

## Estrogen-Receptor Biology: Continuing Progress and Therapeutic Implications

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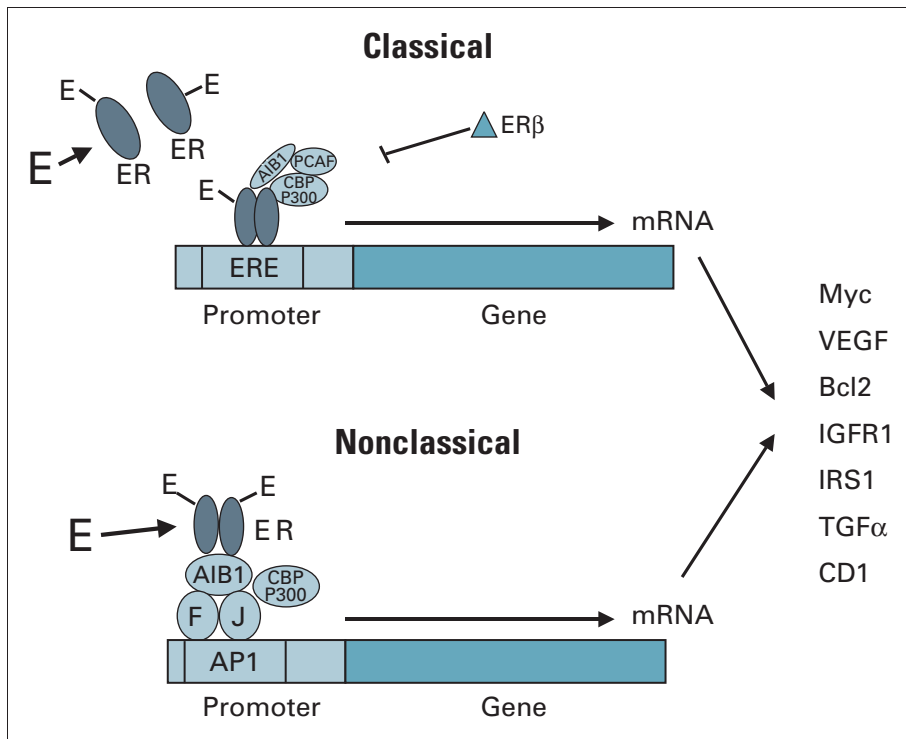
### INTRODUCTION

Endocrine therapies, first used more than 100 years ago, are the most effective treatments for breast cancers expressing the estrogen receptor (ER). All endocrine therapies are designed to block ER function in some way, thereby making them the first targeted therapies used for cancer. Selective ER modulators (SERMs) such as tamoxifen bind ER and partially block its activity. Ovarian ablation, luteinizing hormone-releasing hormone agonists, and aromatase inhibitors reduce the level of estrogen and inhibit ligand-induced activation of ER. Steroidal antiestrogens such as fulvestrant bind ER, more completely block its function, and induce receptor degradation. While all of these therapies are effective in certain patients, *de novo* and acquired resistance remain major problems. New information on the biology of ER provides insight into the mechanisms of treatment resistance and new strategies to overcome it, thereby potentially making these therapies even more effective.

### ER STRUCTURE AND ITS CLASSICAL (GENOMIC) ACTIVITY

There are two ERs, ER $\alpha$  and ER $\beta$ , products of different genes.<sup>1,2</sup> These receptors belong to a super family of nuclear hormone receptors including those for other steroid hormones, thyroid hormone, vitamin D, and retinoic acid. Classically, these receptor proteins function as transcription factors in the nucleus when they are bound to their respective ligands.<sup>3</sup> ER $\alpha$  and ER $\beta$  have similar, although not identical, structure.<sup>4</sup> ER $\alpha$  con-

