechanism of resistance.¹¹³

A novel ER-interacting protein called MNAR/PELPI (standing for modulator of nongenomic activity of estrogen receptor¹¹⁶), which modulates both MISS and NISS activities of ER,^{117,118} was recently cloned. Similarly, the metastasis-associated genes (MTA) family of ER coregulators may also regulate MISS and NISS. MTA1 is a corepressor of genomic ER activity via traditional recruitment of histone deacetylases to ER transcriptional complexes.¹¹⁹ A

naturally occurring variant, MTA1s, not only downregulates nuclear ER activity but, by sequestering ER in the cytoplasm, also simultaneously increases ER cytoplasmic or membrane signaling.¹¹⁴ Interestingly, both forms of MTA1 have been recently identified as targets of growth factor signal transduction.¹²⁰

PR Is a Marker of a Functional ER

There are many theories to explain the generation of ER-positive/PR-negative breast tumors (Table 1). The simplest is that the ER is nonfunctional and unable to stimulate PR production and that the tumor is, therefore, no longer dependent on estrogen for growth and survival. However, some ER-positive/PR-negative breast cancers may simply result from low circulating levels of endogenous estrogens in postmenopausal women that are insufficient to induce PR expression even though the ER pathway is intact. Thus, brief treatment of patients with ER-positive/PR-negative tumors with estrogen can restore PR levels in some of the patients.¹²¹ Additionally, reduced response to tamoxifen may be a result of lower ER levels in ER-positive/PR-negative tumors because response has been shown to be directly related to ER levels.²⁸ However, as a group, patients with ER-positive/PR-negative tumors responded significantly better to estrogen withdrawal than to tamoxifen in the ATAC trial (Fig 3B), indicating that these tumors are also still estrogen dependent and that simple loss of ER function via low levels of estrogen or ER is not the whole explanation for resistance to tamoxifen.

Indeed, an increasing literature suggests that the non-functional ER theory is too simple on its own to completely explain the loss of PR in ER-positive breast cancer and that there must be multiple molecular mechanisms for this phenotype. For instance, a recent study of the transcriptional activity of ER in fresh breast tumor lysates showed that ER is transcriptionally active in some ER-positive/PR-negative breast tumors.¹²⁵ Furthermore, no mutations have been found in the ER DBD of a series of ER-positive/PR-negative tumors.^{126,127} A subset of ER-positive/PR-negative breast tumors do show an inability of the ER to bind DNA, and this

Table 1. Molecular Mechanisms to Explain the Loss of PR in Breast Tumors and Generation of the ER-Positive/PR-Negative Phenotype

Molecular Mechanisms for Loss of PR in Breast Tumors	Reference
Nonfunctional ER	Horwitz et al, ³⁸ Horwitz and McGuire ⁵⁷
Low circulating levels of estrogen	Bloom et al ¹²¹
Hypermethylation of PR promoter	Lapidus et al ¹²⁴
LOH at PR gene locus	Tomlinson et al, ¹²² Winqvist et al ¹²³
Growth factor downregulation of PR	Ciu et al ⁵⁰
SERM or growth factor-induced MISS activity of ER	Schiff et al ⁷⁸
Altered ER coregulator levels or activity	Torres-Arzayus ¹⁵³

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; LOH, loss of heterozygosity; SERM, selective estrogen receptor modulator; MISS, membrane-initiated steroid signaling.

inability to bind DNA may be related to oxidative stress within the tumor.¹²⁸ Although it is possible that there are mutations in other regions of ER or in ER coregulators important for transcriptional activation that could cause loss of ER function, studies to date have failed to find any that can fully explain loss of PR expression in the tumor.

Taken together, these data suggest that a nonfunctional ER is not sufficient to totally explain either the ER-positive/PR-negative phenotype or the reduced efficacy of tamoxifen in these tumors. Other regulatory mechanisms must be considered.

