Published in final edited form as:
Cancer Biol Ther. 2009 March 15; 8(6): 485–496. doi:10.4161/cbt.8.6.7582.

The silent estrogen receptor:

Can we make it speak?

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Keywords

breast cancer; epigenetic silencing; hormone resistance; ER reactivation; growth factors

Introduction

One of the most common cancers in women worldwide, breast cancer is classically an endocrine-dependent cancer.[1] It has been known for over a century that development, progression and metastasis of breast cancer are strongly influenced by hormonal factors.[2] Indeed about two-thirds of breast cancers express the estrogen receptor α (ERα) protein, a key predictor of prognosis and response to endocrine therapy. These cancers are frequently amenable to therapies that target estrogen signaling pathways, including selective estrogen receptor modulators like tamoxifen, selective estrogen receptor downregulators like fulvestrant; and agents that reduce estrogen ligand like aromatase inhibitors and ovarian suppression through luteinizing hormone-releasing hormone (LHRH) agonists (Fig.1). It is likely that these approaches, especially adjuvant tamoxifen, have contributed to the reduction in breast cancer mortality that has been observed in recent years.[3] However, data from clinical studies have suggested that only about 60% of ERα-positive breast cancers respond to hormonal therapy.[4,5] Further, those tumors that lack expression of ERα and the estrogen-regulated progesterone receptor (PgR) are unresponsive to hormone therapy. Thus the problem of acquired or de novo endocrine resistance is a substantial one. Recent molecular and biological advances have contributed to our understanding about potential underlying mechanisms. Here we will focus especially on silencing the expression of ERα as one such endocrine-resistance mechanism and how it might be exploited clinically.

A key contributor to our understanding of estrogen as a critical regulator of growth and differentiation in normal breast tissues and malignant progression has been the elucidation of estrogen signaling pathways. Estrogen functions by binding to two distinct estrogen receptors (ERα and ERβ) to modulate their transcriptional activity.[6] ERα and ERβ exhibit differential transcriptional properties and can form hetero- and homo-dimers.[7] While both isoforms share similar binding affinities for estrogen (E_2), they play different roles in regulation of gene expression. This review will focus entirely on ERα.

ERα mediates both genomic and non-genomic signaling pathways.7 It has two distinct transactivation function (AF) domains, AF1 and AF2, that mediate transcriptional activation. Depending on the cell type, AF1 and AF2 may activate transcription independently and/or

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synergistically. The classic mechanism of action of ERα involves binding of estrogen to ERα, formation of dimers, and localization to estrogen response elements (ERE) on regulatory promoter regions of estrogen responsive genes in association with either coactivators or corepressors to alter transcription of the responsive gene [2] In addition, ERα can also mediate transcription of other target genes through protein-protein interactions with other transcriptional factors[9] like activator protein-1 (AP-1)[10].

In addition to nuclear ERα, plasma membrane and mitochondrial ERα mediate non-genomic signaling.[11–5] Here, ERα may be activated by a ligand-independent mechanism through the phosphorylation of specific sites in AF-1 by kinases and peptide growth factors.[16] ERα in mitochondria bind to ERE-like sequences in the DNA of the mitochondrial (mt) genome and drive the levels of mRNA. mtERα signaling inhibits apoptosis through mt-superoxide dismutase (mtSOD) [17]

Potential Mechanisms for ERα-Negative Breast Cancer

About one-quarter of breast cancers lack ERα expression and such tumors are associated with poorer clinical outcome and are unresponsive to endocrine treatments.[4,5,18] Molecular classification has allowed us to identify at least two types of ERα-negative breast cancer, the human epidermal growth factor (HER)-2 overexpressing subtype as well as the “triple-negative” subtype that lacks expression of ERα, PgR and HER-2. Currently risk factors and pathways for the development or evolution of such cancers are poorly understood.

Breast cancer, like other cancers, results from an accumulation of genetic and epigenetic events. Such changes contribute to the establishment of ERα negative cancer, a hormone resistant phenotype which could result from a variety of mechanisms. These include the classic genetic mechanisms of homozygous deletion of the ERα gene or loss of heterozygosity along with mutation in the remaining allele (reviewed in ref. 19). However, genetic alterations within the ERα gene like insertions, deletions, rearrangements and point mutations have not been historically reported as a major cause of loss of ERα function.[20,21] Instead, ERα-negative cancers are frequently associated with the absence of ERα mRNA expression and it has been suggested that epigenetic alterations at the ERα promoter or alterations in other growth factor and signal transduction pathways may be responsible for downregulation of transcription (Fig. 2).[22] Because these changes are potentially reversible, the use of strategies to modulate these alterations has been proposed.

A contentious issue is whether ERα negative tumors arise de novo or represent an acquired phenotype. Certainly, acquired alterations in multiple signaling pathways in normal or ERα positive cells resulting in clonal selection and evolution into ERα-negative breast cancer cells are one potential mechanism. However, the possibility that self-renewing stem-like cells within breast tumors may contribute to the emergence of the ERα negative phenotype has also been raised. Putative breast cancer stem cells (BCSC) of the $CD44^{+}/CD24(-/low)$ antigenic phenotype that is hypothesized to be stem cell specific were isolated from malignant pleural effusions and ascites samples from breast cancer patients. These were marked by the absence of ERα, PgR and HER2 expression raising the possibility that they play a role in the origin of ERα negative tumors.[23–30] However, this is an area of great controversy as it is also possible that these BCSC may also be clonally selected from a highly heterogeneous tumor with the pressure of time and/or therapy. A lack of availability of good markers for identification of BCSC and the rarity of these cells, if they do indeed exist, represent two limitations in this field at the present time.[31]

In sum, although several theories exist for the origin of ERα negative hormone-resistant breast cancers, there is no consensus about their relative contributions at present. But two compelling lines of evidence attribute loss of ERα expression in breast cancer to crosstalk

between ERα and growth factor signaling pathways and epigenetic silencing of ERα and these areas are reviewed below.

