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Breast Cancer: From Transcriptional Control to Clinical Outcome

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Abstract

Breast cancer is the most common malignancy in women worldwide. The risk of breast cancer in women increases with age, and this is partly attributable to the accumulation of genetic lesions. Growing evidence demonstrates the role played by epigenetic modifiers and the tumor microenvironment in contributing to the increased risk of breast cancer. This chapter provides a comprehensive overview of the epigenetic regulatory signatures that impact the well-studied signaling pathways in breast tissues. Additionally, we will also delve into the therapeutic and diagnostic potential of noncoding RNAs in breast cancer.

Keywords: epigenetic control, noncoding RNA, estrogen receptor α , DNA methylation

1. Introduction

Tumorigenesis is a multistep process that involves accumulation of genetic mutations which confer a selective growth advantage to the cancer cells. However, an emerging area of research suggests that epigenetic changes complement these genetic mutation events and direct the cancer cells towards a full blown malignancy [1–3]. Epigenetic changes refer to the modifications that do not occur on the primary nucleotide sequence of DNA (genetic mutations) but rather affect chromatin structure and function and are reversible in nature. Epigenetic changes involve histone modifications by enzymes that can “write” marks on histone tails such as acetyl and methyl transferases, enzymes that can “erase” these marks such as demethylases and deacetylases and a group of proteins that can “read” the chromatin marks and recruit other proteins to alter gene expression [4].

A recent study in mammary epithelial cells that are on the road to tumorigenic transformation has revealed a coordinated series of events that alter DNA methylation and deregulation of histone marks across large regions of the chromatin [5], thus underlying the need to study these epigenetic modifications to address their diagnostic as well as therapeutic potential in the context of breast cancer. Breast cancer is the most common cause of cancer in women worldwide. It is a complex, heterogeneous disease, thus posing a challenge in the diagnosis and treatment of patients. At the molecular level, based on the gene signature obtained from cDNA microarrays and global mRNA expression studies, breast cancer has been classified into four basic types, namely Luminal A, Luminal B, HER2-enriched, and triple negative/basal-like subtype [6–10]. This classification is based on the molecular characteristics displayed by the tumor, such as hormone receptor status, additional marks such as cytokeratin 5 (CK5) and cell proliferation rate (Ki67 marker¹ status; summarized in **Table 1**). These subtypes, along with displaying unique molecular signatures, also differ in their prognosis and response to treatments. Apart from the aforementioned mRNA markers, recent studies have highlighted the importance of miRNAs in subtyping breast tumors as well as providing directions for diagnosis, prognosis and therapy [11, 12].

However, despite several years of study, a broad-spectrum curative therapy for patients with malignant breast cancer remains elusive. This chapter will focus on key epigenetic regulators including noncoding RNAs identified in breast cancer that affect the hormonal signaling pathways and provide a perspective on combinatorial drug treatments using drugs that target these epigenetic regulators along with tamoxifen, aromatase inhibitors and other conventional therapeutics in specific sub-types of breast cancer.

Breast cancer molecular subtype	Characteristics	Prevalence	Treatment response and clinical outcome
Luminal A	ER positive and/or PR positive HER-2-negative Low Ki67	30–70%	Hormone therapy, chemotherapy; good prognosis and patient survival
Luminal B	ER positive and/or PR positive HER-2 positive (or HER-2 negative with high Ki67)	10–20%	Hormone therapy, chemotherapy; fairly high survival rates, though not as high as Luminal A
HER-2	ER negative PR negative HER-2 positive	10–15%	Trastuzumab and anthracycline-based chemotherapy; generally poor prognosis
Triple negative/basal/basal-like	ER negative PR negative HER-2 negative	5–15%	Platinum-based chemotherapy and PARP inhibitors; generally poor prognosis

Table 1. Summary of the common molecular subtypes of breast cancer with their characteristics, disease prevalence and treatment response [6].

¹Ki67 marker: Ki67 is a nuclear protein which is used as a marker for proliferation. It is associated with ribosomal RNA synthesis and thus serves as a proliferation marker. It is present in all cycling cells (G1, S, G2 and M phase) but it is absent in Go phase cells.

2. Epigenetic alterations in breast cancer

Each cell in our body contains the genetic material in the form of DNA, which is the essential blueprint required for all cellular functions. DNA is packaged into chromatin by wrapping around basic histone proteins to form nucleosomes. These nucleosomes are further condensed into the nucleus to form the chromatin by enzymes that catalyze posttranslational modifications on the histone tails. The chromatin serves to not only condense the DNA within the cellular nucleus but also to control how information in the DNA is retrieved [13]. The histone components of the nucleosomes include a pair of H2A-H2B dimers and a tetramer of H3 and H4 to form the histone octamer around which the DNA is wound. These core histone proteins undergo a wide variety of posttranslational modifications such as acetylation, methylation, ubiquitination, phosphorylation, sumoylation, deamination and ribosylation, to name a few [14]. Since histones regulate accessibility of the DNA to transcription factors and DNA-modifying enzymes, alterations in the structure and posttranslational modifications of histones affects cellular gene expression to a great extent. Enzymes that covalently modify histones, acetyltransferases, methyltransferases and kinases, thus regulate multiple cellular processes that require accessibility to the DNA such as transcription, DNA replication and repair, apoptosis and cell cycle progression [15] (**Figure 1**). It is thus unsurprising that aberrant expression of many epigenetic regulators is prevalent in cancer tissues and contributes to the tumorigenesis process. By altering their epigenetic circuitry, cancer cells overcome the barrier of replicative senescence, accumulate genomic instability and catapult into an organized chaos that is the cancer epigenome (**Figure 2**). This makes it imperative to study the role and activity of proteins involved in epigenetic regulation of gene expression in the context of tumorigenesis. An important attribute of the chromatin-modifying enzymes is that the reactions catalyzed by these molecules such as histone acetylation are easily reversible and thus offer a therapeutic window of opportunity.

Emerging evidence indicates the role played by somatic mutations in the carcinogenesis process. A study by Stephens et al. highlighted the significance of these somatic mutations in the context of breast cancer [16]. Their study which sequenced the genome of 100 tumors for changes in somatic copy numbers and mutations identified point mutations and deletions in known cancer-causing “driver” genes characterized in the context of mammary carcinomas such as *PTEN*, *BRCA1*, *TP53*, *RB1* and *AKT1*. The highlight however was the identification of inactivating somatic mutations in epigenetic regulators such as *ARID1B* and *SMARCD1*, suggesting an altered epigenetic landscape in these tumors [16]. The reversible nature of epigenetic changes and their dynamic role in regulation of cellular gene expression in a tissue specific manner makes them potent tumor stimulating factors and reiterates the need to find suitable “druggable” epigenetic factors to serve both as a biomarker as well as a therapeutic target for the various molecular subtypes of breast cancer [17].

This section will discuss the epigenetic signature, histone posttranslational modifications as well as DNA methylation changes, characterized thus far in the various subtypes of breast cancer and will provide an overview of targeting these chromatin modifiers as a potential combination therapy.

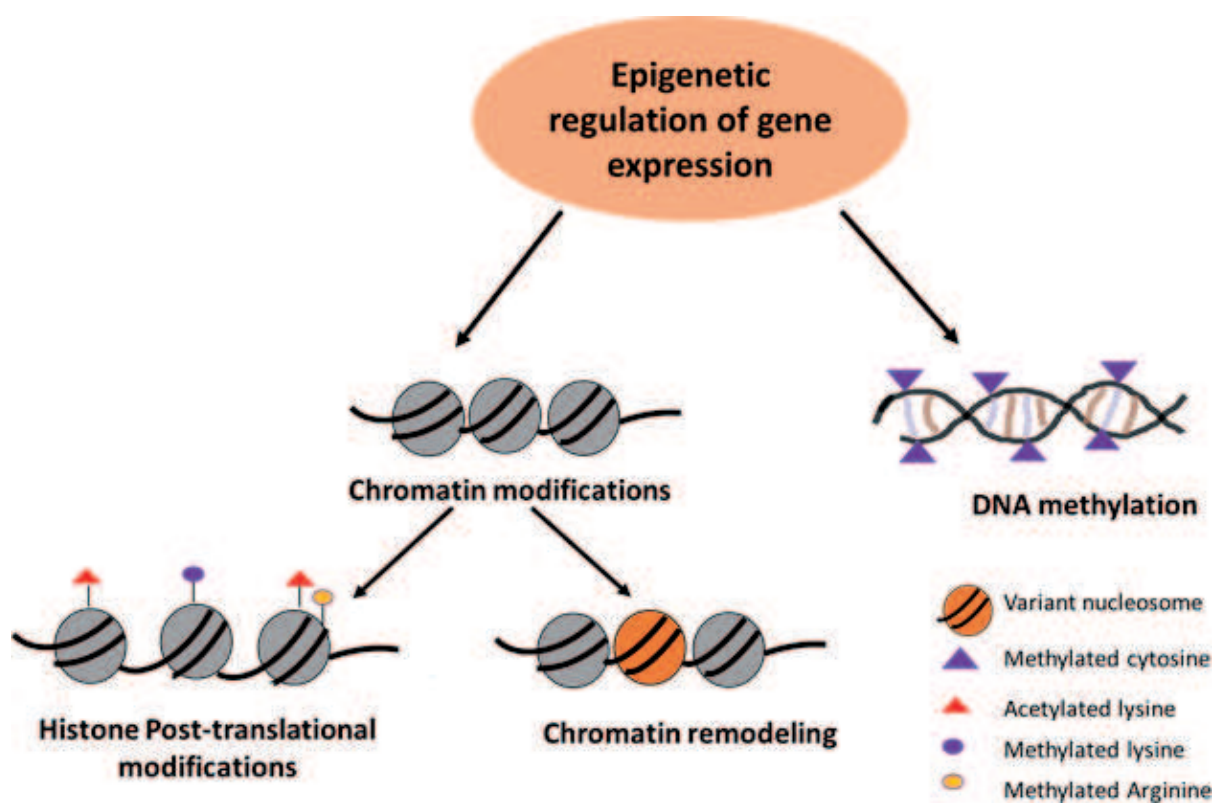


Figure 1. Epigenetic regulatory circuits in cells. A schematic representation of the epigenetic changes which regulate gene expression in normal cells.

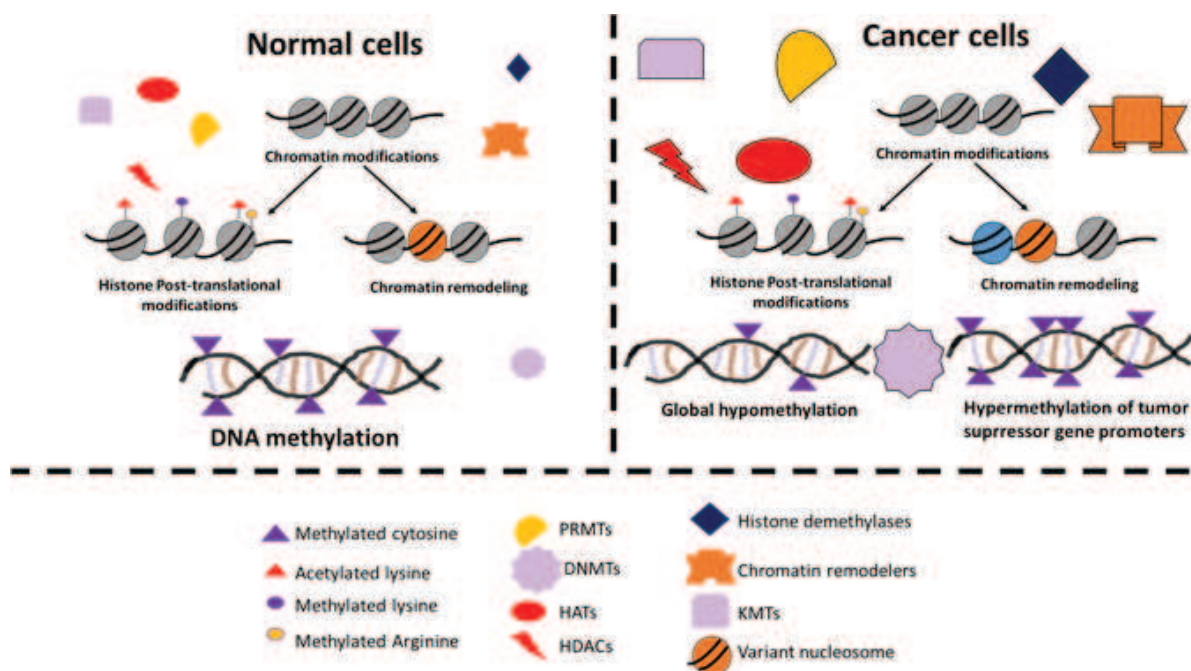


Figure 2. Altered epigenetic pathways in tumorigenic cells. Schematic depiction of the altered epigenetic landscape in cancer cells. Orange nucleosome represents a variant nucleosome which could be introduced as a result of aberrant expression and function of chromatin remodelers. Altered expression and function of HATs, HDACs, DNMTs, KMTs and KDMTs (represented as different sized icons the figure) results in a widespread disarray of the epigenetic marks in cancer cells.