Hypermethylation of the PR Promoter or LOH at the PR Gene Locus

Ferguson et al¹²⁹ have shown that loss of ER in breast cancer is in part a result of hypermethylation and repression of the ER promoter, which prevents ER production. Similarly, the same group found that this suppressive form of epigenetic change may account for the loss of PR in some tumors.¹³⁰ Although no methylation of the PR promoter was found in ER-positive/PR-positive breast tumors, 21% to 40% of ER-positive/PR-negative tumors showed methylation at the PR promoter. Although a definitive cause and effect relationship has not been demonstrated between PR levels and the extent of methylation of the PR gene promoter, the finding in the clinical samples may provide another mechanism that could silence PR expression in ER-positive/PR-negative breast cancer.

Another potential mechanism for loss of PR in breast cancer emerged when two groups reported genetic loss (LOH) at the PR gene locus (chromosome 11q23).^{122, 123, 131} LOH results in a reduction of PR expression as a result of the loss of one copy of the gene and may, in part, account for the PR-negative status of the tumor. The studies indicated that this LOH occurs in approximately 40% of primary breast cancers and is associated with loss of PR protein expression. Importantly, however, both hypermethylation and LOH fail to explain why PR-negative or PR-low tumors are more resistant to SERM therapy than PR-positive tumors because the therapy targets ER and not PR function.

Growth Factor Pathway Downregulation of PR and SERM Resistance

Recent studies by our group and others suggest novel mechanisms for loss of PR expression in breast cancer.⁵⁰ These alternative mechanisms invoke molecular cross talk between ER and growth factor signaling pathways and the relationship between the classical and nonclassical effects of ER in breast cancer cells. On the basis of all emerging evidence, we propose here two alternative, although probably complementary, mechanisms to explain how tumors may become ER-positive/PR-negative and why this results in poor response to SERM therapy.

Growth factor-mediated downregulation of PR. Previous studies of breast cancer cell lines have implicated

growth factor signaling in repression of PR expression.¹³² We have shown that replenishment of ER in a specifically selected ER-negative/PR-negative MCF-7 breast cancer cell subline (C4-12) did not restore endogenous PR expression, although ER did restore estrogen induction of cyclin D1, IRS-1, and IGF-IR levels.¹³³ Interestingly, the C4-12 cells exhibit increased HER2 levels and heregulin signaling (Lee AV, unpublished data). Similarly, in antiestrogen-resistant MCF-7 cells generated by continuous culture of the ER-positive/PR-positive parental cells in antiestrogen-supplemented medium, EGF receptor signaling was enhanced, whereas PR levels were diminished.¹³⁴ Replacement of antiestrogen by estradiol failed to induce PR, whereas expression of other estrogen-responsive genes like *pS2* was significantly elevated. Coincidentally, these antiestrogen-resistant MCF-7 cells, like the C4-12 cells, were grown continuously in medium that was depleted of steroid hormones like estrogen but still retained polypeptide growth factors. Therefore, it is possible that long-term cell culture in growth factor-containing medium in the absence of estrogen might suppress PR expression to undetectable levels.

In a recent report, Konecny et al³⁹ performed in vitro studies comparing ER and PR levels in breast cancer cell lines transfected with HER2. In ZR-75 breast cancer cells, overexpression of HER2 caused PR levels to decrease by 500-fold to those levels normally observed in ER-negative cell lines, whereas ER levels only decreased by half. Moreover, PR expression also decreased significantly in T47D cells overexpressing HER2. Although ER was also reduced, the PR downregulation is probably directly mediated by HER2 signaling because PR expression in T47D breast cancer cells is reportedly independent of ER.¹³⁵ Taken together, these data suggest that growth factors downregulate PR levels independent of ER levels or activity in breast cancer cells, which is a mechanism further substantiated by results from clinical studies (discussed later).