Crosstalk between ERα and growth factor signaling pathways

Epidermal growth factor receptors (EGFR)—The human epidermal growth factor receptor family is comprised of four members: EGFR/ c-erbB1/HER-1, c-erbB2/HER2/neu, c-erbB3/HER3 and c-erbB4/ HER4. Upon ligand binding these transmembrane receptor tyrosine kinases form homo- and heterodimers, resulting in their activation and transduction of signaling into the cell.[32] A role for EGFR family members has been implicated in the development and progression of many human cancers including breast cancer.[33,34] Indeed, one proposed mechanism for the suppression of ERα expression is through the hyperactivation of mitogen activated protein kinases (MAPK) due to EGFR and HER2.[35–45]

EGFR is believed to be a marker of poor prognosis in breast cancer patients.[39,46] A significant inverse relationship between ERα and EGFR expression has been observed in human breast cancer specimens and human breast cancer cell lines.[39,47] It appears that EGFR does not alter ERα gene expression but EGFR mRNA expression is negatively regulated by ERα.[48] ERα-negative breast cancer cells that overexpress EGFR activate the EGF growth signaling pathway and bypass the requirement of E_2 for proliferation. Hence, activation of EGFR may play a role in cell growth in ERα-negative breast cancers and targeting EGFR could be a therapeutic strategy.[31,49]

Overexpression of a second member of the HER family, HER2, is observed in 20–25% of breast cancers, usually as a consequence of HER-2 gene amplification; such tumors are divided between ERα-positive and ERα-negative but ERα positive breast cancers with amplified HER2 possess low ERα and PgR content in comparison to ER positive breast cancers that are HER2 negative.[50,51] Thus, reduced ERα and PgR content is a proposed mechanism of hormonal resistance in HER2 overexpressing tumors, and the possibility that anti-HER-2 targeted therapy might help to restore endocrine sensitivity has been raised.[33,43,51–53]

MAPK—One proposed mechanism for the emergence of ERα-negative breast cancers from an ERα-positive background is hyperactivation of the MAPK pathway; possible triggers for MAPK hyperactivation can include overexpression of EGFR and/or HER2, estrogen withdrawal, or hypoxia. Using engineered cell line models of upregulated growth factor signaling, it has been demonstrated that hyperactivation of MAPK represses ERα expression.[35] However, inhibition of ERα through MAPK is dynamic and reversible, and inhibition of the MAPK pathway in MAPK-overexpressing MCF-7 cells reverses the effects and allows the re-expression of ERα.[35,54] Importantly, either abrogation of the MAPK pathway using MAPK inhibitors or inhibition of upstream signaling from overexpressed growth factor receptors like EGFR or HER-2 using targeted agents in ERα negative breast cancers cell will, in some cases, result in ERα re-expression, restoration of estrogen dependency and sensitivity to hormonal therapy.[54] These effects can be amplified by the concomitant use of a MAPK inhibitor and an epigenetic modifier. For example, treatment of ERα negative breast cancer cell lines with the classic DNMT inhibitor, 5-aza-2'-deoxycytidine (5-azaDc) for three days (which reduces MAPK activity 9.4 fold), followed by inhibition of MAPK signaling (leading to a further 4.4. decrease in MAPK activity) synergistically increased re-expression of ERα protein (1.5-fold in comparison to azaDc alone) at eight hours.[35]

Nuclear factor κB (NFκB)—The NFκB complex is a ubiquitously expressed family of inducible transcription factors.[55–57] Activation of NFκB has been implicated in promotion

of proliferation, motility and invasion and inhibition of apoptosis during mammary gland development. Normally NFκB is inactive and is sequestered in cytoplasm by IκB inhibitory proteins. However, once activated, NFκB translocates to the nucleus and binds to specific DNA sequences to induce transcription of target genes.[58]

Variable NFκB activity has been observed in human breast cancer cell models. NFκB is constitutively active in most ERα negative human breast cancer cell lines (MDA-MB-231 and SKBR3) and shows minimal activity in ERα positive/HER2 negative breast cancer cells (MCF-7 and T47D). Intermediate NFκB activity has been seen in ERα positive/HER2 positive breast cancer cell lines including tamoxifen-resistant BT474 and MCF7/HER2 cells.[58–60] A study in tumors from 81 breast cancer patients with ERα-positive breast cancers treated with tamoxifen showed that higher NFκB activity as measured by higher DNA binding is significantly correlated with lower ERα content. Further, AP-1-DNA binding activity as well as uPA and HER2 protein content showed a strong correlation with NFκB activity and disease-free survival.[58] Although the mechanism is not well understood, inhibition of NFκB activity by estrogen bound ERα is widely proposed.[61,62] Indeed, in human breast cancer cell lines and tissues from malignant tumors, NFκB was predominantly activated in ERα negative tumors (mostly in ERα negative and HER2 positive).[63] It is possible that NFκB activation in ERα negative breast cancer cells is a consequence of enhanced growth factor signaling as both EGFR and HER2 activate NFκB[64] via the PI3-kinase/Akt pathway.[65]

In aggregate, these data suggest that activation of signaling through one or more factors including EGFR, HER2, MAPK and/ or NFκB plays a key role in ERα expression. These factors may act independently or collectively, resulting in ERα loss and altered clinical course. Therefore inhibitors that target MAPKs, EGFR, HER2 and NFκB alone or in combination with standard chemotherapies are rational agents to pursue in a quest to restore the reactivation of hormone sensitivity in ERα-negative breast cancers.

Epigenetic silencing of ERα

Epigenetic events are defined as heritable changes in gene expression without alterations in DNA sequence. Epigenetic silencing involves the interplay of multiple processes including chromatin modifications, alterations in nucleosomal positioning/remodeling, and DNA methylation, resulting in the transcriptional downregulation of target genes. Gene silencing via such epigenetic changes is necessary for regulating many normal biological processes. Dysregulation of these processes that results in aberrant epigenetic control is a common occurrence in many cancers.[66] We, and others, have hypothesized that epigenetic alterations in the promoter of ERα may play an important role in ERα inactivation and hence hormone resistance in breast cancer. Particular areas of focus include the role of methylation of the ERα promoter and the contributions of modifications of histone proteins in the expression and silencing of ERα.

Methylation of ERα promoter—DNA methylation involves a covalent modification of the C5 position of the pyrimidine ring of cytosine residues occurring in CpG dinucleotides. These CpG dinucleotides are primarily found clustered in, and near, the promoter regions of genes. Human cancer cells typically exhibit global hypomethylation and promoter specific hypermethylation, resulting in genomic instability.[67] Aberrant promoter methylation is most commonly implicated in transcriptional repression of tumor suppressor genes in cancer cells. In mammalian cells, DNMT1 is a maintenance methyltransferase while DNMT3a and 3b are de novo methyltransferases.[68–70] Together DNMT1 and DNMT3b account for 95% of genomic methylation.[71] Such promoter specific methylation could serve to block gene transcription directly or indirectly by recruitment of repressor proteins or interference with

recruitment of transcription factors. Reports on aberrant methylation of ERα gene in different cancers date back two decades.[72–74] Indeed, the downregulation of ERα in multiple tissues including breast, colon, lung, ovary, prostate and hematopoietic neoplasms is associated with promoter methylation.[75–77]