tains a DNA-binding domain, a dimerization domain, a hormone-binding domain, and several transcription activating domains. Hormone binding to ER $\alpha$  activates the protein through phosphorylation, dissociates chaperonin proteins such as heat-shock protein 90, and alters its conformation. Several kinases in the growth factor signaling networks can also activate ER and its coregulatory proteins, a process termed ligand-independent activation.<sup>3,4</sup> Hormone-bound ("activated") ER then dimerizes with another receptor, and the dimer binds to estrogen response elements (specific DNA sequences) present in the promoter of estrogen-responsive genes. Promoter-bound ER dimers form a complex with coregulatory proteins such as amplified in breast cancer 1 (AIB1 or SRC3) that coordinately act to influence the transcription of estrogen-responsive genes (Fig 1).<sup>5</sup> Transcription of many genes is increased by estrogen, while transcription of many others is inhibited.<sup>6</sup> The ability of estrogen to downregulate expression of certain genes may be explained by recruitment of corepressor proteins to the ER complex in the context of certain gene promoter sequences. Many of the genes regulated by estrogen are important for cell proliferation, inhibition of apoptosis, stimulation of invasion and metastasis, and promotion of angiogenesis. Although much less is known about ER $\beta$ , it probably has functions which are distinct from ER $\alpha$ , and it seems to have opposing activity on tumor growth.<sup>4,7,8</sup> High levels of ER $\beta$  may help to inhibit tumor growth when the receptors



**Fig 1.** Nuclear initiated estrogen signaling, classical (top) and nonclassical (bottom). Top: estrogen (E) binds estrogen receptor (ER), induces dimerization of the protein, and activates DNA binding to estrogen response elements (ERE) in the promoter of target genes. Coactivator proteins (AIB1, CBP/P300, PCAF, others) are recruited to the complex and gene transcription is activated (classical). ER $\beta$  may antagonize the activity of ER $\alpha$ . Bottom: estrogen-bound ER brings coactivator complexes to other transcription factors such as Fos (F) and Jun (J) to activate gene transcription at other promoter sites such as AP-1. Downregulation of gene expression by estrogen results from the recruitment of corepressors to specific promoters. Proteins encoded by these genes include: VEGF, vascular endothelial growth factor; IGFR1, insulin-like growth factor receptor 1; IRS1, insulin receptor substrate 1; TGF $\alpha$ , transforming growth factor alpha.

are bound by tamoxifen, and studies of clinical samples suggest that ER $\alpha$ -expressing tumors with low levels of ER $\beta$  tend to be tamoxifen resistant.<sup>9,10</sup>

The coregulatory proteins bound to ER on the promoter of target genes may be just as important as the receptor itself in mediating transcriptional activity (Fig 1). Some of these proteins are coactivators that enhance transcriptional activity; others function as corepressors to inhibit this activity.<sup>11,12</sup> Typically, coactivators bind ER when the receptor is bound by estrogen, while corepressors bind when ER is bound by tamoxifen. As described above, corepressors may also bind estrogen-liganded ER on some promoters. Coactivators such as AIB1 (SRC3) recruit acetyltransferases to the promoter site, which help to unwind the DNA, allowing gene transcription to occur.<sup>4,11,12</sup> Reducing the level of AIB1, for instance, significantly impedes ER-mediated effects, not only on gene transcription, but also on tumor growth in experimental models.<sup>13</sup> AIB1 is overexpressed relative to normal ductal epithelium in 65% of breast cancers and is gene amplified in 5%, suggesting an important role in breast cancer development and progression.<sup>14,15</sup> High levels of this protein may also contribute to SERM resistance by enhancing the estrogen agonist activity of these drugs.<sup>16-18</sup> Under specific conditions, such as high HER-2 activity, ER bound by tamoxifen may complex with coactivator proteins such as AIB1 rather than corepressor proteins, resulting in increased estrogen agonist activity of tamoxifen.<sup>18</sup> This estrogen-like activity of tamoxifen not only may contribute to certain types of treatment resistance, but it also

may explain why SERMs can function as antagonists in breast tissue, and also as agonists in the uterus, bone, and cardiovascular system.<sup>19,20</sup> The transcriptional effects of ER on estrogen-regulated genes containing an estrogen response element (ERE) in their promoters have been labeled as genomic activity or nuclear initiated steroid signaling.<sup>21</sup> However, ER has also been shown to regulate gene transcription in other ways.