These unresolved phenomena raise the question of how growth factors modulate PR expression. In exploring this issue, we found that short-term treatment (hours) with IGF-I, EGF, and heregulin all sharply lowered PR levels and progestin-induced PR activity in breast cancer cells.⁵⁰ This is in contrast to other estrogen-regulated genes, such as *pS2*, whose expression is increased by IGF-I.¹⁰⁶ The PI3K/Akt pathway was specifically involved in this growth factor downregulation of PR levels because inhibitors of this pathway could reverse the PR downregulation. In addition, we found that this downregulation of PR is not mediated via a reduction of ER levels or ER activity, suggesting that growth factor regulation of PR is independent of ER. The downregulation of PR protein levels occurred at the level of PR mRNA and was dependent on an intact PR promoter. A recent report suggests that AP-1 may be involved in repression of the PR promoter.¹³⁶ Further studies are needed to fully understand how growth factors inhibit PR promoter activity (Fig 6).

Thus, PR levels may reflect growth factor activity within a tumor. Low or absent PR expression in some tumors indicates high IGF-IR, EGFR, and HER2 activity, and this growth factor action on PR is independent of ER function or levels. As mentioned previously, this increased growth factor signaling might even potentiate NISS on some gene promoters, while PR, at the same time, is repressed. In addition, high growth factor signaling may reduce the ability of tamoxifen to act as an antagonist, resulting in SERM resistance.

Growth factors potentiate nonclassical ER signaling. In addition to direct downregulation of PR mRNA levels by growth factors (see previous section), experimental evidence also suggests that hyperactive growth factor signaling can perturb the classical estrogen-dependent ER function by either stimulating ligand-independent activation or shifting the ER activity in the tumor cells to act predominantly via MISS or other nonclassical modes of signaling (Fig 6). In both of these instances, growth factors may alter the set of genes that are normally regulated by estrogen. PR may be a classic example of such a gene.

Several studies have shown that growth factors can directly modulate ER activity independent of estrogen via phosphorylation of ER itself or via phosphorylation of co-regulators. This has been shown to modulate the ability of SERMs to act as agonists and antagonists. The effect of ligand-independent activation of ER on target gene expression has not been extensively studied. However, it was shown that mutation of ser305 in ER to a glutamic acid (to mimic a phosphorylation site) did result in selective induction of estrogen-responsive finger protein and cyclin D1, suggesting that ligand-independent activation of ER may induce different patterns of gene expression compared with estrogen induction of ER activity.¹³⁷

Data from several preclinical models suggest that both de novo and acquired resistance to tamoxifen is often asso-

ciated with increased signaling from the EGFR/HER2 pathway¹³⁸ and a switch from NISS to MISS ER activity.^{78,114} Importantly, although human breast cancer xenografts that overexpress HER2 are stimulated by tamoxifen as a mechanism of de novo resistance, these tumors are still completely inhibited by estrogen withdrawal.¹¹³ We recently showed that, in these tumors, both estrogen and tamoxifen can induce a rapid (but not transient) phosphorylation and thereby activation of both EGFR and HER2.¹¹³ The increased MISS ER activity and the resultant increase in tamoxifen agonist activity in these HER2-overexpressing cells is entirely dependent on ER cross talk with the growth factor signaling pathway because both phenomena were completely reversed in the presence of specific EGFR/HER2 inhibitors.^{113,139} Thus, in this model, estrogen and tamoxifen may actually decrease PR levels by MISS activation of growth factor signaling (Fig 6). Kumar et al¹¹⁴ and Mazumdar et al^{114,140} have reported that increased HER2 activity results in more ER outside the nucleus, and they have other evidence that classical ER-dependent transcription is inhibited by a mechanism that is HER2 dependent and involves ER sequestration in the cytoplasm via the ER corepressor MTA1s. High levels of membrane and cytoplasmic ER have also been found in breast cancer cells amplified for the *HER2* gene.¹¹⁵ In these cells, a direct interaction between ER and HER2 in the cell membrane is associated with resistance to tamoxifen-induced apoptosis.¹¹⁵ Whether this change in ER localization in the cell also occurs in human tumors requires more study.