2.1. Histone modifications and histone-modifying enzymes in breast cancer

2.1.1. Aberrant histone acetylation

Histone acetyltransferases (HATs) conventionally play an important role in the activation of gene expression by resulting in an open chromatin structure thus providing access for the transcription machinery to the DNA. There are different families of HATs identified thus far and their role in acetylating histones has been extensively studied. Histone acetylation is regulated by the activity of HATs as well as the histone deacetylases (HDACs), which remove the acetyl moieties from lysine residues. The acetylated lysines are read by reader proteins containing bromodomains (such as BRD2, BRD3 and BRD4) and depending on the complexes recruited by these “readers,” gene expression can be switched on or off [4].

In breast cancer, a study by Elsheikh et al. has identified low levels of the histone marks, H3K9Ac, H3K18Ac, H4K12Ac and H4K16Ac, to correlate with poorer prognosis and is associated with basal and HER2-positive tumors. This study has also detailed the status of methylation on H3, which will be discussed in the following sections [18]. This altered epigenetic signature is hypothesized to be due to altered enzymatic activities of the HATs and HDACs, which could be attributed to their dysregulated expression. There are multiple lines of evidence now to support this hypothesis. A ubiquitously expressed acetyltransferase p300/CBP, which is also known to function as transcriptional coactivator, was identified to be overexpressed in breast carcinoma as compared to adjacent normal mammary epithelia. Further, this study also showed that higher expression of p300 as studied by immunohistochemistry from a tissue microarray correlates with poorer prognosis-free survival and increased tumor recurrence [19]. However, it is unclear whether the role of p300 as a histone acetyltransferase or a lysine acetyltransferase (acetylating other non-histone proteins) is involved in this function and remains an interesting avenue for future studies.

Another acetyltransferase, TIP60, belonging to the MYST (MOZ, Ybf1, Sas2, TIP60) family of acetyltransferases is known to undergo mono-allelic losses in breast carcinomas as well as in head and neck tumors [20]. Low nuclear expression of TIP60 as evidenced by IHC correlates with higher tumor grade in breast cancer [20], suggestive of a tumor suppressive role played by this epigenetic regulator. One of the histone targets of TIP60 is the acetylation of Histone H4 at K16. A significant global reduction in histone H4 acetylation and lysine trimethylation has been observed across most cancer types including breast cancer [21]. This loss of mono-acetylation was identified to be due to a reduction in the acetylation status of K16 and not the other putative mono-acetylated lysine on Histone H4 (K5, K8, K12 which are targets of p300/CBP). Other acetyltransferases capable of acetylating K16 on H4 are MOZ (monocytic leukemia zinc finger), MOF (male absent on the first) and MORF (MOZ-related factor). This study also identified the sequence specific loss of recruitment of MOZ, MOF, MORF in cancer cells as compared to the normal cells to the DNA repetitive elements associated with loss of H4K16 acetylation (H4K16Ac) and H4K20 trimethylation (H4K20me3) [21]. In addition, independent studies have identified MOF mRNA and protein expression to be downregulated in breast carcinomas, and this was correlated with the reduced level of H4K16Ac acetylation in these tested primary breast carcinomas [22].

The dysregulated histone acetylation in cancer can also be explained by changes in expression and function of histone deacetylases (HDACs). In breast cancer, HDAC1, HDAC2 and HDAC3 are identified to be differentially expressed as compared to the normal tissue and overexpression of HDAC2 and HDAC3 strongly correlates with a more aggressive tumor type, that is, negative hormone status [23]. This offers the opportunity of treating breast cancers with inhibitors of HDAC to restore acetylation level and suppress the tumorigenesis, and this approach will be detailed in the last part of this section which addresses the therapeutic implication of targeting the epigenetic regulators.

2.1.2. Aberrant histone methylation

Histones can be methylated (mono, di or tri) by enzymes that catalyze the transfer of methyl moiety to the lysine or arginine residues on the histone tails. The enzymes involved are known as histone methyltransferases (HMTs), while another class of enzymes, the histone demethylases (HDMs), is involved in erasing the methyl groups from the histone tails. The dynamic regulation between the HMTs and HDMs regulates the methylation status in the cells, thereby regulating cellular gene expression.

Studies have identified widespread changes in histone methylation in cancer cells as compared to the nontumorigenic counterparts. There is a global reduction in H4K20me3 in multiple cancer types including breast cancer [21]. Global reduction in H4K20me3 was also observed in human breast cancer cell lines compared to the nontumorigenic cells [24]. Further, in an established model of breast cancer in rats, there was a global decrease in H3K9 trimethylation (H3K9me3) and H4K20me3 indicating that these epigenetic dysregulations play an important role in tumorigenesis [25]. In addition, another study has identified low levels of histone methyl marks, H3K4 dimethylation (H3K4me2), H4K20me3 and H4 Arginine dimethylation (H4R3me2) in human tumors, and these were found to correlate with poorer prognosis and more aggressive subtypes of breast cancer such as Luminal and HER2-positive tumors [18]. These global alterations in the level of methylation on histones are suggestive of an imbalance in the expression of methyltransferases as well as the demethylases.

In support of this notion, a variety of histone methyltransferases have been identified to be aberrantly expressed in breast tumors. Frequent overexpression and amplification of the histone methyltransferase NSD3L have been observed in mammary carcinomas, and depletion of this enzyme decreased the invasiveness of breast cancer cells highlighting its potential as an oncogene. However, the targets of NSD3L-affecting tumorigenesis have not been elucidated in detail [26, 27].

Enhancer of zeste 2 (EZH2) a methyltransferase that is a part of the Polycomb Repressive Complex 2 (PRC2) is found to be overexpressed in breast cancer, both at mRNA and protein level. The high expression of EZH2 is correlated with more aggressive cancer and a poor prognosis for patients. Overexpression of EZH2 in normal breast epithelia promotes anchorage independent growth, cell invasion, characteristics of a neoplastic phenotype in these cells, which is dependent on the suppressor of variegation 3-9 (Su(var)3-9), enhancer of zeste (E(z)), and trithorax (Trx) (SET) domain of EZH2 and HDAC activity [28]. This study paved the way

for many other groups to investigate the role of EZH2 enzymatic activity mediated by the SET domain, conventionally known to silence gene expression, in the context of breast carcinomas. H3K27 di and tri methylation are characteristic of Polycomb Group (PcG) target genes and are associated with transcriptional silencing. The PRC2 complex of which EZH2 is the catalytic subunit with the other members being EED and Suz12 is involved in dimethylation and trimethylation of H3K27. The SET domain of EZH2 can function as an N methyltransferase, that is, EZH2 by utilizing S-adenosyl methionine (SAM) as a cofactor can add methyl groups to the lysine residues of substrate proteins. SET domain containing methyltransferases bind SAM and the substrate on opposite sides of the active site of the enzyme, thus SAM can dissociate without interrupting substrate binding to enzyme, resulting in multiple methylations on the lysine residues [29, 30]. In breast cancer cell lines, increased EZH2 expression resulted in the down-regulation of a tumor suppressor, RUNX3. This was identified through chromatin immunoprecipitation to be due to the H3K27me3 at RUNX3 promoter and associated HDAC1, since depletion of EZH2 resulted in the loss of H3K27me3 and HDAC1 from this promoter and increased expression of RUNX3, which was associated with significantly lesser cell growth as compared to the siRNA control [31]. In addition, EZH2 also results in down-regulation of another potential tumor suppressor, FOXC1, a transcription factor that has a role in differentiation and reduces cell migration and invasion. By trimethylating H3K27 at the FOXC1 promoter, EZH2 shuts down the expression of this transcription factor in a highly metastatic breast cancer cell line, MDA-MB-231 [32]. EZH2 is also known to repress RAD51, a protein involved in DNA repair and *CDH1* (E-cadherin), a marker for epithelial cell type, loss of which results in increased invasiveness [33]. However, recent studies have also shown that increased EZH2 expression does not necessarily correlate with the H3K27me3 abundance. In particular, high expression of EZH2 was found in basal-like, HER2-positive and triple-negative tumors, while high H3K27me3 was found in normal-like (ER-negative), HER2-positive and Luminal A type tumors [34]. A possible explanation for this anomaly could be a non-canonical catalytic activity independent function of EZH2. In triple-negative breast cancer (TNBC), it has been reported that EZH2 functions as an activator of NOTCH signaling. EZH2 overexpression could increase *NOTCH1* expression and accelerate mammary tumorigenesis in mice. It can bind to *NOTCH1* promoter, a function that is independent of its ability to methylate histones [35]. This opens new doors to discover other functions of this epigenetic regulator in mediating tumor progression by regulating nonhistone targets or affecting chromatin structure or in a manner independent of its catalytic function.

JARID1C, a histone demethylase, is also known to be upregulated and correlates with increased metastasis in breast cancer lesions compared to the normal counterparts. Mechanistically, JARID1C by modulating H3K4me3 at the promoter of breast cancer metastasis suppressor 1 (*BRMS1*) represses the expression of *BRMS1* and depletion of JARID1C results in reduced migration and invasion of breast cancer cells [36]. Enzymes belonging to the demethylase family of KDM4 are seen to be overexpressed in breast cancer and affect cell proliferation and growth of these cells [37]. KDM3A, a histone demethylase, which demethylates H3K9 mono and di-methyl moieties, works as a positive regulator of estrogen receptor (ER) activity. The catalytic activity of this enzyme is essential for ER target gene expression and growth of the cells, highlighting the significance of this methylation status in promoting tumorigenesis [38].

2.1.3. Other histone modifications

Phosphorylation of histones is another posttranslational modification, which occurs on histone tails and involves the kinase enzymatic activity. Serine, threonine and tyrosine residues on histone tails are known to be phosphorylated. H3S10 phosphorylation which marks the entry of the cell into mitosis is catalyzed by the enzyme Aurora B Kinase. Elevated expression of this kinase in several cancers is correlated with a poor prognosis for survival; however, it is not determined if this is due to the phosphorylation of H3S10 resulting in increased proliferative ability of cancer cells [39]. Ubiquitination is yet another posttranslational modification found on histones. Mono-ubiquitination of H2B (H2Bub1) is found to be globally reduced, and this is true in the context of breast cancer as well. Proteasome inhibition can reduce ER α -mediated transcription, and this was due to reduction in H2Bub1 levels, which correlated with reduced transcription of ER target genes [40].

2.2. Chromatin remodelers in breast cancer

The chromatin compacts the DNA into the nucleus, and this regulates the accessibility of the wound DNA to transcription and repair machinery. One of the ways through which the chromatin is regulated has been discussed in the preceding section and involves extensive posttranslational modifications on histone tails. Apart from this, the locus-specific DNA methylation status can help in the recruitment of enzymes that alter chromatin structure, and this has also been discussed. Another way to regulate chromatin structure and function is by physically altering the nucleosome location or composition, and this process is known as chromatin remodeling. The groups of enzymes involved in restructuring the chromatin by this mechanism are referred to as chromatin remodelers and are further classified into different families depending on the associated cofactors. All chromatin remodelers utilize the energy of ATP hydrolysis to catalyze the reactions that affect histone-DNA interactions [41].

Remodelers are involved in mobilizing nucleosomes across the genome and regulate chromatin organization. They facilitate proper placement of nucleosomes whenever the DNA is accessed, for instance, before and after replication, repair and transcription. Remodelers also slide or evict nucleosomes and can replace them with a nucleosome that contains a histone variant. A common example is the histone variant H2AZ found flanking the transcription start site [41]. All these functions of remodelers suggest the important underlying role played by this group of epigenetic regulators in controlling basic cellular processes such as transcription, chromatin assembly and DNA repair. Thus, it is not surprising that the altered expression or localization of these chromatin remodelers is correlated with tumorigenesis.