#### NONCLASSICAL TRANSCRIPTIONAL REGULATION BY ER

ERs have also been shown to modulate gene expression at alternative regulatory DNA sequences such as AP-1, SP-1, and upstream stimulatory factor sites, as well as other poorly defined non-ERE sites.<sup>22-25</sup> In this circumstance, ER does not function as the major transcription factor but rather is tethered to the specific promoter complex by its interaction with other DNA-bound transcription factors such as c-jun or c-fos, or with other coactivator proteins (Fig 1). In this way, ERs can themselves function as coactivator proteins by stabilizing the DNA binding of the transcription factor complex or by recruiting other coactivators to these complexes. Transcription of several genes important in growth factor signal transduction pathways is regulated in this way.<sup>22,26-28</sup> Proteins encoded by these genes include insulin-like growth factor receptor 1, cyclin D1, myc, and the antiapoptosis factor Bcl-2. Although the relative importance for tumor growth in vivo of this alternative transcriptional process is not clear, recent laboratory studies suggest that it may play a major

role in estrogen-mediated breast cancer cell proliferation and survival.<sup>29</sup> These alternative ER signaling pathways, particularly at AP-1 sites, may also be an important contributor to the onset of hormone therapy resistance in breast cancer, especially to SERMs like tamoxifen. An increase in the level of activated jun N-terminal kinase and phosphorylated c-jun together with increased levels of AP-1 transcriptional activity have been identified in pre-clinical models of tamoxifen resistance and in tumors from patients.<sup>30,31</sup>

### NONGENOMIC ER ACTIVITY

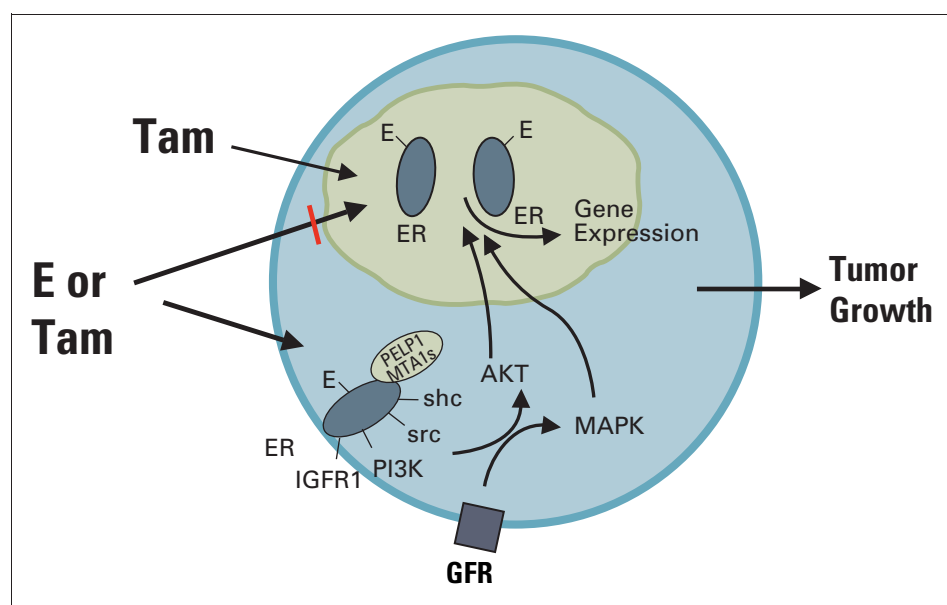
Sixty years ago it was reported that steroid hormones might have very rapid action on cells, too rapid to invoke transcriptional mechanisms.<sup>32,33</sup> Binding sites for estrogen were identified in the membrane of endometrial cells that triggered the induction of cyclic AMP.<sup>33</sup> Later studies have also argued for the presence of ERs outside the nucleus that can mediate rapid signals originating from the membrane or in the cytoplasm.<sup>34</sup> This nongenomic ER action or membrane-initiated steroid signaling (MISS) occurs within minutes of the addition of estrogen. SERMs such as tamoxifen may also activate membrane ER. These receptors have been found in bone, neural, uterine, fat, and endothelial cells.<sup>35</sup>

