In this issue of Clinical Cancer Research, Bayliss et al. (1) report that inhibiting inherent p42/44 mitogen-activated protein kinase (MAPK) activity in estrogen receptor (ER) α–negative breast cancer established cell lines, and in ex vivo tissue and primary cultures of human ER-negative breast tumors, frequently results in re-expression of ER as well as in recovery of tumor cell responsiveness to antiestrogen treatment. The authors have previously shown that in the ER-positive MCF7 cell line, inducing hyperactive MAPK by genetically engineered up-regulation of growth factor signaling leads to a reversible loss of ER expression (2). These intriguing findings highlight the dynamic and heterogeneous nature of ER status in breast cancer and broaden the therapeutic horizon for patients with ER-negative tumors by suggesting that a subset of this group may benefit from a treatment strategy combining signal transduction inhibitors with endocrine therapy.

ER and Endocrine Therapy in Breast Cancer

Two thirds of breast tumors express ER, and the biological effects driven by this pathway are directly involved in breast cancer development and progression. Although the prognostic value of ER is still controversial, ER status is currently a valuable predictive factor for the success of endocrine therapy in breast cancer (3). Current endocrine therapies, aiming at blocking the ER pathway, include either strategies to deprive the receptor of its estrogen ligand by ovarian ablation or aromatase inhibitors, or approaches to directly inhibit the receptor by selective ER modulators such as tamoxifen or by selective ER down-regulators such as fulvestrant, that function as potent antagonists (4) and references therein. The widespread use of systemic endocrine therapy in patients with ER-positive tumors over the past 30 years accounts, at least in part, for the observed decrease in breast cancer mortality in the last two decades (5).

ER is mostly a nuclear receptor that acts as a ligand-dependent transcription factor to regulate genes involved in breast cancer cell proliferation, survival, invasion, and tumor angiogenesis. Besides this classic genomic or nuclear activity of ER, an alternative, rapidly manifested activity of ER, stemming from a fraction of the cellular pool of ER residing in the cytoplasm and/or the membrane, has recently been recognized in breast cancer cells. Through this action, ER can directly or indirectly interact with and up-regulate various growth factor receptor (GFR) tyrosine kinases (e.g., HER1, HER2, and insulin-like growth factor-I receptor) as well as signaling intermediates including membrane proteins, signaling adaptor molecules, and cellular kinases (e.g., caveolin, Shc, MNAR/PELP, and Src; refs. 6–8 and references therein). Conversely, GFR signaling can also modulate and enhance the genomic/nuclear activity of ER. This multilevel bidirectional cross-talk between ER and GFR signaling pathways plays an important role in both acquired and de novo resistance to endocrine therapy in breast cancer (refs. 7, 9–11 and references therein). Recent preclinical and clinical data have further shown that acquired resistance to endocrine therapy is frequently associated with significantly increased levels of HER1, HER2, or other GFR downstream signaling molecules (10, 12, 13) and, occasionally, with a substantial down-regulation of ER (12, 14), and that a combined therapy with HER1/2 inhibitors can significantly improve endocrine therapy outcome (10, 13).

Heterogeneity, Origin, and Biology of ER-Negative Breast Cancer

ER status of breast cancers is highly heterogeneous, with tumors presenting widely different percentages of ER-positive cells that express the receptor protein at mixed intensities. Twenty percent to 30% of breast cancers are considered ER negative (15). The clinically assigned status of ER in a given tumor is somewhat subjective and depends on the methodology and the cutoff of the chosen assay; both are based on clinical correlation with response to endocrine therapy. Using the currently most established assay, immunohistochemistry, even tumors designated ER negative can have a few ER-positive cells (15).

Histologic evidence suggests that over the natural course of breast cancer progression, ER can be lost (16). The rather common phenomenon of apocrine metaplasia of nonmalignant and malignant breast epithelium, which is associated with complete ER loss and up-regulation of the androgen receptor, has also been suggested to be involved in the development of some ER-negative breast cancers (17). The cancer stem cell theory provides another mechanistic explanation for the heterogeneous phenotype of ER status in breast. According to this theory, the molecular nature of the early stem or progenitor cells responsible for the origin of the tumor, and the particular
mutations driving carcinogenesis, account for the diverse phenotypes of breast cancer and ER status (18, 19).