There are several families of chromatin remodelers such as SWI/SNF, INO80 and CHD complexes, all of which are implicated in different cellular processes. Mutations in the SWI/SNF family of chromatin remodelers are found in about 20% of cancers, and some of these mutations could have a gain-of-function phenotype while in the case of breast cancer as well as in leukemia, wild-type SWI/SNF complexes by their diverse protein interactions aid tumor progression [42]. The bipolar function of this important class of chromatin remodelers also implicates the dynamic range of functions of remodelers and the myriad of their cellular interacting

partners, which assist their aberrant functions in cancer cells. In breast cancer, a member of the NuRD complex, a part of the CHD family of remodelers, is known to be aberrantly expressed. Metastasis-associated proteins (MTA-3) are associated with ER-positive breast cancer, and increased expression of these MTA-3 is correlated with increased ER expression as well as invasive behavior. MTA-3 can directly repress *SNAIL* transcription in response to the ER stimulus; therefore, a decrease in either ER expression or MTA-3 expression results in increased metastasis due to aberrant *SNAIL* expression [43].

ARID1A, a member of the human SWI/SNF complex, is known to undergo frequent mutations across many cancer types. In breast cancer, *ARID1A* mRNA expression is seen to be downregulated in tumors as compared to adjacent normal tissues. This decreased expression was correlated with bigger tumor size and with the triple-negative breast cancers. Immunohistochemical staining also revealed a similar correlation between the protein level of ARID1A and tumor stage as well as the triple-negative tumor type [44].

Another member of the SWI/SNF complex, BAF155/SMARCC1 (*BRG1*-associated factor 155/SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily C member 1) has been identified to play a critical role in breast cancer tumorigenesis in concert with a protein arginine methyltransferase coactivator-associated arginine methyltransferase 1 (CARM1). CARM1 is highly expressed in many cancers such as breast and prostate with the levels of CARM1 higher in metastatic breast tumors as compared to the primary lesions. CARM1 is also implicated in the growth and proliferation of breast cancers by functioning as a coactivator of the steroid hormone receptors [45]. The arginine methylation of BAF155 at R1064 by CARM1 recruits BAF155 to a unique set of target genes such as genes in the c-MYC pathway. Intriguingly, there was a higher expression of both total and methylated BAF155 as observed by IHC in metastatic tumors, which were also associated with increased expression of CARM1 in these tumor samples. Patients with higher methylated BAF155 had a higher risk of tumor recurrence and poorer prognosis with a hazard ratio² very similar to the aggressive triple-negative breast tumors [46]. This and other studies show the significance of chromatin reorganization and altered recruitment/function of chromatin remodelers to be an important hallmark of cancer cells and the need to target these reversible epigenetic changes for development of more specific therapeutics.

Other examples of SWI/SNF family implicated in breast carcinogenesis are the Brahma and Brahma-related gene 1 (*BRM* and *BRG1*) that are overexpressed in breast tumors. Knockdown of either *BRG1* or *BRM* reduced the proliferation of breast cancer cells, whereas a combined knockdown resulted in additive effect, suggesting independent pathways regulated by these chromatin remodelers in breast cancer progression [47].

A genetic mutation in *BRCA1* predisposes women to ovarian cancer and significantly increases the risk for development of breast cancer. *BRCA1* is predominantly found in cells in a complex with members of the SWI/SNF family that is involved in chromatin remodeling. *BRCA1*

²Hazard ratio: it is the ratio of the hazard rates of two groups being compared, that is, ratio of how often an event happens in one group compared to the other. In clinical trials, hazard ratios represent survival in a group of patients treated with a drug at any point of time with the other group given a placebo/different treatment. A hazard ratio of 1 indicates no difference in survival while a ratio greater or lesser than 1 indicates one of the groups has a better survival.

directly interacts with BRG1, and this interaction is essential for *BRCA1* transcriptional coactivation function. A dominant negative mutation of *BRG1* or the deletion of exon 11 of *BRCA1* (implicated in cancer) results in abrogation of p53 stimulated *BRCA1* transcriptional activity. This underscores the importance of chromatin organization and remodeling by *BRG1* and *BRCA1* to control *BRCA1* transcriptional activity, loss of which results in increased risk for tumor development [48]. Additionally, interaction of mutant p53 with the SWI/SNF complex leads to open chromatin structure at the Vascular Endothelial Growth Factor Receptor 2 (*VEGFR2*) promoter in breast cancers that leads to the upregulation of *VEGF2*. This aids the growth of cancer cells in two and three dimensional cultures. Thus, mutant p53 by regulating a major family of chromatin remodelers promotes tumor progression [49].

2.3. Aberrant DNA methylation in breast cancer

DNA methylation is another form of epigenetic regulation that involves the addition of a methyl moiety to the 5' cytosine of a CG dinucleotide, which are distributed across our genome and are enriched at the gene promoters to form the Cytosine preceding Guanine (CpG) islands. DNA methylation is conventionally associated with gene silencing due to the steric blocking of transcription factors by the methyl moieties, thereby preventing gene expression. In addition, methyl binding proteins such as MeCP2, MBD2 and MBD3 which can physically interact with both DNA methyltransferases as well as histone methyltransferases (Suv39h1 which adds H3K9me3), HDACs and Heterochromatin protein 1 (HP1), recruit this repressive complex to synergistically shut off gene expression of genes with methylated promoters [50, 51]. The enzymes involved in DNA methylation are the *de novo* methyltransferases DNMT3a and DNMT3b which establish new silencing patterns in response to environmental cues and the maintenance methyltransferase DNMT1 which is responsible for maintaining the heritable silencing patterns [50].

Abnormal changes in DNA methylation patterns are widespread across all cancer types including the breast cancer genome. Paradoxically in cancer, there are two distinct aberrations—a global hypomethylation observed as a result of an increased expression of demethylases and gene-specific hypermethylation events possibly due to the inaccessibility of the demethylases to the chromatin structure, both of which could contribute to tumorigenesis [52, 53].

In breast cancer too, a specific cohort of genes is known to be hypermethylated, and therefore, their expression is turned off. This happens at promoters of potential tumor suppressor genes involved in regulation of cellular proliferation, invasion, and metastasis. A few examples of such genes are *CDH1* (E-cadherin), *BRCA1*, *14-3-3σ*, *ERα*, *ERβ*, *RARβ* and *TIMP3* [54].

The importance of methylation in regulating gene expression in a cell- and tissue-specific manner becomes evident on analysis of breast tumor samples for DNA methylation. Different studies by performing methylation specific PCRs have described the concept of methylation index,³ which is a ratio of genes hypermethylated to the total number of genes studied. It is

³Methylation index (MI): It is a reflection of the fraction of genes methylated. It is a ratio of the total number of genes methylated to the total number of genes analyzed. MI can be used to predict the risk of cancer.

observed that a higher methylation index correlates with a poorer prognosis and increased risk of recurrence of breast cancer [55]. Of more clinical significance is the finding that promoter hypermethylation events can be detected from patient serum samples. In a study by Wong et al., the authors, from peripheral blood samples determined that *BRCA1* promoter methylation increases the risk for development of breast cancer by 3.5 fold [56]. Another study has identified genome-wide differential methylation of CpGs in breast tumors compared to normal breast tissues. Interestingly, this study has also identified the differences in methylation between different molecular sub types of breast tumors, highlighting that the altered epigenetic circuitry could result in a different outcome for the disease. While Luminal B, Luminal A and HER2-positive tumors were extensively methylated in the CpGs, the basal-like tumors showed a distinct methylation pattern compared to the other sub types. The DNA methylation in Luminal A and HER2 tumors was more heterogeneous reiterating that breast cancer as a disease is constantly evolving and dynamic. They identified a distinct signature of DNA methylation in Luminal B tumors which are principally associated with CpG methylation at promoters, while, in contrast, basal-like tumors are marked by hypomethylation events in the gene body. This has led to classifying tumors from Luminal A, HER2 subtypes which exhibit signatures of CpG methylation similar to either Luminal B or basal-like as Epi-LumB or Epi-Basal respectively, highlighting the importance of these epigenetic changes in underlying tumor progression. Both Epi-LumB and Epi-Basal types of tumors were significantly correlated with increased tumor size, and Epi-LumB type tumors were found to be associated with shorter patient survival times [57]. This indicates the potential for the use of DNA methylation events in a clinical context for diagnosis and personalized treatment as well as targeting these methylation marks for discovery of therapeutics.

As discussed earlier, hypomethylation events in cancer are also associated with a poorer prognosis. The demethylation of tumor supportive genes that aid proliferation and metastasis such matrix metalloproteases-9 (MMP9) and urokinase plasminogen activator can in part explain this paradox about both hypomethylation and hypermethylation events in breast cancer increasing tumorigenic potential. Treatment of nonmetastatic breast cancer cells with demethylating agents increases their metastatic potential, while, in contrast, treatment with agents that reverse demethylation decreases the invasive capacity of breast cancer cells [58–60].

Apart from 5-methylcytosine, other methylation modifications on DNA include 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), all of which are regulated by Ten-Eleven Translocation (TET) proteins. TET proteins, in an α -Ketoglutarate and Fe(II)-dependent manner, catalyze the oxidation of the methyl groups on DNA, and these modifications could also function as intermediates in the demethylation of DNA [61, 62]. There are three known TET members identified in mammals, which include TET1, TET2 and TET3. They vary in structure and thus catalyze the oxidation reactions with varying efficiencies [63]. Inactivating mutations in *TET2* have been identified in about 15% of myeloid tumors and in patients with myelodysplastic syndromes [64, 65]. Studies by different groups have identified a significant reduction in 5hmC levels in solid tumors including breast, colon, lung, pancreatic, prostate, colon and gastric tumors [66, 67]. Interestingly, these reductions in 5hmC levels were accompanied by a significant decrease in expression of *TET1*, *TET2* and *TET3* in liver and breast carcinomas compared to the adjacent normal epithelia [66].

In the context of breast cancer, 5hmC levels are known to be deregulated. An example is the lower level of 5hmC mark at the promoter of a prominent tumor suppressor, Leucine zipper, putative tumor suppressor 1 (*LZTS1*) in breast cancer patient samples compared to normal breast tissues from healthy individuals. This results in a lower expression of *LZTS1*, and this lower expression as well as reduced *TET1* expression was correlated with 5hmC levels in the tumors. Further, lower levels of 5hmC were also associated with unfavorable prognosis and lymph node involvement [68]. High mobility group AT-hook2 (*HMGA2*), a chromatin remodeler, which is known to be overexpressed, regulates the expression of *TET* in breast cancers. Knockdown of *HMGA2* in both cell lines and mouse breast tumors induces the expression of *TET1*. *TET1* in turn demethylates its own promoter as well as Homeobox A (*HOXA*) genes such as *HOXA7* and *HOXA9*. This induces the expression of *TET1* as well as the *HOXA* genes which together suppress the breast cancer growth and metastasis in mouse xenograft models [69]. This study also uncovers the potential of using the novel *HMGA2/TET1/HOXA9* axis as a prognostic tool for breast cancer patient survival [69]. Further, under hypoxic conditions, which are a characteristic of many solid tumors including breast tumors, studies have shown an increase in expression of *TET1* and *TET3*. The expression of *TET1* and *TET3* correlated with tumor hypoxia in patient samples and poorer prognosis and survival. Under hypoxic conditions, *TET1* and *TET3* proteins demethylate promoter of *TNF- α* and thus activate the *TNF- α* -p38-MAPK signaling pathway and thereby contribute to tumor progression *in vitro* and *in vivo* [70].

The complexity of epigenetic regulation by DNA methylation is evident from these numerous studies. All the studies indicate the possibility of using DNA methylation as well as DNA hydroxymethylation as predictive biomarker for breast cancer especially for early detection of these tumors. Evidence for this is provided by numerous studies which highlight that methylation signatures are more correlated with clinical patterns as compared to the gene expression and suggest a combination of these to expand the current classification and clinical prognosis predictions. Specifically, methylation pattern of promoters of *RASSF1A*, *MAL*, *SFRP1*, *BCAP31*, and *BRCA1* can be used in combination with other gene promoters to increase specificity and statistical power to predict clinical outcome [71, 72].