A precise cellular localization of these nongenomic ERs and the mechanisms by which they signal is still somewhat controversial (Figs 2 and 3). Nevertheless, many studies, using a variety of techniques including confocal microscopy, suggest that a small pool of ERs is located in the plasma membrane and cytoplasm.<sup>36-38</sup> Full length ER, an alternatively spliced truncated form of ER, and other membrane receptors distinct from classic ER have

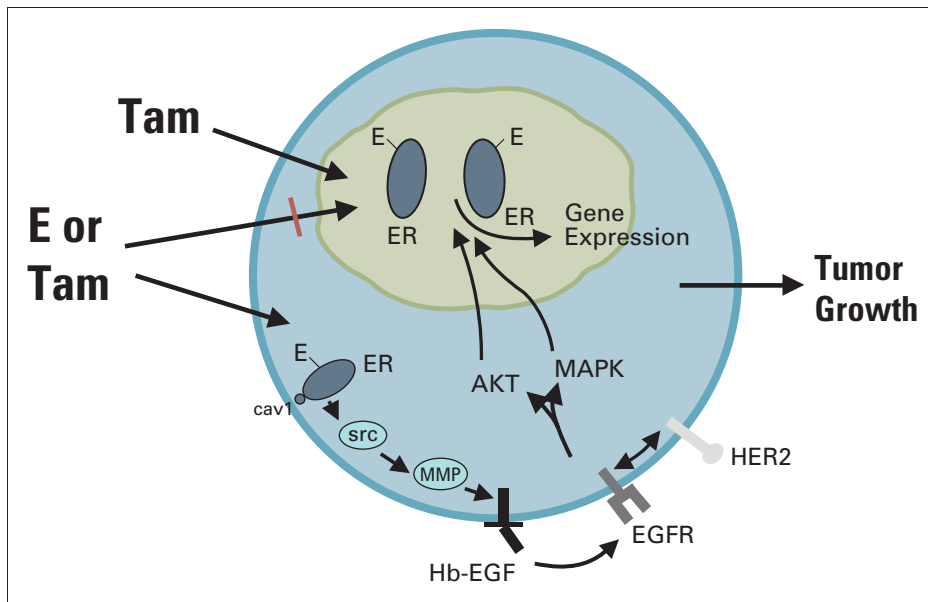
been implicated in several studies.<sup>36-39</sup> The MISS activity of ER results in activation of growth factor receptors, cellular tyrosine kinases, mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3 kinase, and Akt (protein kinase B) -signaling enzymes and adaptors such as adenyl cyclases and Shc.<sup>40</sup>

Mechanisms by which estrogen activates membrane ER function are beginning to be clarified. Direct interactions between ER $\alpha$  have been observed with a variety of membrane-signaling molecules including the insulin-like growth factor 1 receptor, the p85 regulatory subunit of PI3K, Src, and Shc, a protein which may directly couple ER to a variety of growth factor tyrosine kinase receptors (Fig 2).<sup>41-43</sup> Activation of these pathways by estrogen sends powerful cell survival and cell proliferative signals via activation of Akt and MAPK. In addition, these kinases can phosphorylate ER and its coregulators to augment nuclear ER signaling. Phosphorylation of these proteins can also increase the estrogen agonist-like activity of tamoxifen and other SERMs.<sup>18</sup>

Another potential mechanism for the MISS activity of ER has been well studied and involves indirect activation of the epidermal growth factor receptor (EGFR; Fig 3).<sup>38,44</sup> ER bound to caveolin 1 in the cell membrane acts as a G-protein-coupled receptor in response to estrogen- or tamoxifen-binding to directly or indirectly interact with and activate specific G proteins. The subsequent activation of c-Src rapidly activates matrix metalloproteinases, which then cleave heparin-binding epidermal growth factor (EGF) from the membrane. This form of EGF then binds to surface EGFR in an autocrine or paracrine manner to activate the receptor and its downstream kinases including ERK 1/2 MAPK and Akt. The pure antiestrogen