Acquired resistance to both long-term estrogen deprivation and tamoxifen may also be associated with a shift from NISS to MISS ER activity. We and others have shown that tumors that acquire resistance to estrogen withdrawal become hypersensitive to estrogen as a mechanism of resistance and that acquired tamoxifen resistance is a result of a

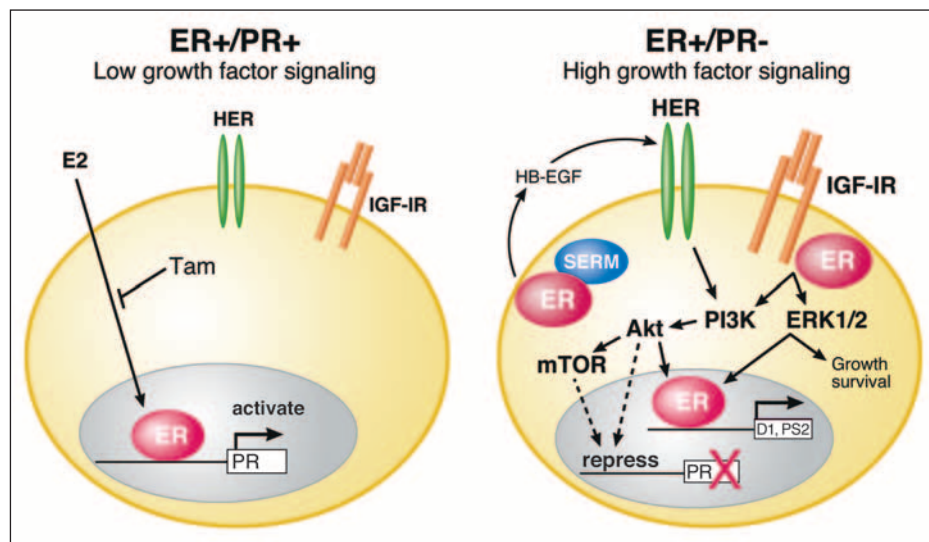


Fig 6. Growth factor reduction of progesterone receptor (PR) via direct inhibition of PR gene transcription and induction of membrane-initiated steroid signaling estrogen receptor (ER) signaling. Schematic illustrating the ability of growth factor signaling to downregulate PR levels. HER, human epidermal growth factor receptor; IGF-IR, insulin-like growth factor-I receptor; Tam, tamoxifen; E2, estradiol; HB-EGF, heparin-binding epidermal growth factor; SERM, selective estrogen receptor modulator; PI3K, phosphatidylinositol 3'-kinase; ERK1/2, extracellular regulated kinase 1/2; mTOR, mammalian target of rapamycin.

switch to tamoxifen-stimulated growth.¹⁴¹⁻¹⁴³ The resistant tumors continue to express high levels of ER and can still be inhibited by the SERD fulvestrant (ICI 182,780). However, the enhanced response to the agonistic effects of both estrogen and tamoxifen does not occur at the level of ER-mediated transcription of classical ER-dependent genes.^{134,144} Expression of classically estrogen-dependent genes, including PR, is in fact reduced in the tamoxifen-resistant tumor cells. Resistance in these models is associated with upregulation of growth factor receptors (EGFR and/or HER2) and/or their downstream signaling pathways.^{141,142,145} Together, the experimental data suggest that at least some scenarios of acquired resistance to estrogen deprivation and tamoxifen are predominantly driven by the MISS activity of the ER.