Elevated DNMT1 protein levels have been reported in breast cancer tissues and MCF-7 breast cancer cells relative to normal human mammary epithelial cells (HMECs).[78] Initial cell culture studies also suggested that DNMT1 mRNA and protein levels are typically higher in ERα negative than ERα positive breast cancer cell lines.[76,79] DNMT1 protein expression is cell cycle-dependent in normal and ERα positive breast cancer cells, while its expression was felt to be elevated and cell cycle independent in ERα negative breast cancer cells.[80,81] Dysregulation of DNMT1 in breast cancer appears to reflect increased DNMT1 protein stability, perhaps due to the destruction of the N-terminal region of DNMT1 that is required for its proper ubiquitination and degradation.[78,82] A role for DNMT3b is suggested by a study in tissue samples from breast cancer patients. Here, overexpression of DNMT3b mRNA (30%) predominated over DNMT1 (5.4%) and DNMT3a (3.1%) and DNMT3b overexpression was significantly related to ERα negativity and poor relapse free survival in the patient samples.[83]

The ERα gene is comprised of eight exons and spans 300 kb of the chromosome 6q25.1 locus.[84,85] Like many nuclear receptor genes, the ERα gene has multiple promoters (A–F) that can give rise to isoforms with differentially spliced 5’ UTR regions.[86] Examination of normal and malignant breast cell lines and tissue identified three ERα gene transcripts arising from distinct promoters, including the proximal promoter and two distal promoters located approx imately 2 kb and 21 kb upstream (promoter B and promoter C respectively).[87–90] The 5’ upstream region of ERα has areas that are heavily CG-enriched,[91,92] and aberrant methylation has been observed here in multiple human cancers.[21,22,76,93–97] Methylation of CpGs at both the proximal promoter and distal promoter B has been associated with altered ERα expression in human breast cell lines as well as in primary breast cancer specimens. In ERα-positive breast cancer cell lines the ERα promoter is predominately unmethy-lated at both the proximal promoter and distal promoter B, whereas methylation of both these regions is observed in ERα-negative breast cancer cell lines (Table 1).[98–101] This is also observed in human breast cancer specimens where ERα CpG island methylation of both the proximal promoter and distal promoter B is far more common in primary ER-negative human breast cancers than in ERα-positive breast cancer specimens.[101–104]

Increased prevalence of aberrant methylation of ERα 5’CpG islands has been reported as human breast cancer progresses from ductal carcinoma in situ (34%) to invasive ductal carcinoma (52%) to metastatic/locally advanced disease (61%) in a total of 111 human breast tumor samples.[93,105] In addition, the ERα repression typically observed in BRCA1-linked breast cancers is associated with methylation of critical CpG sites within the ERα promoter, and patients with methylated ERα are three times more likely to be BRCA1-methylated than patients with unmethylated ERα.[102,106,107] However, no clear correlation has been observed between ERα methylation and ERα gene expression in PgR+ve breast tumors. This discrepancy may reflect that exon 1A of the ERα promoter rather than the promoter region itself was selected for this study.[108] DNA methylation is also an important mechanism for ER-β gene silencing in breast cancer and treatment with DNMT inhibitors restores ER-β expression in ER-β-negative breast cancer cell lines.[103,109]

Although methylation appears to play an important role in ERα downregulation it is not the only mechanism involved in ERα silencing. Increasing evidence suggests that methylation is closely associated with histone deacetylation.[13] Alteration in steps that precede methylation

including loss of transcriptional coactivators and factors that block methyltransferases are other proposed causes.[110]

Histone modifications—DNA is wrapped around structural proteins called histones to form nucleosomes. These histones may be modified in a variety of ways to alter the accessibility of the associated DNA. Direct modifications can include acetylation, methylation, phosphorylation and ubiquitination of residues in the aminoterminal tail of core histones. A variety of proteins are responsible for the addition of these groups, includingx histone methyltransferases, demethylases, deacetylases and acetyltransferases. Additionally, histone/DNA associations may be regulated by ATP-dependent chromatin remodeling factors which adjust nucleosome positioning. The resulting histone code can alter chromatin structure, regulate the accessibility of the packaged DNA to transcriptional activator proteins, and thereby modulate gene expression.[111]

Histone methylation, which is regulated by histone methyltransferases (HMTs) and demethylases, occurs on the lysine and arginine residues of the histone tails. Lysine residues can be mono-, di- or tri-methylated. Histone methylation on different residues appears to provide binding sites for specific transcriptional regulators thereby positively or negatively regulating gene expression. Methylation of lysine 4 and 6 of histone H3 (H3K4 and H3K6) is associated with transcriptional activation while methylation of lysine 9 of histone H3 (H3K9) is associated with transcriptional silencing.[112–118] SUV39H1, which methylates H3K9, has been identified at the ERα promoter in several breast cancer cell lines (Fig. 3 and Table 1).[119] Additionally, the silenced ERα promoter in ERα negative human breast cancer cells is associated with methylated H3K9 while the active promoter in ERα positive cell lines is associated with methylated H3K4 by chromatin immunoprecipita-tion assay (Table 1).[120]

Acetylation of histones (primarily H3 and H4) on the lysine residues of the N-terminal tails is maintained by a dynamic balance in the activity of the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). It is proposed that decetylation represses gene expression prior to long-term silencing by DNA methylation.[121] HDACs fall into several classes including class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9 and 10) and class III (sirtuins or SIRT1–7).[122] Because multiple HDACs exist, there has been much interest in which HDACs might play a role in the epigenetic silencing of ERα. This has been only partially studied. HDAC1 has been shown to be associated with the silenced ERα promoter in ERα-negative breast cancer cell lines; other HDACs were not evaluated in these studies.[123,124] Indeed, overexpression of HDAC1 in MCF-7 cells induces loss of ERα and increases cell proliferation and colony formation, all of which could be reversed by trichostatin-A (TSA).[125,126] That HDAC2 is more relevant than HDAC1 or HDAC6 in regulation of ERα and PR expression and potentiation of the effects of tamoxifen in both ERα negative and ERα positive human breast cancer cell lines was reported by Bicaku et al.[127] In sum, though, a comprehensive analysis of the role of individual HDACs in ERα expression and silencing has not been completed.

It has recently been reported that HDACs also regulate the functions of non-histone proteins including transcription factors and regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins in a pattern similar to histone proteins.[128] Alteration in acetylation status of these proteins affects their DNA-binding affinity, stability and/or subcellular organization, leading to alterations in signal transduction.[122,129] Another unexpected target is DNMT1. HDAC inhibition results in DNMT1 degradation through the ubiquitin-proteosomal pathway. HDAC inhibition results in hyperacetylation of the molecular chaperone heat shock protein 90 (Hsp90) that forms a chaperone complex with

DNMT1. This leads to its dissociation from DNMT1 followed by ubiquitination and degradation of DNMT1.[82] Therefore it appears that HDAC inhibition not only results in chromatin modifications that favor an active chromatin but also potentially promotes gene reactivation through degradation of DNMT1.