**Fig 2.** Membrane-initiated estrogen signaling. Estrogen (E) activates nuclear estrogen receptor (ER) and ER in or near the membrane. Tamoxifen (Tam) antagonizes nuclear activity but activates membrane ER. Membrane ER binds to growth factor signaling elements such as insulin-like growth factor receptor 1 (IGFR1), the p85 subunit of phosphatidylinositol 3 kinase (PI3K), Src, and Shc. Proteins like PELP1 or MTA1s bind ER and sequester it in the cytoplasm to increase membrane activity. Estrogen then activates growth factor signaling just like a growth factor binding to its membrane receptor (GFR), which then activates key molecules such as Akt or mitogen-activated protein kinase (MAPK). These kinases can phosphorylate and activate ER and its coregulators to enhance nuclear effects on transcription.



**Fig 3.** Binding of estrogen (E) or tamoxifen (Tam) to membrane estrogen receptor (ER) activates Src, which then activates matrix metalloproteinases (MMP), which in turn cleave heparin-binding epidermal growth factor (Hb-EGF) from the membrane. Hb-EGF activates adjacent EGF receptors (EGFRs). Dimerization of EGFR with another receptor or with HER-2 activates signaling through Akt and mitogen-activated protein kinase (MAPK). These kinases then enhance nuclear ER signaling. cav1, caveolin-1.

fulvestrant does not activate membrane ER in this way; however, SERMs such as tamoxifen do activate membrane ER in a manner similar to estrogen.<sup>18,45</sup> The membrane effects of ER, like its genomic activity, may be cell, receptor subtype, and ligand specific, and it may also be influenced by the growth factor signaling milieu being much more prominent, for instance, in breast cancers overexpressing EGFR or HER-2.<sup>18</sup> Stimulation of the MISS activity of ER by tamoxifen and other SERMs may, in part, explain the resistance to these agents sometimes observed in HER-2-overexpressing tumors.<sup>18,46,47</sup>

Other proteins may also play a role in the MISS activity of ER. MNAR/PELP1 (modulator of nongenomic activity of the estrogen receptor) modulates both the genomic and membrane effects of ER (Fig 2).<sup>48</sup> This protein may help to sequester ER in the cytoplasm/membrane and may be an important linker to Src. These ER-interacting proteins have also been reported to bind pRb in an estrogen-dependent manner.<sup>49</sup> This interaction enhances progression of breast cancer cells into S phase and may explain why overexpression of this protein has prognostic significance in breast tumors.<sup>48</sup> Another protein in the metastasis-associated gene family (MTA1) functions as an ER coregulator.<sup>50</sup> MTA1 is a corepressor of genomic activity of ER. Its naturally occurring variant, MTA1s, downregulates nuclear ER activity by sequestering the protein in the cytoplasm. This trapping of ER in the cytoplasm increases its nongenomic activity by facilitating interactions with membrane components.

In summary, considerable data now indicate that ER has at least two major functions. It serves as a transcription factor for estrogen-regulated genes and a coactivator for other transcription factors in the nucleus, and it functions

outside the nucleus and in the plasma membrane to activate growth factor signaling. In some breast tumors, particularly those with highly active growth factor signaling pathways such as HER-2 amplification, a vicious cycle is established in which estrogen activates growth factor signaling and the growth factor signaling pathway further activates ER. Estrogen in such tumors would be expected to be a dominant factor by activating multiple pathways important in tumor progression. This molecular crosstalk has important implications for the treatment of breast cancer. As an example, estrogen-deprivation therapy with aromatase inhibitors should be more effective than SERMs in HER-2 amplified tumors by shutting off both the nuclear-initiated steroid signaling and MISS activities of ER.