The underlying mechanisms responsible for the change in the balance between MISS and NISS ER activities are largely unknown. An increase in levels of cytoplasmic and/or membrane ER can lead to hyperactivation of MISS ER activity.^{110,146} This increased non-nuclear ER may result from an increase in total cellular ER protein, as found for example in tumors resistant to estrogen deprivation,¹⁴⁷ or from an active cytoplasmic ER-sequestering mechanism.¹⁴⁰ Cytoplasmic sequestration of ER would then reduce its genomic nuclear activity. In addition, increased levels of growth factor receptors, as well as other signaling molecules that interact with the ER and mediate its nongenomic signaling, might also directly enhance the nongenomic ER activity. ER and many of its coregulatory proteins, such as AIB1, are phosphorylated by a number of kinases at distinct amino acid residues in the proteins. It is also possible that phosphorylation at these specific sites could modulate the MISS and NISS activity of the ER.

Therefore, in tumors in which MISS activity is the dominant mode of ER signaling, estrogen regulation of tumor cell proliferation and survival may no longer rely primarily on ER acting as a transcription factor for the classical set of estrogen-dependent genes. Instead, estrogen or tamoxifen binds ER and, via its MISS functions, activates growth factor receptors and their downstream signaling molecules. Such tumors, if analyzed based on their classical ER-dependent gene patterns (eg, PR status), may portray an expression profile that will misclassify them as tumors with a nonfunctional ER (eg, ER-positive/PR-negative). Furthermore, because MISS signaling may originate from ER molecules confined mainly to the membrane and the cytoplasm, some of these tumors could even be mistakenly classified as ER negative by immunostaining.¹¹⁴ More sensitive methods for detecting and quantifying this non-nuclear pool of ER are needed to address these questions.

Mechanistically, however, these tumors with high ER MISS activity should still be highly dependent on estrogen and, therefore, should still be highly sensitive to estrogen

withdrawal therapy. In contrast, SERMs like tamoxifen stimulate at least some of the MISS actions of ER including activation of growth factor receptors and downstream effectors such as Akt and ERK1/2.¹⁴⁸ The sensitivity of these tumors to SERMs may differ substantially from tumors with low growth factor activity in which ER functions primarily as a nuclear transcription factor and in which tamoxifen would be expected to function as an antagonist to block growth. The switch from predominant NISS ER activity to MISS ER activity could also explain the ER-positive/PR-negative phenotype in tumors with active growth factor signaling pathways and why aromatase inhibitors are superior to tamoxifen in such patients.³⁷

CLINICAL IMPLICATIONS OF GROWTH FACTOR REGULATION OF PR LEVELS

PR As a Predictive Marker of Both ER and Growth Factor Action

In theory, PR may be a better indicator than ER for predicting response to SERM therapy because levels of PR reflect the combined and integrated effects of ER and growth factor activity. As stated earlier, several growth factor pathways can activate ER, and ER, in turn, can potentiate these same growth factor signaling pathways.⁷⁴ Interestingly, the trimeric G protein and integrin pathways, which are also capable of triggering PI3K/Akt activation, are also involved in breast cancer development and can cross talk with ER and growth factor signaling. Thus, more precisely speaking, PR may serve as an indicator for intracellular kinase networking elicited by overall extracellular stimulations.

Although there are many ER-regulated genes, PR is unique from the perspective of its distinctive mode of regulation by estrogen and growth factors. Cyclin D1, for example, is induced by estrogen, IGF, and EGF.^{112,149,150} Another well-known estrogen-induced gene, *IRS-1*, is decreased by IGF-I¹⁵¹ but increased by EGF.¹⁵² Hence, PR may be an ideal marker as a readout of both ER and growth factor action and, thus, may aid in the prediction of response to various hormone or growth factor receptor-inhibitor therapies, with the caveat that other mechanisms for PR loss reviewed earlier and false-negative PR assays as a result of low circulating estrogen levels in some older women need to be considered. A 24- to 48-hour treatment with estrogen before biopsy to induce PR levels may help to identify this group.