2.4. Targeting epigenetic regulators in breast cancer

Breast cancer, especially the triple negative subtype, is highly aggressive and needs an exhaustive list of treatment options to be made available for the patients. Understanding the dysregulated epigenetic circuitry has now made it possible to search for cures targeting the reversible marks put by the epigenetic regulators. Furthermore, methylation and acetylation marks as discussed earlier have shown immense potential to serve as candidate biomarkers, highlighting the need to monitor their levels for early diagnosis and treatment. The use of these biomarkers and screening of patients for potential biomarkers also facilitates in improving the individualized therapy and personal medicine, moving away from the conventional “one size fits all” to cater to the needs of the individual patients.

Current treatment strategies for breast cancer include surgery for removal of local tumors, adjuvant therapy in the form of chemotherapy, hormone therapy and targeted therapy.

Treatment of basal-like breast tumors involves treatment with EGFR inhibitors and PARP inhibitors [73]. However, they all suffer from drawbacks mainly due to unprecedented side effects of these drugs. The two most studied therapeutic agents, which regulate epigenetic factors, are DNA methylation inhibitors and histone deacetylase inhibitors and will be detailed in this section. The major challenge for “epi-drugs” is to recapitulate the efficient action from cell-based studies in the clinical context.

The two most used DNA methylation inhibitors are 5' Azacytidine (5-Aza) and 5-aza-2-deoxycytidine (decitabine). Treatment of ER-negative breast cancer cells with 5-Aza reactivates the expression of ER at both mRNA and protein level. In addition, preclinical evidence suggests a useful role for DNMT inhibitors (DNMTi) in breast cancer treatment. Nanomolar (nM) dose of DNMTi has resulted in reactivation of silenced tumor suppressors such as *ER*, *BRCA1* and *PTEN* in breast cancer cell lines [74]. However, there are no available clinical data for the efficacy of these drugs in breast cancer. These drugs though having improved survival of patients with myelodysplastic syndrome and low blast count AML (lower number of immature blood cells called myeloblasts or blasts for short which are not normally found in the blood) have disappointingly not shown much promise in solid tumors. Other nucleoside analogs like zebularine and antisense oligo to specifically inhibit DNMT1 (MG98) are in clinical development. There have been clinical studies that used a combination of HDAC inhibitors (HDACi) and DNA demethylating agents and have shown promise. A phase I study used a combination of decitabine and vorinostat (HDACi) in cancer patients with advanced disease and showed stabilization of the disease in seven of the 22 evaluable patients of which 2 were patients with breast cancer [75].

HDAC inhibitors function by inhibiting the activity of the enzymes responsible for catalyzing the removal of acetyl moieties from proteins, the HDACs. HDACs are divided into four classes, and current HDACi therapy focuses on inhibitors for Class I and Class II HDACs that include HDACs 1–10. The only HDAC inhibitor that has FDA approval is Vorinostat. HDAC inhibitors result in increased acetylation of histones which is associated with reactivation of tumor suppressor genes such as p21 and p27 which in turn have the potential to inhibit tumor cell growth [76]. Vorinostat can inhibit the proliferation of breast cancer cells irrespective of their ER status. Treatment of vorinostat concomitantly with another HDACi, LAQ824, sensitizes ER positive cells to tamoxifen therapy by downregulating expression of phosphorylated and total Akt (also known as Protein Kinase B [PKB] and originally identified as an oncogene from the AKT-8 retrovirus). HDAC inhibitors such as Vorinostat used in combination can enhance the effect of tamoxifen in the hormonal strategies to treat breast cancer, whereas the mechanistic studies are still exploring the pathways involved in reversal of resistance to hormonal therapy. There are several ongoing phase II trials of combination of HDACs such as vorinostat, entinostat and valproic acid (VA) with tamoxifen, chemotherapeutic agents such as epirubicin and paclitaxel, which show promising results in treatment of the metastatic disease [75].

In a notable exception to the use of HDAC inhibitors, a study found that HDAC inhibitor valproic acid (VA) stimulates the self-renewal and expansion of normal hematopoietic stem cells [77]. In addition to this, VA enables cells to be reprogrammed to induced pluripotent

stem cells [81, 82]. VA was found to have a differential effect on breast cancer cells that were differentiated *in vitro* compared to breast cancer cells that had stem cell-like characteristic, in that it radiosensitized the already differentiated cells as compared to radioprotecting the cells that had stem cell-like characteristic [83, 84]. An HDAC inhibitor as such, therefore, can lead to cancer stem cells being formed by dedifferentiating the cells that have non-stem cell-like characteristic to the ones that have the phenotype of stem cells. Chen H. et al. treated the patient-derived breast cancer cells and highly metastatic cell lines with HDAC inhibitors and found that the capacity to initiate tumor formation was high in cell lines that had non-stem cell-like characteristic, and the signaling pathway found to be involved was the WNT/ β -catenin [83]. Therefore, in summary, it is extremely important that clinical studies using HDAC inhibitors should be done with extreme caution, and all possible effects should be taken into consideration before combinatorial use in trials.

Intriguingly, these epigenetic regulators and the key aberrantly regulated pathways in breast cancer including ER α signaling share a complex dynamic, which influences the treatment regime and also directs resistance to certain therapeutics. This interplay between epigenetic control and signaling from cell surface receptors has been detailed in the following section.

3. Epigenetic control of signaling pathways in breast cancer

A cell's response to external stimuli requires the activation of a signaling cascade. These signaling cascades can be either linear or multinodal where different signal transduction pathways converge resulting in the translocation and integration of these signals into the activation or repression of gene expression [78]. Signaling pathways crosstalk among each other to regulate the gene expression patterns by modulating downstream effectors such as transcription factors, cofactors and histone modifiers. This coordinated activation of signaling pathways impacts the epigenetic landscape and plays a major role in translating a signaling event into a long-lasting molecular and phenotypic change. Analyzing the relationship between cell signaling and epigenetics is of utmost importance, as it will help us extend our vision on how a cell is able to integrate information from external and/or internal stimuli to gene expression regulation through chromatin modifications.

The combined action of a cell-type-specific transcription factor and signal effectors on regulatory elements of the genome is strongly influenced by the chromatin landscape of a given cell, resulting in the establishment of a dynamic interplay between signaling pathways and the epigenetic machinery leading to the development of different cancer types including breast cancer. Globally, most of the frequently mutated somatic genes are *ER*, *HER2*, *AKT* and *MAPK*, and these are regulated by epigenetic modifications suggesting the interplay of these regulatory networks in breast cancer tumorigenesis [79].

In this section, we will discuss the interplay between signaling pathways and epigenetic regulators with special emphasis on estrogen receptor signaling. We will highlight how chromatin modifications triggered by extrinsic signaling in breast cancer play a critical role in pathological events leading to tumorigenesis.

3.1. Epigenetics of estrogen receptor signaling

Epigenetic changes can be defined as stable molecular alterations of a cellular phenotype that are heritable during somatic cell divisions but do not involve changes in the DNA sequence. Epigenetic regulation is critical in normal growth and development and closely coordinates the transcriptional expression of genes. Estrogen refers to a family of hormones responsible for the development and regulation of the female reproductive system and secondary sexual characteristics. Estrogen is produced by the ovaries and in smaller amounts by the adrenal cortex, testes, and fetoplacental unit [80]. Although estrogen is considered to be a female hormone, it is present in both sexes. Estrogen is found in three naturally occurring forms, such as estrone (E1), estradiol (E2) and estriol (E3). Another type of estrogen called estetrol (E4) is produced only during pregnancy. The steroid 17 β -estradiol is the most potent and prevalent estrogen among the group. Estrogen is known to play an important role in a variety of biological processes. It is involved in growth, differentiation, development of brain and has an important role in reproduction [87]. Estrogen plays an important role in controlling hormonal effect; therefore, high levels of estrogen increase the risk of the development of breast cancer as high levels increase the transcription of genes known to be involved in the cell cycle regulation and metabolism pathways [88, 89].

Estrogen diffuses across the cell membrane where it binds and activates its receptor, the estrogen receptor (ER) that plays an important role in the action of estrogen. The biological effects of estrogen are mediated by its binding to the structurally and functionally distinct estrogen receptors (ER α and ER β) [81]. ER α is a member of the steroid/thyroid hormone and vitamin A/D nuclear receptor super family [82]. ER α plays a role in regulation of genes in a diverse set of target cells that are involved in the estrogen-activated pathway and is therefore also referred to as a nuclear receptor that is activated by ligand. In addition to playing a role in normal development, ER α and its ligand 17 β -estradiol have been known to be involved and are implicated in the progression of breast cancer [91]. The function of ER β has been detailed recently; however, studies to determine its role in breast cancer development and/or prognosis are still ongoing. The role of ER β in breast cancer remains elusive, but the presence of ER α at the time of diagnosis is used as an indication for endocrine therapy. Pathological estrogens have been associated with a higher risk of breast cancer as estrogen stimulation induces modifications of histones at the promoter region of ER α gene such as phosphorylation, methylation and acetylation by interacting with various enzymes of the epigenetic pathway that induces these histone modifications [88, 92]. These enzymes if deregulated lead to neoplastic transformation driven by ER α [88].

3.1.1. Mechanism of ER α -mediated histone modifications

The transcriptional outcome of ER α is regulated by a dynamic interaction of histone-modifying enzymes and associated coregulators. The multiprotein complexes containing ER α , its coactivators such as p300/CREB-binding protein (p300/CBP), p300/CBP-associated factor (PCAF) [83] and histone-modifying enzymes such as acetylases/deacetylases and methylases/demethylases assemble in response to hormone binding, resulting in transcriptional regulation

[84]. ER α exerts a positive feedback loop on expression of *CYP19A*, which is involved in the synthesis of estrogens in human placenta, thereby promoting induction of its own gene at the transcription level and contributing to local estrogen synthesis by promoting increased acetylation in the *CYP19A* promoter [85]. ER α also enhances the recruitment of metastasis-associated 1 protein (MTA1), a component of the histone deacetylase and nucleosome remodeling complex (NuRD), in a ligand and growth factor signaling dependent manner, resulting in the attenuation of ER α signaling [86]. MTA1 interacts with histone deacetylases directly and hence behaves as a corepressor. Dysregulation of MTA1 leads to cell migration, formation of colonies in semisolid media [97], mammary carcinoma development in transgenic mice [98] and breast cancer cells growth in some experimental observations. In addition, inhibiting the expression of MTA1 protein also led to growth inhibition and reduced invasion of highly metastatic breast cancer cells MDA-MB-231, making it an important molecule in breast tumorigenesis [99].

ER α signaling pathway has traditionally been known to be involved in the activation of genes involved in transcription; however, recent observations using experimental techniques such as microarray and ChIP have found that in transcriptome of more than half of ER α target genes regulated by ER α are repressed [100]. Different chromatin modifications at the ER α target genes as well as recruitment of different regulators of transcription may account for differential regulation by ER α . One of the examples of this regulation is the repressor of estrogen receptor activity (REA) and its binding partner EZH2. EZH2 is an important corepressor that is upregulated during the progression of different cancers, a process accompanied by the silencing of various genes. Interestingly, EZH2-mediated repression of cellular genes was attenuated on inhibition of histone deacetylase activity, implying a dependence of EZH2 targets on acetylation status of histones as well as chromatin remodeling [87]. In another study by Jene-Sanz et al., it was found that EZH2 targets tumor suppressor genes, because EZH2 overexpression not only repressed a significant number of genes but also resulted in increased metastasis [88]. Therefore, the combined interaction of EZH2 with the repressor of estrogen receptor is needed for ER α 's recruitment to specific target genes and repression of estrogen-dependent transcription [89]. The inhibition of EZH2 by siRNA was found to be responsible for an increase in estrogen-dependent transcription.

ER α also modifies chromatin organization by affecting the acetylation and deacetylation of conserved lysine residues present in histone tails. Specifically, the coactivators of ER α possess histone acetyl transferase activity and are known to associate with and modulate functions of specific acetyl transferases. In addition, ER α -mediated deacetylation is accomplished by recruitment of histone deacetylases (HDACs), which are recruited indirectly to ER α target genes through multisubunit corepressor complexes. ER α also utilizes corepressor complexes such as nuclear receptor corepressor (NCOR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) that associate with histone deacetylases [90]. Studies employing siRNA targeting histone deacetylases and corepressors indicated that one such histone deacetylase, HDAC6 functions with a corepressor, ligand-dependent corepressor (LCOR) on some ER α target genes as part of a feedback loop to regulate estrogen-dependent gene regulation in breast cancer cells [91]. The expression levels of HDAC6 correlate with better prognosis and response to endocrine therapy in breast cancer patients. Thus, ER α is known to achieve several histone modifications at target gene promoters using several coregulators.