#### IMPLICATIONS OF GROWTH FACTOR RECEPTOR/ER CROSSTALK IN RESISTANCE TO ENDOCRINE THERAPY

Overexpression of EGFR and its family member HER-2 potentiates the nongenomic ER activity in response to both estrogen and tamoxifen.<sup>18</sup> While membrane ER can activate HER-2 signaling, the kinase cascade downstream of HER-2 can phosphorylate and activate ER and its coregulatory proteins.<sup>18</sup> ER is known to be activated by a variety of kinases in the growth factor pathway including ERK 1/2 and p38 MAPKs, cyclin-dependent kinase 2, cyclin-dependent kinase 7, c-Src, protein kinase A, pp90rsk1, and Akt.<sup>4</sup> Phosphorylation at many of these specific sites on the protein enhances the transcriptional activity of ER even when bound by tamoxifen, and, therefore, it may play a role in endocrine therapy resistance.<sup>18,51</sup> Phosphorylation of ER coactivators and corepressors is also functionally important. Phosphorylation

of the corepressor N-CoR causes the protein to exit the nucleus, making it unavailable to repress ER transcriptional activity.<sup>52</sup> Phosphorylation of coactivators such as AIB1 (SRC3) increases ER-dependent transcription.<sup>18,51,52</sup>

As a result, a potent transcriptional coactivator complex is formed, which in some model systems can convert tamoxifen-bound ER into an estrogen agonist rather than an antagonist.<sup>18</sup> Similar to ER, a variety of signaling kinases can phosphorylate coactivators such as AIB1.<sup>4,53</sup> It is not surprising that increased signaling from these kinases is associated with de novo or acquired hormone resistance in breast cancer given the profound modulation of ER function that they cause.<sup>18,46</sup>

Compelling clinical and experimental evidence suggest that increased expression of EGFR and/or HER-2 is associated with a poor response to tamoxifen.<sup>18,54-56</sup> In addition, data from experimental models suggest that resistance to estrogen-deprivation therapy such as treatment with an aromatase inhibitor often occurs through an adaptation to an estrogen hypersensitive phenotype, which may involve activation of the ERK 1/2 MAPK pathway.<sup>57</sup> Initially, however, aromatase inhibitors would be expected to be better than tamoxifen in tumors overexpressing growth factor receptors, since the aromatase inhibitor reduces ligand activation of both the nuclear and membrane ER while tamoxifen activates its membrane activity. Several clinical studies now support this idea and have demonstrated a superiority of aromatase inhibitors over tamoxifen in this setting (Table 1).<sup>58-60</sup>

Additional clinical evidence for a role for this receptor crosstalk in the induction of hormone treatment resistance comes from a recent study that demonstrated poor disease-free survival for patients receiving adjuvant tamoxifen, whose tumors express high levels of both