Molecular classification of human breast tumors based on their intrinsic global expression patterns also emphasizes the heterogeneous molecular nature and biology of breast cancer in general and of ER-negative tumors in particular. Although constantly being refined, this approach has already corroborated several biological subtypes, including the luminal (A and B groups), basal-like, HER2-positive, and normal breast subtypes (20). Importantly, ER-negative tumors have been found in almost all of these categories, although at different percentages. Recognizing the power of this molecular technology, current efforts are focused on further delineating the subclassifications of ER-negative tumors with the purpose of shedding light on key issues regarding the origin and biology of ER-negative breast cancers and identifying novel pathways and targets to improve our presently limited therapeutic strategies for these tumors.

**Regulatory Mechanisms for ER Loss and the Generation of an ER-Negative Phenotype**

ER expression is tightly regulated in breast epithelial cells under both normal and pathologic conditions (21). Loss of ER expression causes tumor growth that is no longer dependent on estrogen, frequently resulting in a more aggressive phenotype and resistance to endocrine therapy. Down-regulation or a complete loss of ER may occur at multiple levels, from the gene to the protein, and by multiple mechanisms (Table 1).

Although ER gene amplification has been recently suggested as a common mechanism underlying ER overexpression in breast cancer (22), current data, although somewhat limited, suggest that loss of heterozygosity or mutations in the ER gene locus do not play an important role in ER expression loss (22–24).

In contrast to the genomic/gene level, ER expression and its loss are clearly controlled at the epigenetic level. Hypermethylation of CpG islands within the ER promoter, as observed in 25% of ER-negative breast cancers, has been repeatedly documented as an epigenetic mechanism to transcriptionally silence the ER gene (25, 26). Because both DNA methyltransferases and histone deacetylases play a crucial role in maintaining the transcriptionally repressed state of genes, inhibitors to these enzymes have been used as a therapeutic strategy for restoring ER expression as well as sensitivity to endocrine therapy in preclinical models of ER-negative breast tumors (ref. 27; Fig. 1 and see later discussion).

ER transcriptional regulation is also controlled by other cis- and trans-acting elements that may contribute to ER loss in ER-negative tumors. These elements consist of several cell-specific promoters and an array of abundantly expressed and tissue-specific transcription factors (21), including the ER protein itself acting as an autoregulator (28). Posttranscriptionally, tissue- and cell-specific alternative splicing that generates various ER protein isoforms with different activities, cellular localizations, and stability properties, has been further suggested as a potential mechanism contributing to ER loss (21, 24). Regulation of the ER mRNA stability by sequences at the 3′ untranslated region of the mRNA and, as recently shown, also by specific micro-RNA, represents an additional layer of complexity in controlling ER expression and, potentially, in mediating ER loss (21, 29).

At the protein level, manifold posttranslational modifications, including phosphorylation, acetylation, sumoylation, and ubiquitination, not only modulate the different activities of ER but also affect its stability and turnover, which, under specific circumstances (e.g., hypoxia; ref. 30), may result in a complete loss of ER.

**Hyperactive GFR Signaling as a Molecular Determinant of ER Loss: The Dynamic Nature of ER Status and Clinical Implications**

High levels of GFRs, particularly of HER1/2, have long been recognized to inversely correlate with ER expression level, and ER-negative tumors have been found to frequently overexpress GFR signaling (31, 32). Additionally, overexpression of these and other GFRs and their signaling intermediates can also result in resistance to endocrine therapy (ref. 13 and references therein).

To further investigate this interaction between GFR signaling and the ER pathway, El-Ashry’s group has previously constructed, using the ER-positive MCF7 breast cancer cells, an in vitro model of hyperactivated MAPK signaling by exogenous up-regulation either of the GFRs HER1 and HER2, or of a constitutively active Raf or MAP/extracellular signal-regulated kinase kinase (MEK; ref. 2). These genetically engineered cell lines display estrogen-independent growth as well as resistance to antiestrogen therapy and, somewhat unexpectedly, show a complete loss of ER mRNA and protein, due to both transcriptional repression and enhanced protein degradation.