Studies on ER α target gene regulation have introduced a new degree of complexity, wherein a combination of interactions between ER α and histone acetyltransferases, histone deacetylases, histone methyltransferases, coactivators, corepressors and transcription factors reveals a complex histone code that regulates promoters involved in breast cancer cells proliferation. A dynamic process of DNA methylation is also known to be involved in the control of the cyclic expression of ER α target genes. In a significant fraction of breast cancers, the absence or loss of ER at the time of diagnosis or treatment is due to aberrant methylation of CpG islands, cytosine-guanine-rich areas that are located in the 5' regulatory regions of the ER α gene [92, 93]. Methylation/demethylation of CpG sites on promoters following estrogen stimulation revealed the importance of DNA methyltransferases control on estrogen-dependent gene expression. Interestingly, ER β has been found to play a role in the establishment of new and stable methylation. All these results provide strong evidence that estrogen target gene expression is tightly regulated by multiple highly dynamic machinery affecting estrogen receptor in both a transcriptional and an epigenetic manner.

Current endocrine therapy for ER α -positive cancer involves modulating the ER α pathway using antiestrogens (AEs) or aromatase inhibitors (AIs). ER α 's ability to modulate epigenetic changes by regulating writers, erasers and readers of epigenetic modifications provides a unique therapeutic opportunity to design novel drugs and small molecular inhibitors for treating ER α -positive cancers [88] (Figure 3).

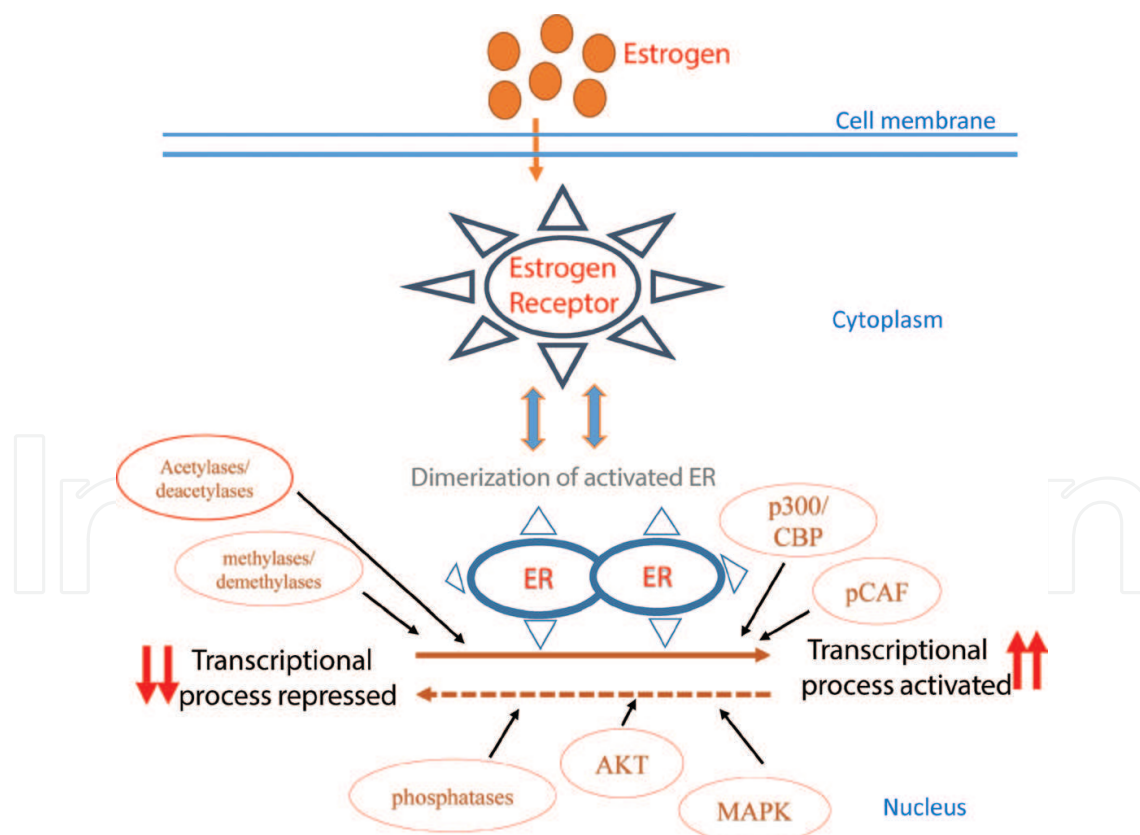


Figure 3. Regulation of epigenetic modifications by ER α . Estrogen signaling activates a set of kinases *via* its extra-nuclear signaling that modifies histone tails or influences the recruitment and function of histone modifying enzymes. In addition, ER α -driven transcription also involves a coordinated interaction of ER α with acetylases/deacetylases and methylases/demethylases. ER α if deregulated affects tumor progression and its associated therapeutic resistance.

3.2. Linking chromatin to the downstream signaling effectors

Eukaryotes utilize the chromatin landscape as its epigenetic template within the nucleus of living cells to promote gene transcription in response to environmental signals. Different classes of chromatin-associated enzymes or kinases that play important role in modulating chromatin structure within the human genome have been discovered recently. These signal transduction kinases play a pivotal role as chromatin-anchored proteins in eukaryotes, relaying signals from the cytoplasm to the nucleus and direct the association of chromatin-bound transcription complexes at activated targets in the nucleus [94]. These interactions serve to integrate the hormonal signals into a network of coordinated programs, and it is the outcome of this integration that specifies the nature, intensity and duration of the cellular response.

Estrogen and progesterone, two of the hormones known to play a role in breast cancer progression influence a variety of functions *via* their respective signaling cascades. These steroid hormone receptors (SHR) are known to interact with hormone-responsive elements (HREs) in the promoter/enhancer region of target genes, thereby affecting the epigenetic landscape of the cell [95]. SHRs can also activate genes lacking HREs by interacting with other sequence-specific transcription factors bound to their target sequences [96]. In addition to nongenomic interactions that involve the activation of *PI3K/Akt* pathways by the interaction of the ER α with the regulatory subunit of PI3K, SHRs can also induce direct genomic effects by binding to different regulatory elements of the genome and inducing the downstream effector response. Not much has been reported or is known on how progesterone receptor integrates the signaling pathways at the epigenetic level. A study by Ballare et al. using a synthetic progesterone, progestin, found that some of the kinases activated by progestin in the cytoplasm phosphorylate the progesterone receptor (PR) and form a complex with the activated receptor. This complex is recruited to the target sites where the kinases modify the protruding core histone tails and the linker histones. These modifications lead to the displacement of linker histones and a repressive complex, by recruiting specialized ATP-dependent remodelers such as switch/sucrose nonfermentable (SWI/SNF) [97]. In addition, other specialized ATP-dependent remodelers displace histone H2A/H2B dimers from the promoter nucleosome, enabling synergistic access of other transcription factors and additional receptor complexes to previously hidden binding sites on the surface of a histone H3/H4 tetramer particle [95]. It is only after completion of these initial chromatin remodeling steps that complexes containing mediator and RNA polymerase along with associated basal transcription factors are recruited, and further steps in transcription can take place. Thus, these signaling pathways of progestin action converge on the chromatin to enable gene regulation in the case of breast cancer.

3.2.1. Role of kinases in the epigenetic signaling network

Cross talk between signaling kinases and chromatin remodelers are critical for eliciting inducible transcriptional programs that include differentiation of cells, their ability to invade and migrate and to form cancer stem cells. Epigenetic approach targeting breast cancer stem cells (CSCs) may prove to be a good therapeutic option since not much has been known about the cross talk between these signaling kinases and chromatin remodelers. In an exception, one study found the chromatin-associated role of an evolutionarily conserved protein kinase

C (PKC) family protein, PKC- θ . After nuclear translocation to the nucleus, PKC- θ plays a role in generating a T cell-induced immune response by influencing the transcription of genes involved in generating the response that also include some microRNAs [112]. Aberrant expression of this kinase may lead to uncontrolled cell growth leading to tumors, inflammatory disorders or an aggressive form of breast cancer leading to cancer metastasis [113].

PKC- θ is present mainly in ER-negative basal-like breast cancer lines, localized in the nucleus, and an increased nuclear PKC- θ results in epithelial to mesenchymal transition (EMT). Experiments such as ChIP using pan-PKC- θ -specific antibody was performed, and it was found that PKC- θ occupies the proximal promoter region of *CD44* gene in EMT models. Additionally, ChIP analysis demonstrated that RNA polymerase II and PKC- θ coexist on the promoter of CSC-inducible gene suggesting that PKC- θ exists as part of a transcription complex in the mesenchymal state [98]. Thus, active PKC in primary breast cancers tethers the transcription complex to EMT and CSC-inducible genes, and the expression of this complex is found elevated in cancer stem cells leading to breast cancer. The PKC pathway also cooperates with the transforming growth factor β (TGF- β) pathway and collaborates with the NF- κ B pathway to promote a distinct transcriptional program of inducible EMT and CSC signature genes [99]. Using the p50 and p65 heterodimer (the subunits of NF- κ B pathway), the transcription complex of activated PKC- θ is bound to the chromatin of some inducible genes that are involved in the EMT process. The role of each of the subunit is such that p65 subunit recruits the PKC- θ transcriptional complex to the promoter region of *CD44* and *IL-6* and the p50 subunit is involved in the recruitment of PKC- θ transcriptional complex to only the promoter region of *IL-6* but not to *CD44*. In a cellular system if PKC- θ is knocked out, it is observed that PKC- θ is not only important for maintaining a permissive state for *IL-6* and *CD44* at the chromatin level but also for the enrichment of certain epigenetic marks such as H3K4me3 and H3K9ac [113]. Using genome-wide analysis, distinct cohorts of inducible PKC- θ sensitive genes in the mesenchymal state that are directly tethered to chromatinized PKC- θ were identified. Some of the genes were found to be EMT regulators and some involved in progression of cancer, suggesting that PKC- θ occupies a position upstream making it a novel regulator of the EMT process and in the progression of cancer. Thus, the chromatin bound PKC- θ engages with factors that play a role in establishing a permissive chromatin state, thereby contributing a new dimension toward the understanding of EMT/CSC process in breast cancer. Targeting CSCs remains an underdeveloped area of cancer therapy; however, a novel epigenetic mechanism using specific inhibitors will pave the way for novel “epitherapeutic” strategies.

Some other examples of signaling pathways influencing the epigenetic circuitry include the NF- κ B pathway. Tumor necrosis factor α (TNF- α), an important effector of the NF- κ B pathway, is known to induce expression of a lysine demethylase, KDM4D in macrophages and dendritic cells. Enzymes belonging to the demethylase family of KDM4 including KDM4D are overexpressed in breast cancer and affect cell proliferation and growth of these cells [98]. Another lysine demethylase of KDM4 family, KDM4A, has been known to be involved in transcriptional regulation, where it may either stimulate or repress gene transcription [100]. The latter function involves the association with nuclear receptor corepressor complex or association with histone deacetylases. KDM4A is also known to form complexes with ER

and to stimulate its activity. Accordingly, depletion of KDM4A in ER-positive breast cancer cells leads to a decrease in the expression of ER targets such as the *c-JUN* and Cyclin D1 oncogenes and reduced cell growth [101]. Similarly, KDM4A knockdown inhibited proliferation of ER-negative MDA-MB-231 and ER-positive MCF7 breast cancer cells [102, 103], suggesting that KDM4A is critical for growth of both ER positive and negative breast cancers. Another example is the progesterone-activated extracellular signal-regulated protein kinase 1/2 (ERK1/2) pathway in breast cancer, which phosphorylates both the progesterone receptor and the downstream kinase, mitogen and stress-activated protein kinase 1 (MSK1), forming an active ternary complex that mediates the phosphorylation of histone H3 at serine 10 in breast cancer cells [104]. This initial step triggers the recruitment of histone modifiers and chromatin remodeling complexes, which ultimately leads to displacement of histones H1 and H2A/H2B supporting the role of chromatin remodeling complexes for transcriptional activation of progesterone responsive genes.