**Table 1.** Tumor Response to Neoadjuvant Aromatase Inhibitor or Tamoxifen As a Function of HER-2 Status

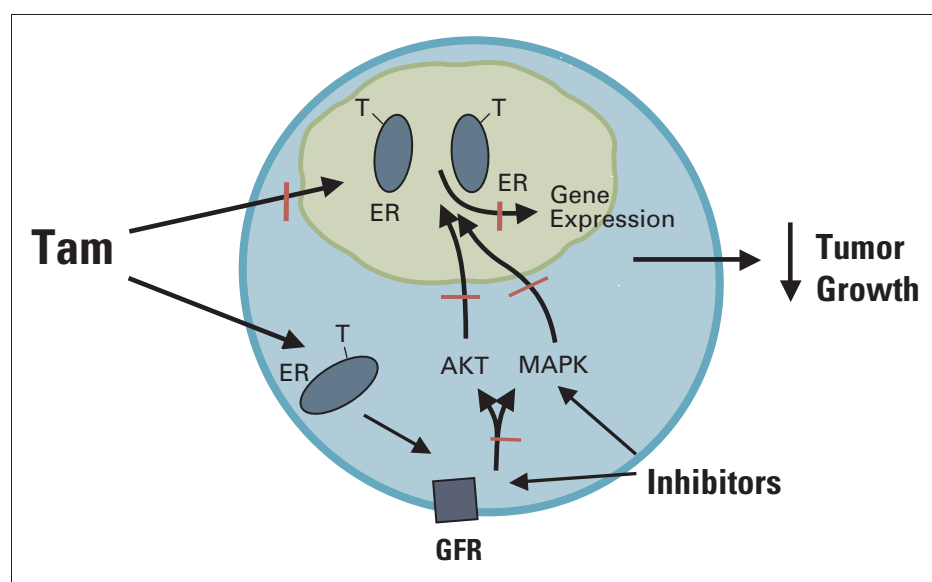
Study	Response Rate				
	AI		Tam		
	No. of Patients	%	No. of Patients	%	
Ellis <sup>59</sup>	ER+/HER-2+	15/17	88	4/19	21
	ER+/HER-2-	55/101	59	42/100	42
Smith <sup>58*</sup>	ER+/HER-2+	7/12	58	6/22	27
	ER+/HER-2-	28/68	41	64/137	47
Zhu <sup>60</sup>	ER+/HER-2+	12/16	75	ND	ND
	ER+/HER-2-	7/20	35	ND	ND

Abbreviations: AI, aromatase inhibitor; Tam, tamoxifen; ER, estrogen receptor; ND, not determined.

\*Tam and AI plus tam groups combined.

HER-2 and the ER coactivator AIB1.<sup>46</sup> Poor outcome was not observed when only one of the two proteins was overexpressed, indicating an interaction between them. AIB1 is phosphorylated by kinases in the HER-2 pathway. Furthermore, AIB1 and HER-2 are often overexpressed together in breast cancer, and laboratory evidence suggests that these signaling molecules via the molecular mechanisms described above would significantly reduce the estrogen antagonist activity of tamoxifen. This finding needs confirmation in tissues from a larger randomized trial to more fully assess the potential clinical significance. Meanwhile, disrupting the interaction between ER coactivators and ER itself, or blocking their activation, offers potential new treatment strategies.

Although ER was first identified more than 30 years ago, we are still trying to clarify and understand its multiple roles in normal physiology and in disease. In breast



**Fig 4.** Combination therapy with tamoxifen (Tam; or other endocrine therapy) to block estrogen receptor (ER) and a growth factor inhibitor (receptor tyrosine kinase inhibitor, blocking antibody or downstream inhibitor) to block the stimulatory effect of Tam on the membrane ER to activate growth factor signaling. This combined therapy blocks two key pathways (ER and growth factor receptor [GFR]) and restores Tam antagonist activity.<sup>18</sup> MAPK, mitogen-activated protein kinase.

cancer there is convincing evidence that ER does not act alone to stimulate tumor growth; rather, a complex interacting network operates to ensure the viability of the cancer cells. Understanding this network will offer therapeutic advantages. If the crosstalk between ER and growth factor receptor pathways is the cause of endocrine therapy resistance in some patients, then ER-targeted therapies combined with growth factor receptor inhibitors or inhibitors of more downstream kinases is a novel strategy worth investigating (Fig 4). Preclinical models demonstrate that growth factor receptor tyrosine kinase inhibitors or anti-receptor antibodies can restore tamoxifen's antagonist activity in HER-2 overexpressing breast cancer cells.<sup>18,47</sup> Preliminary data suggest that they can also delay acquired resistance to estrogen-deprivation therapy in such tumors.<sup>61</sup> Clinical trials are underway to test these strategies

in patients. Other clinical trials are also needed to evaluate various signaling elements from the multiple networks that crosstalk with and modulate ER activity as predictive markers for initial hormonal therapy. In the future, a molecular profile of these various components in a given patient's tumor immediately before treatment might permit the individualization of both the initial type of hormonal therapy and the appropriate signaling inhibitor needed to block de novo or acquired resistance, a strategy that should improve the treatment of this disease.

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