Components of corepressor complexes—Apart from the engagement of DNMT and HDAC in epigenetic silencing, a variety of other DNA binding proteins and co-repressors have roles in the regulation of chromatin structure and transcription including the methyl CpG binding domain proteins (MBDs) and the metastasis associated proteins (MTAs).[130,131] These proteins appear to serve as epigenetic regulators that read or interpret methylation rather than directly modifying DNA methylation; they are of interest as they might serve as promoter specific components of corepressor complexes.[130,131] Chromatin immunoprecipitation (ChIP) assay was used to carry out comparative mapping of the ERα promoter for localization of MBD family proteins in MCF-7 and MDA-MB-231 cells. MBD1, MBD2 and MeCP2 but not MBD3 were easily detected at the ERα promoter in ERα-negative MDA-MB-231 cells but not in the ERα-positive MCF-7 (Fig. 3 and Table 1). The pattern of MBD localization at the ERα promoter paralleled that of DNMTs and HDACs, indicating that MBDs may also be involved in epigenetic silencing of ERα. Indeed treatment with either DNMT or HDAC inhibitors simultaneously released these MBDs, DNMTs and HDACs from the ERα promoter as shown through ChIP in conjunction with ERα reexpression. The findings that DNMTs, HDACs and corepressor complex elements like MBDs may be acting individually and in unison is a current model under investigation for epigenetic silencing of ERα.[120]

The role of other components of corepressor complexes like MTA family proteins on epigenetic silencing of ERα is also under investigation. The three main members of this family; MTA1, MTA2 and MTA3, are reported to play a role in breast cancer invasion and metastasis. MTA1s, an isoform of MTA1, is cytoplasm-specific and sequesters ERα in the cytoplasm and has been implicated in hormone resistance in breast cancers.[132]

Other factors—It has also been suggested that the transcription and methylation pattern of ERα could be determined by protein-protein and protein-DNA interactions around specific sites of the promoter. Studies by Macaluso and colleagues have identified specific regions of the ERα promoter that are differentially bound by members of the retinoblastoma family including pRb1/p105, p107 and pRb/p130 and suggest that these associations may induce modifications of local chromatin structure possibly leading to increased DNA methylation. This group has additionally identified regionspecific binding of pRB2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRB2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 complexes in ERα positive and ERα negative breast cancer cells respectively (Fig. 3 and Table 1).[119,133]

Potential Strategies to Reactivate ER α Expression and Activity

Preclinical data suggesting that ERα expression can be modulated by the upregulation of growth factor signaling pathways and/or epigenetic silencing naturally lead to the hypothesis that abrogation of growth factor signaling pathways with targeted agents and/or reactivation of epigenetically silenced ERα with epigenetic modifiers can re-express functional ERα and perhaps restore hormone sensitivity. The rationale and experience for both of these approaches are reviewed below.

Growth Factor Signaling Inhibitors

The utility of inhibitors of the HER family or MAPK to abrogate ERα silencing has been explored in human breast cancer cell lines and transient human tumor explant cultures. Inhibition of growth factor signaling directly with the MAPK/ERK inhibitor, U0126 or indirectly by upstream inhibition of EGFR by gefitinib or HER2 through trastuzumab resulted in functional ERα expression in several ERα negative breast cancer cell lines with varying levels of EGFR and HER2 expression including SUM 149 (high levels of EGFR), SUM 229 (EGFR overexpressor) and SUM 190 (EGFR and HER2 overexpressor). Treatment with these compounds sensitized all the breast cancer cells studied (except SUM 149 that has high NFκB activity) to 4-hydroxytamoxifen. These studies were extended to show that short term ex vivo treatment of ERα-negative human breast cancer explants with the MAPK inhibitor, U0126, led to enhanced ERα mRNA expression in six of ten specimens. Further, U0126 treatment of a cell line established from one of these ERα-negative tumors led to re-expression of ERα and sensitization of the cells to tamoxifen or fulvestrant.[54]

However, treatment of two ERα negative breast cancer cell lines with a basal phenotype, SUM 102 and SUM 159, that are hypermethylated at the ERα promoter with the MAPK inhibitor did not lead to re-expression of ERα, even though MAPK was downregulated. These data suggest that MAPK inhibition alone may not be sufficient to restore ERα expression in some cell lines with hypermethylation of the ERα promoter.[54]

Because NFκB potentiates tumorigenesis in ERα negative breast cancer cells, the possibility that NFκB inhibitors might also be exploited to reverse ERα silencing is being explored.[134] Agents that target upstream NFκB signaling and downstream IκB degradation mechanisms, including antioxidants like pyrrolidine dithiocarbamate, proteasomal inhibitors like MG-132 and PS-341; and sesquiterpene lactones like parthenolide (PA) that specifically inhibit IKK are under evaluation.[135–138] Natural compounds such as betullinic acid, sauchinone, rocaglamide, panepoxydone, helenalin, octylcaffeate and CAPE that also inhibit NFκB activity are worthy of study.

Epigenetic Therapeutics

Given the wealth of evidence that implicates a role for epigenetic silencing of ERα as well as the myriad of epigenetic modifiers that are available or under development, the strategy of using epigenetic modifying agents to re-express ERα with therapeutic intent has received much attention. Epigenetically targeted therapies that are available for human use include the DNMT inhibitors, 5'azacyti-dine (azaC) and 5-aza-2'-deoxycytidine also known as decitabine (5-azaDc), and the HDAC inhibitor, vorinostat or suberoylanilide hydroxamic acid (SAHA); they are approved by the US Food and Drug Administration for the treatment of myelodysplastic syndrome and refractory cutaneous T cell lymphoma respectively.

DNMT Inhibitors

Several investigators have demonstrated that treatment of ERα-negative human breast cancer cells with azaC or 5-azaDc leads to ERα mRNA and protein expression.[35,120,123,139–141] There remains uncertainty about the exact mechanism.

Direct evidence demonstrating the role of CpG island methyla-tion of the ERα promoter in the epigenetic silencing of ERα gene expression in ERα negative breast cancer cells was presented by Ferguson et al.[139] Treatment of MDA-MB-231 or Hs578t cells with azaC or 5-azaDc was associated with demethylation of the ERα CpG island and ERα mRNA and protein re-expression. Functionality of the reactivated ERα was shown by expression of

ERα response genes like PgR as well as enhanced activity of a reporter gene linked to ERE when cells were grown in estrogen-containing conditions.[139] ChIP assays of the ERα promoter in MDA-MB-231 cells that re-express ERα after 5-azaDc treatment showed depletion of DNMT1 protein and release of DNMT1, DNMT3b, MBD1, MBD2 and MeCP2 from the ERα promoter.