PR measurement is certainly useful, but it is clear that new methodologies and assays need to be developed that measure the new activities of ER, such as MISS. Currently, ER positivity is scored by pathologists based on nuclear staining (using antibodies optimized to detect only nuclear

ER), a situation that may result in misclassification of tumors that have low levels of cytoplasmic or membrane ER staining that can be detected with other antibodies. In addition, the realization that ER controls a major network of genes suggests that a more comprehensive analysis of the ER-controlled transcriptome may yield more beneficial information than simply ER and PR alone. Expression array profiling of these tumors may help to subclassify patients for treatment purposes.

Treatment of ER-Positive/PR-Negative Tumors by Estrogen Withdrawal, SERDs, or Combined SERM and Antigrowth Factor Therapy

Laboratory and clinical evidence indicate that SERMs may be less effective in the presence of high growth factor signaling. In this situation, growth factor–mediated phosphorylation of ER and its coregulators can cause SERMs to show full agonist activity, stimulating tumor growth via NISS, MISS, or both forms of ER activity. In this situation, the most effective way to block ER activity is to fully withdraw any ligand from the ER, rather than by providing a SERM that is seen as an agonist. Therefore, ER-positive/PR-negative tumors, which may show high growth factor signaling, may best be treated by estrogen withdrawal using an aromatase inhibitor, as seen in the ATAC trial (Fig 3). Another effective treatment for these tumors may be the targeted degradation of ER via SERDs. Finally, the combined use of SERMs and antigrowth factor therapies may be beneficial. Although this has yet to be proven in clinical trials, recent preclinical data suggest that the combination of a SERM and an EGFR/HER2 inhibitor is beneficial in ER-positive HER2-overexpressing breast cancer cells.^{113,138} In this situation, ER and growth factor cross talk results in tamoxifen resistance, with tamoxifen exhibiting estrogenic agonist activity and stimulation of tumor growth. However, treatment with an EGFR or HER2 inhibitor restores the antagonist action of tamoxifen. After the excitement of these preclinical studies, several clinical trials are now underway combining growth factor receptor tyrosine kinase inhibitors and antibody antagonists with SERMs or aromatase inhibitors.

One extension of our studies suggests that ER-positive/PR-positive tumors must not exhibit strong growth factor signaling. This would imply that inhibitors of growth factor signaling cascades will show little benefit in patients with these tumors and will only work effectively in tumors that are PR negative (and maybe only a subset of these tumors)

and if administered in combination with endocrine therapies to block ER-related growth signals. Combinations of a SERM plus a growth factor pathway inhibitor might still be considered for ER-positive/PR-positive tumors because, during treatment, there may be an increase in growth factor activity as a mechanism of resistance, and subsequent loss of PR may occur in some of these tumors that are not completely eradicated. It is tempting to speculate that adjuvant tamoxifen for several years followed by an aromatase inhibitor to treat resistant cells or initial therapy with tamoxifen combined with a growth factor inhibitor might be better than an initial use of an aromatase inhibitor in ER-positive/PR-positive tumors. In contrast, for ER-positive/PR-negative tumors, initial treatment with an aromatase inhibitor may be most favorable. Careful clinical trial design and tissue collections for future molecular studies are needed to address these questions.

SUMMARY

Tremendous progress has been made in the treatment of breast cancer. A wealth of knowledge acquired in the last decade in the field of molecular endocrinology and tumor biology has advanced our understanding of the mechanisms of ER action and has led to substantial improvements in the efficacy of cancer treatment and prevention. However, there remains the challenge of developing treatments that are as effective against metastatic breast cancer as they are now for early-stage breast cancer and that will prevent or overcome resistance to endocrine therapy. Loss of PR may be a marker of aberrant growth factor signaling and, consequently, one mechanism for antiestrogen resistance. We anticipate that ER-positive/PR-negative breast cancer probably relies on more than one mechanism for its aggressive phenotype, and understanding why PR-negative tumors respond poorly to endocrine treatment with SERMs could pave the way for the development of better therapeutic strategies.

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Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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