<table>
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<td>LOH at ER gene locus</td>
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Abbreviations: LOH, loss of heterozygosity; UTR, untranslated region; miRNA, micro-RNA.
(Fig. 1). Yet, this acquired ER-negative phenotype is reversible, and abrogation of the MAPK activity by inhibitors of GFRs or downstream kinases, or by dominant negative constructs, rapidly restores ER expression and activity. Although the mechanisms by which elevated MAPK signaling leads to ER down-regulation are still largely unknown, in a subsequent study, El-Ashry's group highlighted the involvement of the nuclear factor-κB transcription factor in this process (33). Molecular profiles identifying a shared "MAPK signature" between these hyperactive MAPK cell lines and human ER-negative breast cancers further support the clinical relevance of this model and reinforce hyperactive MAPK signaling as an underlying mechanism behind the ER-negative phenotype (34).

In the report published in the current issue, Bayliss et al. (1) go on to investigate whether inhibition of MAPK activity stemming from intrinsic hyperactive upstream signaling in both established and primary cultures of human ER-negative tumors can also restore ER expression and sensitivity to endocrine therapy. The findings of this study suggest that MAPK blockade can restore ER expression in a subset of ER-negative tumors and reestablish endocrine sensitivity in some of these, thereby supporting the potential role of a combined MAPK inhibition/endocrine therapy in ER-negative breast cancer patients (Fig. 1). In tumors in which sensitivity to endocrine therapy is not restored despite ER reactivation, additional therapy targeting alternative signal transduction pathways may be of value. Finally, because hypermethylation of the ER promoter may interfere with reactivation of the ER gene in a subset of ER-negative tumors, adding histone deacetylase inhibitors or other agents capable of inducing DNA demethylation to inhibitors of MAPK or other signaling transduction intermediates in these cases may be an effective strategy to restore ER expression and consequently response to endocrine therapy (Fig. 1).
Recent intriguing data from a few clinical reports clearly support the above molecular scenario and justify further clinical development of these novel treatment strategies for patients with ER-negative tumors. In a recent report of patients with HER2+/ER- advanced breast cancer treated with trastuzumab, ER reexpression was identified in 3 of 10 patients after 9, 12, and 37 months of therapy, and endocrine treatment with an aromatase inhibitor in two of these patients led to a long-term response for >3 years in one of them (35). Similarly, enhanced ER expression levels were also noted in several patients post-neoadjuvant trastuzumab (36). Finally, a recent study focusing on the mechanisms of resistance to the dual HER1/2 tyrosine kinase inhibitor lapatinib in HER2-overexpressing tumors also documented treatment-induced enhancement of ER signaling and/or expression, which in the preclinical setting, was proved to be the escape mechanism underlying the acquired resistance to the anti-HER2 lapatinib therapy (37). A simultaneous inhibition of both the ER pathway by endocrine therapy and HER2 by lapatinib in this model system prevented the development of acquired resistance.

**Conclusion and Future Perspective**

ER-negative breast cancer is a heterogeneous disease and may not be a fixed phenotype. ER expression can be lost or restored depending on a number of variables including other signaling events and networks in the tumor. The shifting paradigm, recognizing the dynamic and reversible nature of ER status (and perhaps also of HER1/2 status) in breast cancer, may increase treatment choices for patients with this devastating disease. Therefore, we should no longer view ER for diagnostic and therapeutic purposes as a simple independent variable separate from the rest of the cellular and tumor signaling networking. The strategy of combining anti-MAPK signaling with endocrine treatments to recover ER as a functional target in a subset of ER-negative breast cancers, as successfully addressed in the current article (1), emphasizes the practical relevance of considering the ER status as a dynamic and not a fixed variable for the designing of new treatment strategies for breast cancer patients. Because a comparable role in regulating ER expression and/or signaling has been also attributed to the phosphatidylinositol 3-kinase/Akt pathway, a similar therapeutic approach with specific inhibitors to this pathway in the context of ER-negative tumors should also be investigated (37, 38). Adaptation of advanced multiplex technologies for comprehensive assessment of key signaling networks in any given tumor as clinical standardized tools holds the promise of facilitating personalized medicine for breast cancer in general, and specifically, of enhancing the identification of patients whose ER-negative tumors may revert to an ER-positive phenotype, allowing an endocrine treatment to be effective under appropriate combination therapies.

Finally, the multilevel cross-talk between ER and the HER pathway in breast cancer, which includes an inverse correlation of expression and functionality between these two pathways, suggests that sensitivity or resistance to targeted therapies against either of these pathways may rely, at least in part, on reactivation of the other pathway. Thus, a strategy of combining therapies against both pathways might often be superior to treatments targeting only one of these two pathways.

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The Dynamics of Estrogen Receptor Status in Breast Cancer: Re-shaping the Paradigm

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