However, other factors might also be in play because of a decrease in DNMT levels due to either adduct formation resulting in activation of apoptotic pathways or failure to repair the adducts. A role for inhibition of DNMT1 specifically in the reactivation of silenced ERα in ERα negative breast cancers was evaluated through an antisense oligonucleotide (ASO98) against DNMT1. ASO98 treatment (but not treatment with a scrambled oligonucleotide) led to specific suppression of DNMT1 mRNA and protein expression in MDA-MB-231 and Hs578t cells, both of which have epigenetically silenced ERα. Such treatment resulted in expression of an ERα whose function could be inhibited with fulvestrant. However, there was no change in the methylation status of ERα by MSP analysis. This suggests then that re-expression of ERα after DNMT inhibition is not solely through ERα promoter demethylation but may also result from disruption of repressive transcription complexes on the ERα promoter mediated through DNMT1.[142]

New DNMT inhibitors are under development. One such agent is zebularine. Unlike 5-azaDc and azaC, zebularine is highly stable and shows little toxicity in preclinical testing models although it is associated with hepatic and renal toxicity in primates.[143] Treatment of ERα negative MDA-MB-231 cells with zebularine resulted in ERα reactivation in association with conversion to an active chromatin at the ERα promoter as evidenced by ChIP. A limitation of zebularine is that relatively high doses of zebularine are required for ERα re-expression. However, little cytotoxicity is seen with these doses, making zebularine a potential candidate for combination therapy with other DNMT inhibitors or other types of agents.[144] Alternatively novel zebularine-related inhibitors may emerge via structure-activity studies.

The possibility that natural products, especially dietary constituents, might serve as epigenetic modifiers is also under study. For example, catechol-containing dietary polyphenols and DNMTs use the same source of S-adenosyl methionine (SAM) for their reactions and treatment with polyphenols can deplete SAM and increase the levels of S-adenosyl-L-homocysteine (SAH), which is a potent inhibitor of DNA methylation.[145–149] Two common coffee polyphenols, caffeic acid and chlorogenic acid, are natural DNA methylation inhibitors, and treatment of MDA-MB-231 and MCF-7 cells with these compounds partially inhibited methylation of the promoter region of retinoic acid receptor- β (RAR-β) by non-competitive inhibition of DNMTs through increased formation of SAH.[150] However, the effect of these drugs and other potential demethylating dietary components on ERα reactivation is unexplored.

HDAC Inhibitors

Treatment with any of several hydroxamic acid HDAC inhibitors including trichostatin A (TSA), LBH589 and vorinostat can lead to re-expression of ERα in ERα-negative cells like MDA-MB-231 cells. TSA treatment led to a time and dose-dependent re-expression of ERα mRNA with no alteration of ERα promoter methylation. Rather TSA-activated gene transcription was associated with increased sensitivity of the ERα promoter to DNase 1 treatment (indicative of active chromatin) suggesting that inactive chromatin is a major factor for epigenetic silencing.[151] ChIP assay showed that TSA treatment led to accumulation of acetylated H3 and H4 and release of HDAC1 from the ERα promoter.[120]

Detailed studies with LBH589 show reactivation of ERα mRNA and protein expression occurs as early as 24 hours after treatment and re-expression was stable for at least 96 hours after withdrawal of treatment. ChIP assay of the ERα promoter showed that LBH589 released DNMT1, HDAC1 and the H3-K9 methyltransferase SUV39H1 from the ERα promoter. These changes were associated with an active chromatin conformation manifested as accumulation of acetylated histone H3 and H4, decrease in methylated H3-K9, and impaired binding of heterochromatin protein 1 (HP1α) at the promoter.[124] Thus, studies with several HDAC inhibitors have shown that re-expression of ERα through HDAC inhibition does not alter the methylation status of the ER promoter. It appears that HDAC inhibitor-induced re-expression of the ER is likely mediated through the reorganization of heterochromatin associated proteins without a change in the promoter hypermethylation.[124,151] Such ERα is functional as LBH589 treatment of MDA-MB-231 cells enhanced tamoxifen sensitivity.[124] However, HDAC inhibitors are likely to have a multitude of other effects in ERα-negative breast cancer cells as well including promotion of DNMT1 degradation by interrupting DNMT1 association with HSP90 as discussed earlier.[82] In addition, the possibility that HDAC inhibitors might sensitize ERα-negative cell lines to tamoxifen by activation of ER β has also been proposed.[152] It is noteworthy that HDAC inhibitor-induced ERα expression has not been uniformly observed by all investigators; this may reflect differences in tissue culture cells that have evolved over time or variations in culture conditions.[127,153]

Of note, the treatment of ERα-positive human breast cancer cell lines with HDAC inhibitors like MS-275, vorinostat, LBH529 or TSA decreases ERα expression and may also sensitize these cells to tamoxifen. Indeed Bicaku et al. have reported that HDAC inhibitor treatment of human breast cancer cell lines of diverse phenotypes with regard to ERα and HER-2 expression can sensitize these cells to tamoxifen, presumably by multiple mechanisms.[127] One potential mechanism appears to be that treatment with several different HDAC inhibitors can induce hsp90 hyperacetylation, thereby decreasing its binding to ERα, leading to increased polyubiquitylation and depletion of ERα levels.[123,124,127,153,–155] The role of individual HDAC isoenzymes on ERα and PgR expression was evaluated using pharmacological inhibitors or siRNA. This study suggests that depletion of HDAC1 and HDAC6 was associated with downregulation of ERα but not PgR in ER-positive cells while depletion of HDAC2 downregulated ERα and PgR and potentiated tamoxifen's effects.[127]