In the case of ER α signaling, ER activates a number of kinases in the extranuclear compartment including protein kinase B (AKT) and extracellular signal-regulated protein kinase. In ER-positive breast cancers, mitogen-activated protein kinase (MAPK) pathway exerts an effect at the level of ER-induced transcription as well as at the level of the cell cycle regulation. Estrogen stimulates cell proliferation by activation of MAP kinase, either through rapid, nontranscription effects or by increasing growth factor production and consequently MAP kinase expression. Hormonal stimulation also promotes alterations in the phosphorylation of specific residues in histone tails *via* modulation of these extra-nuclear kinases. Estrogen-ER α signaling activates MAP kinase cascades in breast cancer specifically the one involving ERK-1 and ERK-2 that transmit and amplify signals involved in cellular proliferation [105]. ER α activates ERK2, resulting in its chromatin binding and enabling ERK2 modulation of estrogen-dependent gene expression and proliferation in breast cancers. This convergence of ERK2 and ER α at the chromatin level is also known to activate an oncogenic kinase AuroraA/B to directly affect nuclear receptor activities [106]. The Src-AKTs, which are involved in phosphorylation of Histone H1 and Src-MAPK pathways, are also activated by ER α signaling. Downstream substrates of these kinases such as the ones that phosphorylate histone H1 and core histones are therefore influenced by ER α signaling at the chromatin level [122]. In addition, the expression of several phosphatases such as PP1 and PP2A is also regulated by the ER α signaling. In one of the studies, these phosphatases were identified as key negative regulators of steroid receptor coactivator 3 (SRC-3). SRC-3 is a coactivator and an oncogene, whose phosphorylation transforms it into a powerful coregulator. It was shown that PDXP and PP2A dephosphorylate SRC-3 and inhibit its ligand-dependent association with estrogen receptor, thus regulating the oncogenic cell proliferation and invasion functions of SRC-3 in breast cancer cells [107]. These observations therefore suggest that ER α -extranuclear signaling has the potential to modulate epigenetic modifications. The direct communication between the extracellular environment and the regulation of gene function may be even more widespread and warrants greater study. It could involve many kinases that are known to regulate gene expression indirectly *via* signaling cascades. In addition, the signaling to chromatin may change the role of these kinases and may rationalize the use of chromatin-modifying enzymes as important cellular targets.

3.3. HDACs and signaling pathways

Sustained and increased hormone and growth factor receptor signaling in breast cancer cells contributes to resistance toward endocrine therapy. It has become important to modulate the signaling pathways so as to design an attractive strategy in overcoming potential resistance to endocrine therapy. In the case of breast cancer, down regulation of ER α expression is one of the mechanisms behind the acquisition of endocrine resistance. Histone deacetylases (HDACs) are important epigenetic regulators and are overexpressed in multiple cancers, including breast cancer. Specifically, histone deacetylase 1 (HDAC1) is an important epigenetic regulator involved in transcriptional regulation through modification of chromatin organization [82]. Although, HDACs are primarily known to repress gene expression as part of corepressor complexes, recent findings by Smith et al. have established a link between HDACs inhibition and repression of gene expression, suggesting that they might also function as coactivators [108]. In some cases, as for the regulation of ER α , HDACs inhibitors (HDACi) can have both positive and negative impact on transcription, depending on the cell context. In breast cancer cells, trichostatin A (TSA), a potent and reversible HDACi, produced a strong decrease in ER α accumulation independent of the presence or absence of ER ligands. The effect was dose dependent and was not restricted to TSA since a similar regulation was obtained with different HDACi, suberoylanilide hydroxamic acid (SAHA), which is structurally similar to TSA [109]. Regulation by TSA takes place at the transcriptional level and therefore the use of different HDACi decreases the expression of ER α in ER-positive breast cancer cells. In another study, it was found that the use of HDACi reactivates the expression of the receptor in ER-negative cells and the treatment resulted in dose-dependent and time-dependent re-expression of ER α mRNA [110, 111]. This was speculated to be due to the loss of ER α expression or that TSA could potentiate the effect of DNA methyltransferase inhibitors such as 5-aza-2-deoxycytidine, treated together on the re-expression of the ER α protein [112]. Activation of the silenced ER α by HDAC1 inhibition and partial re-expression of ER α by TSA treatment may provide a possible therapeutic treatment for patients with advanced breast cancer, restoring estrogen-mediated signaling and growth. Thereby, inhibition of HDAC1 expression or activity may provide a new strategy for breast cancer therapy.

The phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway plays a critical role in multiple cellular functions including metabolism, proliferation, growth, and survival [113]. Studies have found PI3K/mTOR pathway to be a promising target in breast cancer [114]. The p70 S6 kinase (S6K1) is one of the best-characterized downstream targets of mTOR and plays an important role in protein translation and cell proliferation [115]. The mTOR inhibitor rapamycin, tested as an anticancer drug, rapidly dephosphorylates and inactivates S6K1. S6K1 is amplified in 10–30% of breast cancer cell lines, and its overexpression is associated with poor prognosis in breast cancer patients. PI3K inhibitors are able to regulate the expression of ER α through the activity of S6K1, as in cells that have S6K1 overexpression, rapamycin can increase both mRNA and protein levels of ER α , promoting the acetylation of its promoter [114].

In some cases, HDAC1 activity and its binding to the ER α promoter is required for the rapamycin-dependent upregulation of ER α expression. Thus, when S6K1 is active and HDAC1 is hyper-phosphorylated, it results in decreased expression of ER α , whereas in the presence of rapamycin or cell starvation, S6K1 activation and mitogen dependent HDAC1 phosphorylation

is ablated, increasing the level of ER α in breast cancer cells. Thus, the mitogen-dependent phosphorylation of HDAC1 inhibits the positive transcriptional regulation of the deacetylase on ER α expression [116]. Since both HDACs and mTOR inhibitors are known to have anti-proliferative effect in breast cancer cells, their combinatorial treatment shows promise (**Figure 4**).

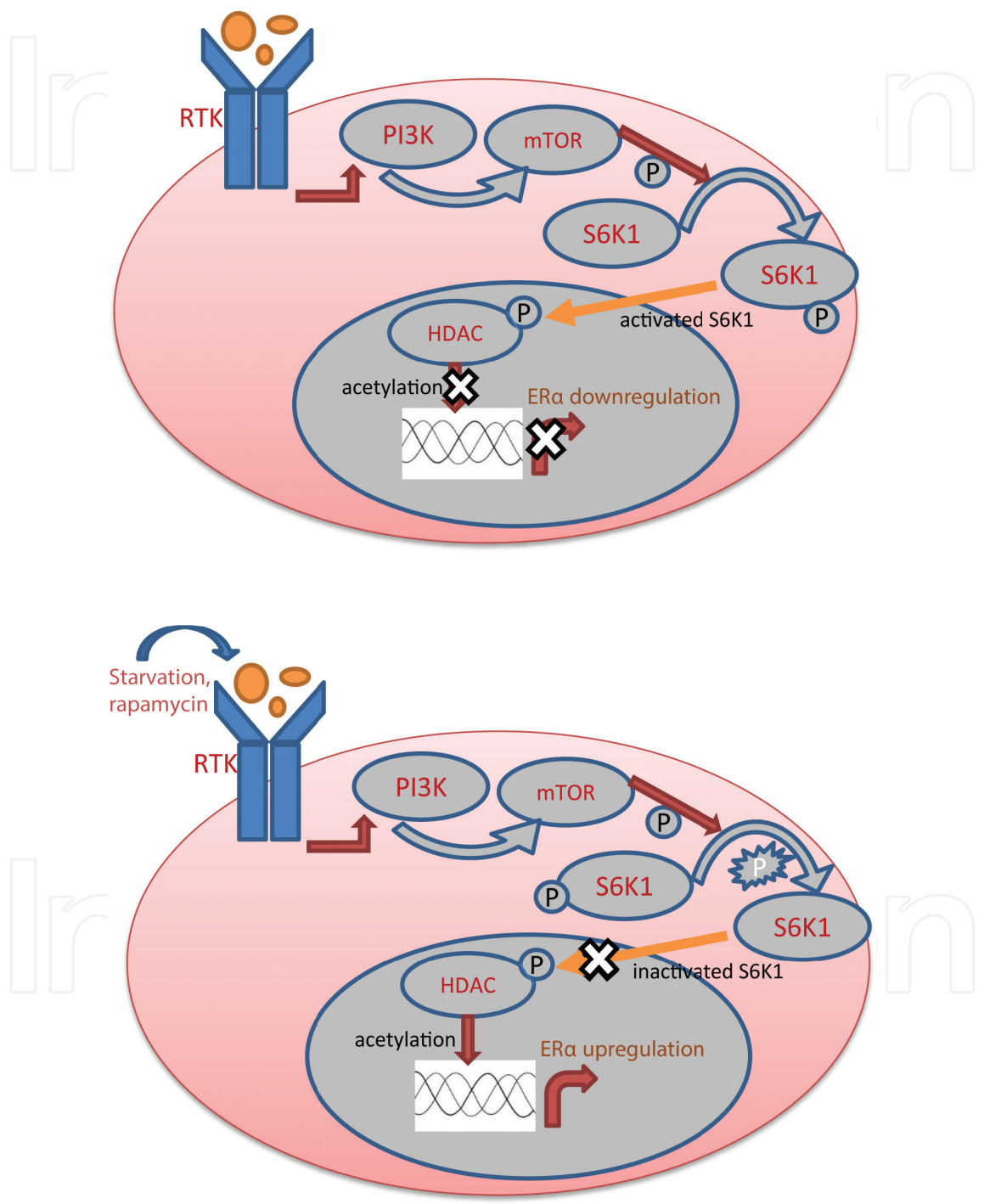


Figure 4. Mitogen-mediated HDAC1 phosphorylation and ER α transcriptional regulation. PI3K/mTOR pathway is activated by the RTK. Subsequently, S6K1 activation controls HDAC1 phosphorylation and thereby reduces acetylation of the ER α promoter and gene expression. In the case of cell starvation or when rapamycin is present, S6K1 is not active and is not able to phosphorylate HDAC1, promoting acetylation of ER α and its gene expression.

3.3.1. *The role of antiestrogens in epigenetic silencing*

Corepressors are associated with deacetylase activity through the recruitment of HDACs, and these HDACs possess different functional domains responsible for deacetylase activity and interaction with other proteins. The amount of histone acetylation is therefore determined by an equilibrium between acetyltransferases and deacetylases, and that the ratio of corepressors to coactivators is the modulator of transcription in any given context [112]. The ligand-dependent activation of steroid hormones receptor regulates a variety of gene expression. Binding of an agonist leads to the activation of transcription, whereas an antagonist does the opposite, leading to inhibition. ER α bound to an anti-estrogen is unable to activate transcription, and this may be due to the recruitment of a repressor complex with HDAC activity [117] making the use anti-estrogens a feasible treatment option. However, the use of anti-estrogens is limited due to the associated side effects or the development of resistance. Moreover, HDAC activity has also been associated with gene silencing in some eukaryotes [117]. This gene silencing associated with HDAC binding at the ER α promoter could be due to the direct targeting of HDAC to estrogen-responsive elements (EREs), thereby mimicking or modulating the effects of the anti-estrogens [118]. The specific sites of the action of HDACs are therefore associated with the binding of corepressors and in-turn lead to the reversible silencing process, thus a potential therapeutic option. An example of this phenomenon was highlighted in one of the studies where treatment of MCF-7 cells with an antiestrogen hydroxytamoxifen (OHT), induced silencing of estrogen-responsive genes [118]. Similarly, the estrogen-dependent expression of the ER α was partially silenced after 3 months of OHT treatment and OHT-resistant cell growth appeared simultaneously. It was found that histone deacetylase activity was involved in the repressive effect by its binding to estrogen-responsive elements (ERE) and the antiestrogen effect might be very similar, if not identical to the ERE-targeted HDAC activity [118].

3.4. Promoter DNA methylation and signaling pathways

DNA methylation profiles of many genes have been linked with cancer initiation and progression [119]. As discussed earlier, in the case of DNA methylation, the most extensively studied mechanism of epigenetic control is global hypomethylation that leads to genome instability. At the same time, hypermethylation of promoter regions has been detected in a vast majority of tumor suppressor genes, which are strongly associated with tumor development. Hypermethylation events can occur early in tumorigenesis, involving the disruption of pathways that may predispose cells to malignant transformation. Gene silencing by hypermethylation of promoter genes is an important mechanism of carcinogenesis and has great potential for cancer prevention and therapy [120].