Combination Epigenetic Modifier Approaches

Initiation and maintenance of epigenetic silencing is a consequence of complex interactions between DNA methylation and histone modifications to determine chromatin structure and transcriptional status.[66] This implies that interventions that target both DNA methylation and histone modifications might be more effective than either strategy alone in altering transcription. It is reported that simultaneous demethylation and histone deacetylation inhibition with 5-azaDc and TSA synergistically re-expresses genes commonly hypermethylated and silenced in colorectal cancer cells.[156] Similarly, treatment of ERα negative MDA-MB-231 human breast cancer cells with both DNMT and HDAC inhibitors resulted in synergistic functional ERα reactivation compared with either agent alone. Treatment with 5-azaDc alone induced the ERα transcript by 30–40-fold, while TSA increased ERα mRNA expression by ten-fold. But treatment with the two drugs in sequence led to a 300–400-fold increase in ERα mRNA expression in these cell lines. This was associated with a marked decrease in DNMT1 protein expression and activity, partial ERα CpG island demethylation and increased acetylation of H3 and H4.[102,140] Combination studies in MDA-MB-231 and Hs578t cells of 5-azaDc with another HDAC inhibitor, Scriptaid, for 18 hours also showed an enhanced effect on cell growth inhibition, increased acetylation of H3 and H4, and synergistic activation of ERα mRNA and protein when

compared to either individual drug given alone.[141] ChIP assays have permitted a detailed dissection of changes in the protein complexes in the ER promoter with monotherapy or combined DNMT and HDAC inhibitor therapy. For example, in MDA-MB-231 cells dissociation of MeCP2, MBD1, MBD2, DNMT1 and DNMT3b was more evident after combination therapy with 5-azaDc and TSA as compared to individual drug treatments. The association of acetylated histones H3 and H4 was no greater with combination therapy than with TSA alone but combination therapy led to greater alterations in the association of methylated histones than either drug alone.[120] Whether these types of combinations lead to enhanced sensitivity to targeted agents like tamoxifen over treatment with a single DNMT or HDAC inhibitor has not been carefully explored.

Future Studies

A number of opportunities for new research in this area exist. First, the availability of two DNMT inhibitors and several drugs that are felt to have HDAC inhibitory effects (including vorinostat) has facilitated the first breast cancer clinical trials with these agents. As with all biological agents, significant questions remain about the appropriate clinical scenarios for testing of a new agent and the definition for success. A traditional Phase II study of vorinostat in women with refractory breast cancer has shown disease stability in a fraction of patients but no evidence of response by classic criteria.[157] A Phase II study of vorinostat and tamoxifen in women with metastatic breast cancer has established the safety of the combination and now evidence for efficacy is being sought.[158] Unfortunately this single arm design does not permit an evaluation of the role of the combination over single agent therapy. Finally a “window” study of vorinostat for three days before definitive breast surgery for women with newly diagnosed untreated breast cancer has completed accrual. This brief duration of therapy will not allow any evaluation of tumor response but tissues and blood recovered before and after therapy should provide an excellent platform for confirmation of biomarker modulation observed in the preclinical studies and discovery of new targets.

While these proof of principle clinical trials with established drugs are in progress, much work is also focused on developing new agents and identifying new targets in the epigenetic pathways. As mentioned earlier one such agent is zebularine. Although its clinical development is in doubt, other epigenetically targeted agents are under evaluation. For example, polycomb group proteins like histone methyltransferase EZH2 are often overexpressed in cancers. EZH2 is reported to be associated with H3 trimethylated lysine 27 (H3K27me3) at the promoters of several silenced genes in colon, prostate and breast cancer.[159–161] Whether such polycomb proteins play a role in ERα silencing is not yet understood. Lysine demethylase inhibition has also emerged as a strategy for modulating epigenetic gene silencing and certain polyamine analogues appear to function as lysine demethylase inhibitors. Their use has been associated with the re-expression of epigenetically silenced genes in colon cancer models; whether these same agents that are in some cases known to inhibit human breast cancer cell growth might also re-express epigenetically silenced ERα is not known. In addition, the silencing of other components of corepressor complexes like the MBDs, which read and interpret DNA methylation without modifying DNA, resulted in reactivation of specific genes associated with MBDs.[130] Development of drugs that target MBDs and other components of corepressor complexes localized on the ERα promoter also potentially represent a novel strategy for ERα re-expression with minimal toxicity.

Finally, although most work in the field of epigenetic modulation focuses on the treatment of established cancer with synthetic drugs, it is also imperative that research into non-drug interventions, especially dietary components, continue. These approaches are especially

attractive as they would theoretically be devoid of toxicity and widely applicable through the diet. Evidence that caffeic acid and EGCG might function as DNMT inhibitors is emerging.[162] In addition, it is reported that dietary components like sulphoraphane, the active chemopreventive compound from cruciferous vegetables, acts as an HDAC inhibitor in vitro in human breast cancer cells.[163] That these dietary components might act in concert is suggested by a report that treatment with EGCG and sulphoraphane can synergistically enhance transcriptional activation of AP-1 in HC-29 cells.[164] In addition, a well established negative relationship exists between the micronutrient selenium and breast cancer.[165] Indeed, breast carcinoma cell lines are more sensitive to selenium induced apoptosis than non-tumorigenic mammary epithelial cells.[166] It has recently been reported that sodium selenite, a selenium derivative, is both a DNMT and HDAC inhibitor in LNCaP prostate cancer cells. Exposure results in demethylation and reactivation of genes commonly silenced in cancers like glutathione-S-transferase, adenomatous polyposis coli and cellular stress response 1.[167] In sum these studies hold promise for the development of dietary components as epigenetically targeted agents although their value in breast cancer generally and estrogen receptor expression specifically is untested. It seems likely, however, that the value of such approaches will be appreciated largely in the prevention rather than in the treatment setting.

Finally, several reports have speculated that a methyl-deficient diet may be a risk factor for breast cancer since CpG methylation is an early event in cancer incidence and a methyl-deficient diet can result in CpG methylation.[149] Because the epidemiological evidence specifically points to a relationship between a methyl-group deficient diet and incidence of ERα negative breast cancer, it was postulated that such dietary deficiency would be associated with epigenetic silencing in the ERα gene. A small epidemiological study in African-American women was not able to establish that link however.[148]

Acknowledgments

This work was supported by NIH CA88843, Department of Defense Predoctoral Award BC050707 and the Breast Cancer Research Foundation.

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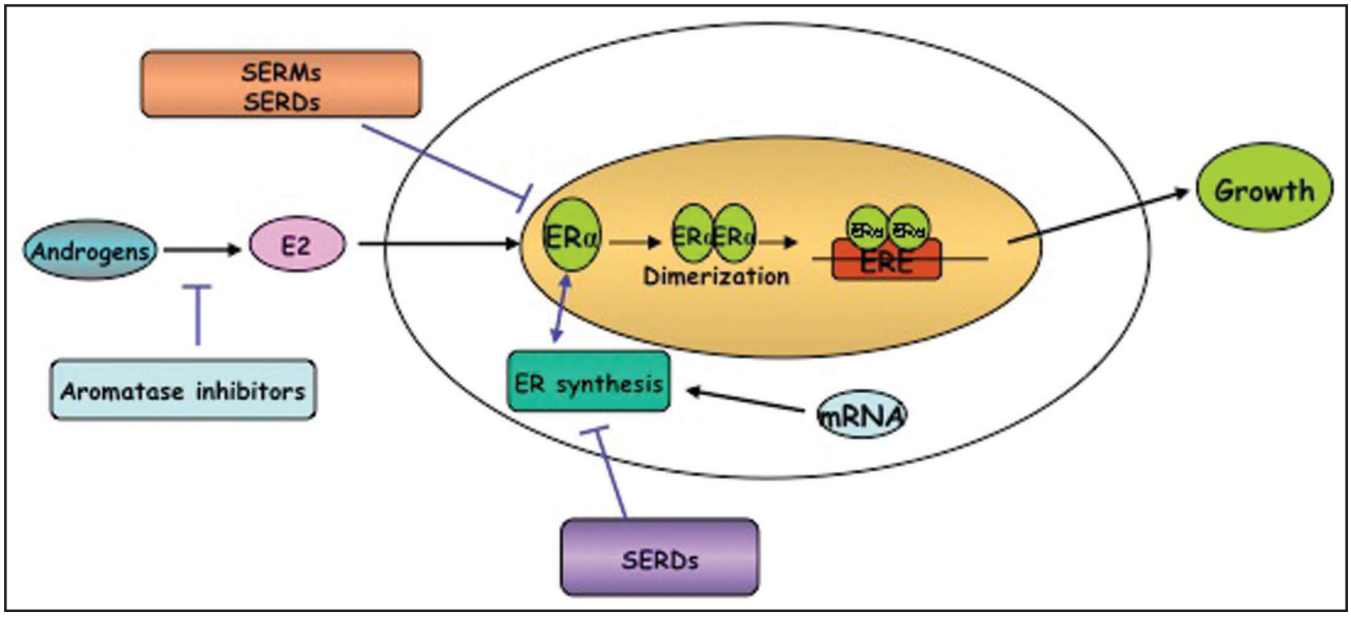

Figure 1.
Therapeutic strategies to block estrogen receptor function: Aromatase inhibitors like letrozole, anastrozole and exemestane inhibit the enzyme leading to decreased E2 levels. Selective ER downregulators (SERDs) like fulvestrant bind to ER and sequester it, thereby inhibiting ER-mediated gene transcripton and disrupting ER nuclear localization through the ubiquitin-proteosomal pathway.

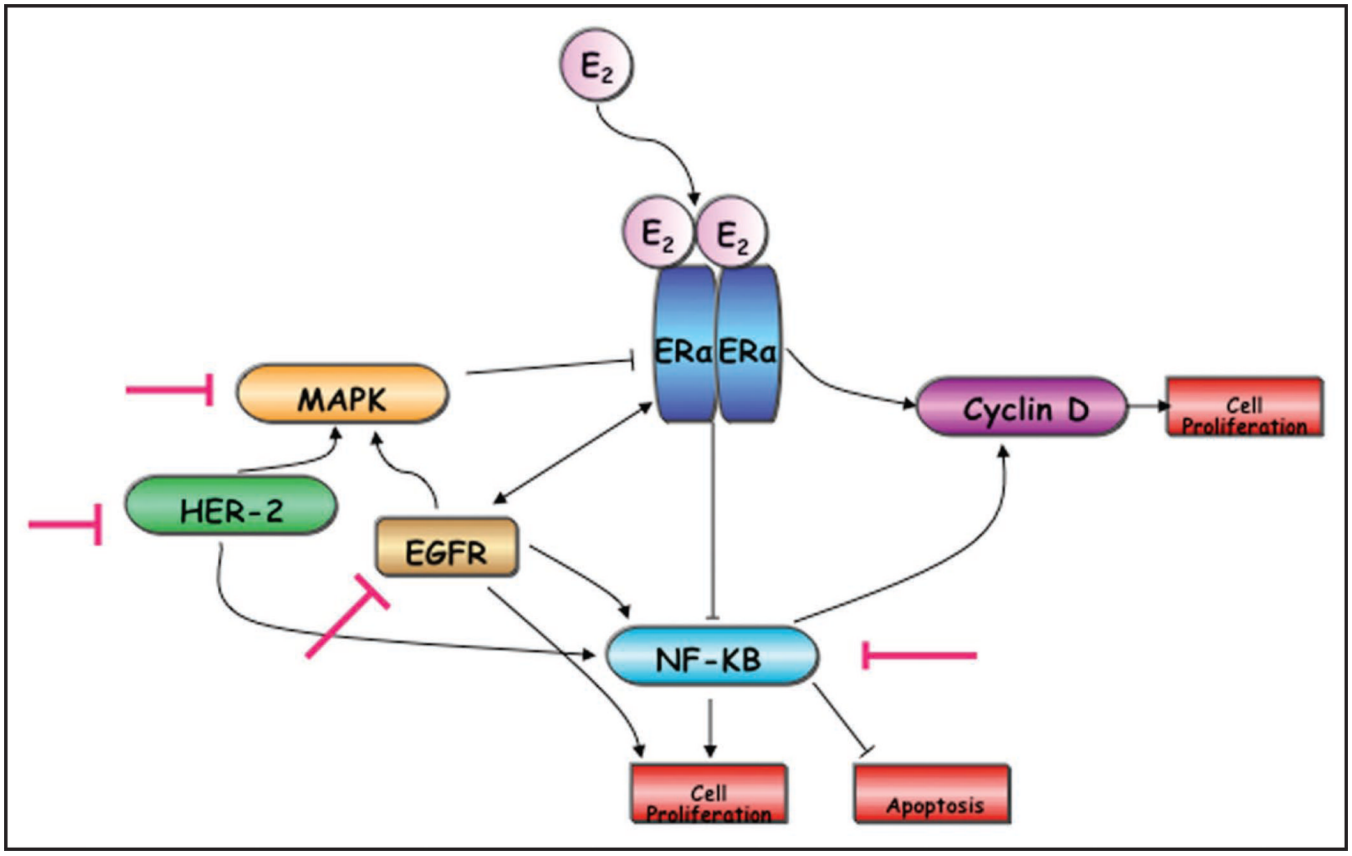

Figure 2.

Crosstalk between estrogen receptor-α (ERα) and growth factor signaling: overexpression of growth factors is a proposed cause for suppression of ERα expression. E2 bound ER modulates expression of estrogen response genes like cyclin D while its non-genomic targets include inhibition of nuclear factor-κB (NFκB). Activation of epidermal growth factor receptors like EGFR and HER2 (as a consequence of ERα downregualtion) triggers activation of mitogen activated protein kinases (MAPKs) and NFκB. MAPKs are implicated in repression of ERα expression and in enhanced ERα independent cell proliferation. Red T symbol indicates potential targets for inhibition that may result in ERα re-expression.