In the case of breast cancer, the distribution of aberrantly methylated regions in the genome was found to be nonrandom and concentrated in relatively small genomic regions spanning up to several hundred kilobases. DNA hypermethylation also leads to aberrant regulation of the Wnt pathway in breast cancer, and an overstimulated Wnt signaling is a hallmark of different breast cancer tumor subtype [121]. Functional loss of negative Wnt regulators by epigenetic gene silencing, through DNA methylation of the tumor suppressor gene-associated promoters, has been found to contribute to the activation of aberrant WNT/ β -catenin

signaling [122]. Recent studies have also found impaired regulation of Wnt-antagonists by promoter hypermethylation in breast cancer. The growing list of epigenetically silenced WNT antagonists involved in human cancers indicates an important role for epigenetic inactivation events in tumor initiation and progression [123]. For examples, some Wnt proteins like WNT1, WNT2 and WNT3A are overexpressed in breast cancer, acting as oncogenic activators for canonical Wnt signaling [124]. In contrast, WNT5A acts as a tumor suppressor inhibiting tumor cell proliferation, antagonizing the WNT/ β -catenin signaling and is thereby silenced by tumor-specific methylation [125]. In parallel, epigenetic inactivation of Wnt gene family members, WNT7A and WNT9A, through promoter methylation, has been reported as well [126]. As epigenetic dysregulation of WNT/ β -catenin signaling frequently contributes to tumor pathogenesis, identification of aberrant epigenetic events that activate WNT/ β -catenin signaling may provide useful biomarkers for cancer detection and prognosis. Some Wnt proteins like Wnt1, Wnt2 and Wnt3A are overexpressed in breast cancer, acting as oncogenic activators for canonical Wnt signaling [124]. In addition, WNT5A acts as a tumor suppressor inhibiting tumor cell proliferation, antagonizing the WNT/ β -catenin signaling and is thereby silenced by tumor-specific methylation [125]. In parallel, epigenetic inactivation of Wnt gene family members, WNT7A and WNT9A, through promoter methylation, has recently been reported [126]. As epigenetic dysregulation of WNT/ β -catenin signaling frequently contributes to tumor pathogenesis, identification of aberrant epigenetic events that activate WNT/ β -catenin signaling may provide useful biomarkers for cancer detection and prognosis.

In addition, hypermethylation of the gene promoters of Wnt repressors was observed in various cell lines and tissues. The epithelial adhesion molecule E-cadherin (encoded by *CDH1*) also acts as a negative regulator of WNT/ β -catenin signaling by affecting the intracellular localization of β -catenin. Epigenetic silencing of *CDH1*, by promoter methylation has been observed in breast cancer, leading to aberrant activation of WNT/ β -catenin signaling. The *APC* promoter (adenomatous polyposis coli) of the WNT/ β -catenin signaling pathway has also been found to be hypermethylated at the CpG island in ~35–50% of breast cancer tumors and cell lines [127]. The methylation of *APC* gene is a cancer-specific change and may disrupt the regulation in the APC/ β -catenin pathway in breast cancers, making it a common mechanism of the inactivation of tumor suppressor gene in primary breast cancer.

Histone methylation is also known to play a key role in ER α -mediated activation of target genes. Recent studies found that histone demethylase KDM1 and ER α coregulator proline-, glutamic acid- and leucine-rich protein-1 (PELP1) plays a role in regulating histone methyl marks at ER α target genes [128]. PELP1 deregulation alters histone methylation at ER α target genes, contributing to hormone-driven tumor progression and resistance to treatment.

3.4.1. The synergistic role of HDACs and DNA methylation in breast cancer

Patients who have ER-negative breast cancer seldom respond to endocrine therapy. One of the mechanisms to explain the loss of estrogen receptors expression is the methylation of cytosine at the 5' regulatory region of the gene at the CpG island [133]. CpG island in ER α genes is highly methylated in ER-negative breast cancer but remain unmethylated in normal breast

tissue and many ER-positive tumors as well as ER-positive cancer cell lines. This abnormal methylation pattern could account for transcriptional inactivation of the ER gene and subsequent hormone resistance in some human breast carcinomas. The functional importance of this finding is demonstrated by the fact that treatment of ER-negative human breast cancer cells with the demethylating agent, 5-aza-2'-deoxycytidine (AZA), led to reactivation of *ER* mRNA and functional ER protein [129].

An abundant chromosomal methyl CpG-binding protein was the first protein identified to link methylated DNA and a HDAC-containing transcriptionally repressive complex for gene silencing. More recently, the well-known maintenance methyltransferase, DNMT1, was found to interact physically with HDAC through its N terminus, thereby leading to a transcriptionally inactive complex that represses transcription [130]. Thus, the loss of ER expression in some breast cancers is associated with transcriptional repression through HDAC activity on the methylated *ER* gene, linking HDAC activity closely to DNA methylation of *ER* α promoter and thereby helping in understanding the associated resistance to endocrine therapy.

Recent studies also demonstrated that combination therapy involving HDAC inhibitors with DNA methyltransferase-1 (DNMT1) inhibition is synergistically effective in inducing apoptosis, differentiation and/or cell growth arrest in many cancer types including breast cancer. The combination was also synergistic in inducing re-expression of *ER* α in *ER* α -negative breast cancer cells. Expression of *ER* α is induced by 5-aza-2'-deoxycytidine (DNMT1 inhibitor) and trichostatin A (HDAC inhibitor) in *ER*-negative breast cancer. Studies at the preclinical level indicate that sensitivity of *ER*-negative breast cancer cells could be restored to endocrine therapy by the use of AZA and TSA both *in vitro* and *in vivo*. When HDAC inhibitors such as vorinostat were used in combination with decitabine, the capacity of breast cancer cells to proliferate and to form colonies was inhibited significantly as compared to when either drug was used alone [146]. Histone methylation could also be a druggable target as some therapeutic benefits have been observed during the preclinical studies.

4. Coding and noncoding RNAs in breast cancer

4.1. Current techniques for detection of breast cancer

The genetic signature identified from gene expression arrays has been incorporated into five different breast cancer prognostic platforms. As an improvement over the classical *ER*/*PR*/*HER2* status, a panel of eight genes has been identified to classify the different breast cancer subtypes [131]. This panel includes the genes *ER*, *PR*, *HER2*, *CK5*, *CK14*, *p53*, *MKI67* and *EGFR*. Cytokeratin 5 (*CK5*) and cytokeratin 14 (*CK14*) genes expressed by basal/myoepithelial cells are used to characterize basal-like TNBC [132]. *EGFR* is frequently upregulated in TNBC cases with a basal phenotype and can be targeted for therapy. Ki-67 is a marker for proliferating cells. Ki-67 and *p53* expression can be used to distinguish Luminal A from Luminal B tumors. The different prognostic tests for breast cancer, namely, the 21-gene Oncotype DX[®] [133], 70-gene MammaPrint[®] [134], and 50-gene PAM50 [135] detect the presence of these vari-

ous mRNA biomarkers in patient samples. The need for additional markers for breast cancer subtype classification and further treatment regime arises from the observation that while Oncotype Dx and MammaPrint are the only FDA-approved RNA-based assays, they only share one gene in common (*MKI67*), besides *ER* and *HER2*.

Less than 2% of the human genome is translated into proteins. However, around 97% of the genome is transcribed, indicating that most of transcripts are not translated. Initially described as “transcriptional noise,” increasing evidence in the past few years has helped identify the regulatory functions of these “noncoding RNAs.” Noncoding RNAs are classified as small noncoding RNAs and long noncoding RNAs (lncRNAs). Small noncoding RNAs include miRNAs, small-interfering RNAs (siRNAs) and piwi-interacting RNAs measuring <200 nt in length. LncRNAs as the name suggests are “long,” ranging in length from 200 nt to 200 kb. Noncoding RNAs, both small and long, have been shown to regulate critical cellular functions such as transcriptional and posttranscriptional regulation which in turn modulate cell growth and differentiation [136]. Thus, it is no surprise that the aberrant expression of several noncoding RNAs has been observed and attributed to various diseases, including cancer.

Given that noncoding RNAs comprise the vast majority of the human transcriptome and evidence of their essential role in gene regulation, it is important that this largely unexplored class of molecules be studied in the cancer context more closely. Some miRNAs and lncRNAs implicated in breast cancer initiation, progression and metastasis have been summarized in **Figure 5**.

4.2. MiRNAs in breast cancer

MiRNAs are 18–24 nt in length noncoding RNA molecules that regulate gene expression by mRNAs degradation or inhibition of protein synthesis. MiRNAs have been shown to regulate numerous physiological processes such as differentiation, development and cell death as well as pathophysiological processes such as cancer biology, progression and prognosis. The aberrant expression of miRNAs in cancers can lead to an abnormal expression of their target genes thereby contributing to cancer etiology. Mounting evidence suggests a significant role of miRNAs in breast cancer classification, prognosis, as potential biomarkers for disease progression as well as treatment [137].

4.2.1. MiRNAs and breast tumor initiation

Mammary gland epithelia comprise different cells including mammary stem cells (MaSCs)/ basal cells, luminal progenitors and mature luminal cells. Several subtypes have been described among breast cancers, including claudin-low, basal, luminal, normal-like and ERBB2-enriched subtypes. These distinct molecular subtypes derive from different “cells of origin,” that is, cells that acquire the first oncogenic events in the initiation of breast tumorigenesis [138, 139]. The close association between cell lineage targeting and the resulting cancer phenotype suggests that lineage-restricted mechanisms that normally operate during the mammary gland development and homeostasis may contribute to tumorigenesis. Some miRNAs have been recently identified