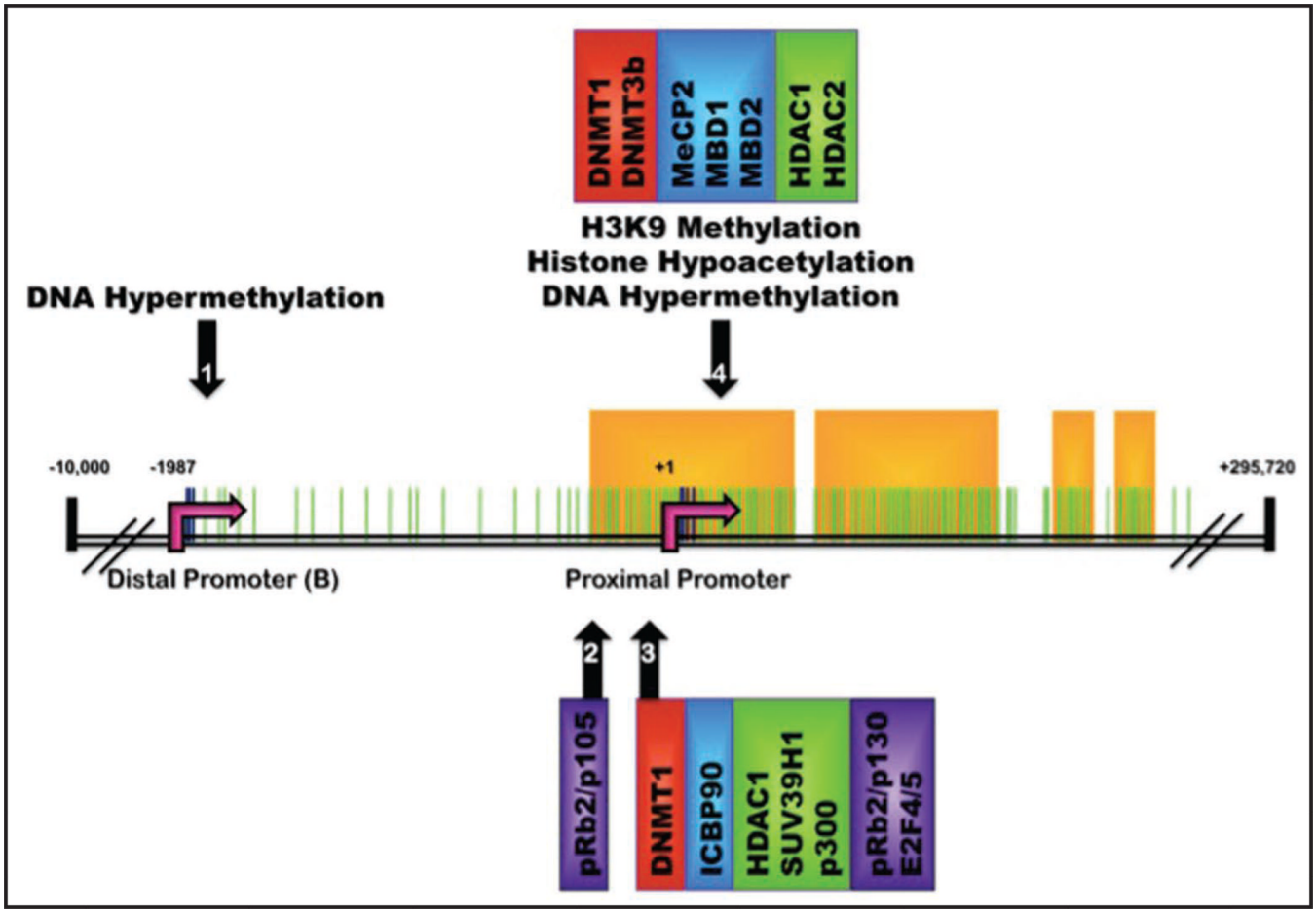

Figure 3.

Methylation sites and epigenetic complexes identified at the estrogen receptor α promoter: Schematic of the ERα gene (ESR1) on chromosome 6q25.1. CpG dinucleotides located on ERα promoter region from −2000 to +2000 are depicted by green lines. Specific CpG residues demonstrated to be sites of potential CpG methylation are marked by dark blue lines and are situated at the distal (B) and proximal promoters (identified by pink arrows at −1987 and +1 respectively). Yellow boxes designate predicted CpG islands (as defined by www.urogene.org/methprimer) which identify four regions of dense CpG population located at the proximal promoter (nucleotides −280 to +359) and just upstream of the proximal promoter (residing at nucleotides +443 to +1140; +1380 to +1427; and +1463 to +1563). Promoter regions determined to contain specific DNA and histone modifications are marked by numbered black arrows. Epigenetic regulators associated with each specific promoter region include DNA Methyltransferases (red boxes), Methyl-CpG-Binding Proteins (blue boxes), histone modifying enzymes (green boxes) and other transcriptional repressors (purple boxes).

Table 1

Epigenetic Regulation of ERα in Normal and Malignant Human Breast Cell Lines

Cell line (ref)	ER expression	Methylation proximal promoter	Methylation distal (B) promoter	Cell line origin	Epigenetic factors associated with specific ER promoter regions
MCF10A (98, 99)	−	−	NE	F	NE
MCF12A (98)	+/−	−	NE	F	NE
MCF7 (100, 119, 120, 133)	++	−	+/−	IDC	<2> pRB2/p105, p107 <3> pRB2/p130-E2F4/5-HDAC1-SUV39H1-p300 <3> ICBP90
T47D (101)	++	−	−	IDC	NE
ZR-75-1 (101)	+	−	+	IDC	NE
SKBR3 (102)	−	+	NE	IDC	NE
Hs578t (19)	−	+	NE	IDC	NE
MDA-MB-468 (19)	−	+	NE	IDC	NE
MDA-MB-231 (19, 101, 119, 133)	−	+	+/−	IDC	<2> pRB2/p105, p107 <3> pRB2/p130-E2F4/5-HDAC1-SUV39H1-DNMT <3> ICBP90 <4> DNMT1, DNMT3b, MeCP2, MBD1, MBD2, HDAC1, HDAC2

NE, not examined; F, fibrocystic disease; IDC, invasive ductal carcinoma. Epigenetic factors categorized by color; DNA Methyltransferases (red), Methyl-CpG-binding proteins (blue), histone modifying enzymes (green) and other transcriptional repressors (purple). <1>,<2>,<3> and <4> designate specific promoter regions (as identified by the black arrows in Fig. 3) found to be associated with unique sets of epigenetic factors.