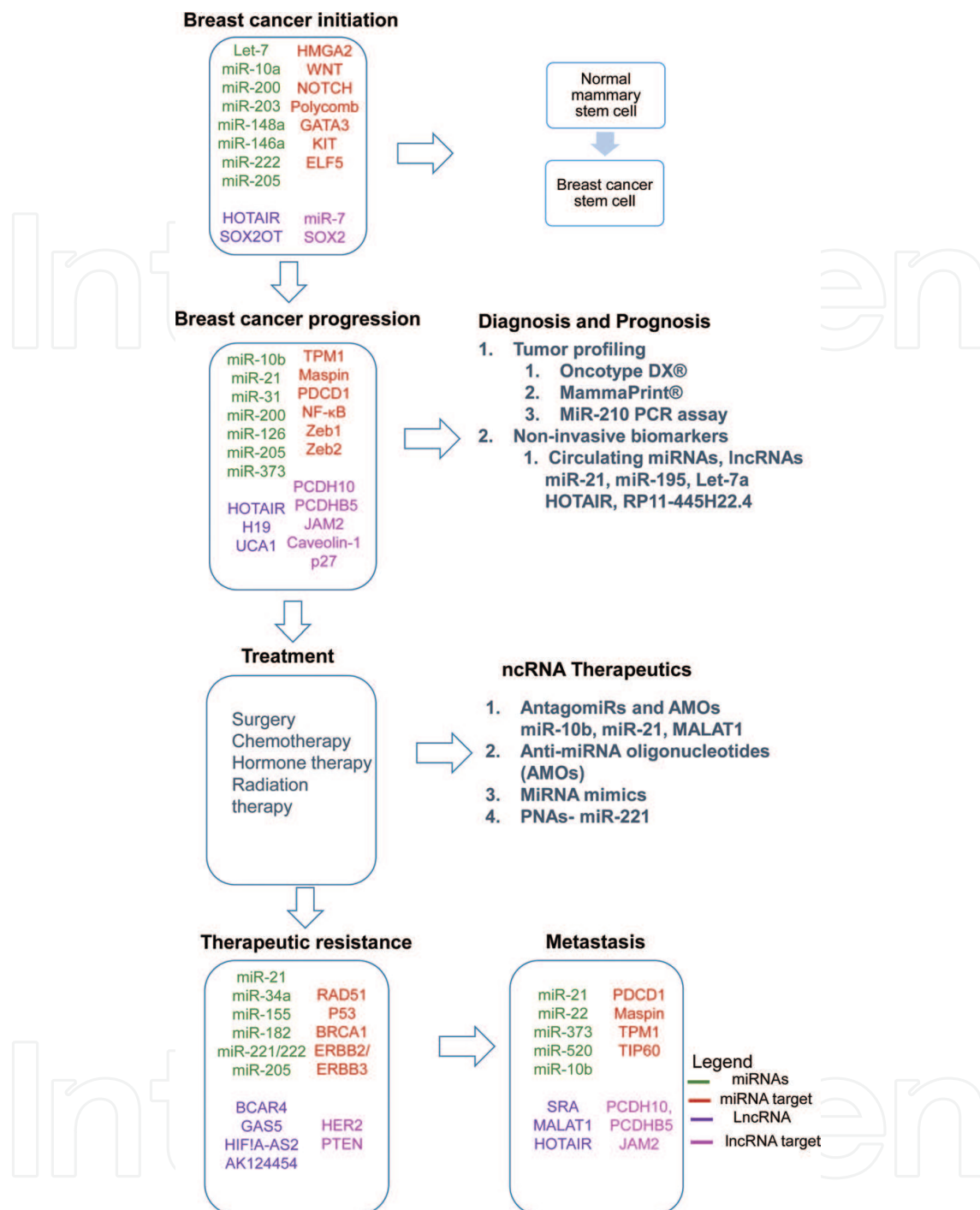


Figure 5. MiRNAs and lncRNAs implicated in breast cancer initiation, progression and metastasis. Several miRNAs and lncRNAs controlling key oncogenes such as *HMGA2* among others are downregulated in the breast cancer stem cells (BCSCs) leading to proliferation and self-renewal of these cells and breast cancer progression. Downregulation of tumor suppressor miRNAs such as the miR-200 family leads to an upregulation of the EMT markers *ZEB1* and *ZEB2*, thus aiding tumor proliferation and invasion. A number of mRNA markers are displayed by tumor cells at this stage aiding in their subtyping and prognosis. Additionally, several noncoding RNA biomarkers have also been identified including noninvasive circulating ncRNAs which closely correlate with patient prognosis. Further, several miRNAs and lncRNAs contribute to the hormonal resistance displayed by breast cancer cells *via* targeting tumor suppressors such as *PTEN*, cell cycle genes such as *p27* or members of the hormone signaling pathways such as *ERα*, thereby leading to more aggressive and metastasized cancer. Currently, a number of novel and safe therapeutic options are being researched to aid the conventional treatment options to help ameliorate breast cancer.

as potential “keepers” of this lineage-restricted identity. Thereby, aberrant expression of these miRNAs has been implicated in breast cancer molecular subtypes. Unique miRNA signatures characterize each step of the mammary differentiation hierarchy in the normal mammary gland (MaSCs/basal cells, luminal progenitors, mature luminal and stromal cells). MiRNA networks, also known as miRNome, are responsible for governing lineage commitment and cellular differentiation in the mammary tissue. MiRNAs act by targeting lineage-specific mRNAs thus regulating lineage-specific gene expression [140]. For example, the expression of miRNAs implied in MaSCs functions and pathways (WNT, NOTCH and Polycomb groups) such as miRNA-10a, miRNA-200a/b, miRNA-203 and miRNA-148a is restricted to the luminal subpopulation. Conversely, miRNA-146a, miRNA-221/222 and miRNA-205, known to regulate genes expressed in the luminal lineages (*BRCA1*, *GATA3*, *KIT* and *ELF5*), are restricted to the MaSCs population. Integrating these miRNA signatures with both transcriptomics and histone marks analysis has revealed that key developmental miRNAs are epigenetically regulated by global changes in histone methylation during differentiation [140]. By comparing miRNA signatures of normal breast epithelial cells with breast tumors, many miRNA-mRNA networks deregulated in cancer cells have been identified. Therefore, these miRNAs may potentially represent new biomarkers and targets. Furthermore, the miRNome of breast tumors allows the classification of tumors into molecular subtypes and can predict the patient’s outcome [141–143].

4.2.2. *Oncogenic and tumor suppressor-like miRNAs in breast cancer*

Due to amplification of chromosomal regions of miRNAs, certain miRNAs may be overexpressed in cancer. If these miRNAs target TSGs, it would downregulate the TSGs leading to malignant growth. Hence, such potentially cancer-causing miRNAs are called oncomiRs. Conversely, oncosuppressor miRNA genes are frequently located in fragile loci, which are hotspots for deletions, mutations and promoter methylation. Genetic aberrations in such loci may result in downregulated miRNA expression and a concomitant increase in expression of oncogenes. These alternations of miRNA lead to tumor formation by inducing cell proliferation, invasion, loss of apoptosis, and angiogenesis. Thus, miRNAs can act both as oncogenes as well as TSGs [144, 145].

4.2.2.1. *OncomiRs in breast cancer*

MiR-21 is a prominent oncomiR which is upregulated in breast cancer. The targets of miRNA-21 include *BCL-2* (regulates apoptosis), *PTEN* (regulates cell survival) and *PDCD4*, *TPM1* and *MASPIN* (involved in tumor progression, invasion and metastasis). Thus, overexpression of miR-21 in breast cancer supports tumor growth [146, 147]. MiR-155 is an oncomiR, with an increase in expression in breast cancer, where it targets tumor suppressor gene *SOCS1* [148].

4.2.2.2. *Tumor suppressor-like miRNAs in breast cancer*

Let-7 is an important tumor suppressor miRNA with a decrease in expression in breast cancer. It targets the Ras pathway and regulates cell proliferation, adhesion and migration [149]. Targets of let-7 include *HMG2A* (responsible for maintenance of stemness of stem

cells), *lin-28* and *PEBP1* (oncogenes involved in cancer progression and metastasis) [150, 151]. Thus, a loss of let-7 leads to an upregulation of these oncogenes resulting in breast cancer stem cell renewal and cancer progression.

4.2.3. *MetastamiRs in breast cancer*

Metastasis is a complex multistep process, which includes the formation of tumors at sites distant from the primary site of the cancer. The term 'metastamiR' refers to as a metastasis-associated miRNA [152]. Several miRNAs such as miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c and miR-205 have been implicated in controlling metastasis in breast cancer [152]. Different metastamiRs have been shown to both promote and inhibit metastasis and regulate key steps in the metastatic program. Key players of the miRNA biogenesis pathway are also targeted by miRNAs thereby controlling metastasis. For instance, in breast cancer patients, it was found that miR-103/107 family targets *Dicer1* to decrease its expression, and as a consequence, several miRNAs were downregulated [153].

4.2.3.1. *MetastamiRs (metastasis-promoting miRNAs)*

MiR-21 is a metastamiR targeting several TSGs in breast cancer. MiR-21 downregulates TSGs *PDCD4*, *TPM1* and *MASPIN* to increase breast cancer invasiveness and metastasis [146, 147, 154]. MiR-10b is an example of another oncomiR, which induces invasion and metastasis in breast cancer xenograft models when overexpressed in nonmetastatic breast tumors [33, 155–157]. MiR-373 and miR-520c are able to initiate breast cancer cell migration and invasion *in vitro* and *in vivo*, which implicates these miRNAs as metastasis-promoting miRNAs [158]. It has been shown that miR-22 targets *TIP60* (HIV-1 Tat interacting protein), a lysine acetyl transferase, in breast cancer and stimulates the expression of EMT genes. Furthermore, analysis of gene expression and survival data from the TCGA dataset and gene expression omnibus (GEO) database revealed that patients with high *TIP60* and low miR-22 expression were associated with good survival, whereas patients with low *TIP60* and high miR-22 levels showed poorer prognosis for survival. This suggests that *TIP60* and miR-22 could act as prognostic marker in breast cancer disease progression and that targeting the *TIP60*–miR-22 axis could lead to an effective therapeutic strategy for metastatic breast cancer [159].

4.2.3.2. *Metastasis-suppressing miRNAs*

Tavazoie et al. [160] demonstrated that restoring the expression of those miRNAs whose expression is lost in malignant breast cancer cells suppresses lung and bone metastasis in metastatic breast cancer. Restoration of expression of miR-335 inhibited metastatic cell invasion while miR-126 restoration reduced overall tumor growth and proliferation. MiR-146a and b target *IRAK1* and *TRAF6* to down regulate NF-κB signaling and inhibit invasion and migration of breast cancer cells [161]. MiR-497, whose expression is downregulated in breast cancer samples, has been shown to induce apoptosis of breast cancer stem cells (BCSCs) by targeting Bcl-w. Additionally, its expression has been shown to be negatively correlated with tumor size, metastasis stage and HER2 status in breast cancer [162]. EMT is an important

property of malignant cancer cells wherein the epithelial cells lose cell-cell contact allowing them to be motile and thus metastasize to distant organs. The miR-200 family is known to regulate EMT by targeting the EMT markers, *CDH1* or E-Cadherin, a marker for epithelial phenotype, *vimentin*, *ZEB1*, which regulates EMT as seen in *in vivo* studies by promoting metastasis of tumor cells in mouse model [163, 164] and *ZEB2*, which are expressed in mesenchymal cells and thus mark the mesenchymal phenotype [165]. Furthermore, it has been shown that *ZEB1* regulates EMT in human breast cancer by promoting metastasis of tumor cells in mouse model [163, 164]. The miR-200 family by targeting *ZEB1* and *ZEB2* down-regulates their expression, thereby tipping the balance toward the epithelial phenotype [165, 166]. Gregory et al. Demonstrated that the miR-200b family is downregulated in response to the cytokine, transforming growth factor- β (TGF- β), which induces EMT. The authors further demonstrated that ectopic expression of the miR-200 family is able to inhibit EMT, thereby affecting breast cancer progression [167].

4.2.4. Regulation of signaling pathways by miRNAs in breast cancer

4.2.4.1. ER signaling

Among the two classes of estrogen receptors, the estrogen receptor- α (ER α) is overexpressed in approximately 75% of breast cancer cases. Increased signaling through ER α in mammary stem cell induces continuous replication of these cells, thereby increasing the risk of tumorigenesis. Tumor-suppressive miRNAs, such as miR-145 [168], miR-17/20 family, miR-193b, miR-206 and miR-302c, inhibit the ER signaling activated proliferation of mammary epithelia, by targeting either the ER receptor α or its coactivator AIB1 [169, 170]. MiR-206 is upregulated in ER-negative breast cancer but downregulated in ER-positive breast cancer [171]. MiR-17-5p targets AIB1, a coactivator of ER α [172]. The let-7 family of miRNAs is known to regulate the expression of both ER α 66 and ER α 36 (a novel short form of the ER α protein) in breast cancer. In breast cancer, let-7 is known to be downregulated, resulting in an upregulation of its targets, ER α 66 and ER α 36. ER α 66 is predominantly nuclear in expression, where it regulates the transcription of *c-Myc*, *CCND1* and *pS2*, while ER α 36 activates MAPK/ERK signaling pathway. Overexpression of let-7 miRNAs can negatively regulate these pathways by inhibiting the phosphorylation of ERK and Akt. Further, ER α 36 protein levels were found to be upregulated in a tamoxifen-resistant MCF-7 breast cancer cell line, indicating that ER α 36 might play a role in mediating resistance to tamoxifen therapy in breast cancer. However, overexpression of the let-7 family members in tamoxifen-resistant MCF-7 cells significantly decreased ER α 36 protein level further increasing tamoxifen sensitivity in these cells [173]. These studies demonstrate the regulation of the ER signaling pathway and development of tamoxifen resistance in breast cancer by let-7 miRNAs, hence hinting at the possibility of developing novel therapeutic strategies.

4.2.4.2. HER2 (ERBB) signaling

In breast cancer, ERBB2/HER2 is found to be amplified and/or overexpressed in up to 30% of patients, correlating with poor prognosis. Further, abnormal HER signaling induces cell proliferation [174]. HER2 and HER3 are targeted by miR-125a/b thereby inhibiting breast cancer

growth [175]. HER3 receptor is also targeted by miR-205 inducing cell cycle arrest thereby inhibiting cell proliferation in breast cancer [176].

4.2.5. MiRNAs regulating breast cancer stem cells

Human breast cancer stem cells (BCSCs) were first isolated by Al-Hajj et al. [177] as cells displaying a different set of cell surface markers CD44⁺/CD24^{low} as compared to normal mammary gland stem cells. Comparison of BCSCs with normal mammary stem cells revealed a differential expression of miRNAs. MiR-200c, let-7, miR-30 and miR-34 were observed to be downregulated, whereas miR-181 and miR-495 showed an increased expression in BCSCs. Let-7 inhibits the stem cell self-renewal in both normal and CSCs of breast and the down-regulation of let-7 in breast cancer, thereby leading to the formation of BCSCs by unchecked self-renewal and undifferentiated status of mammary gland stem cells [60]. Moreover, let-7 is also known to target many oncogenes such as *HMGA2*, *k-Ras*, *p-RAS* and *ERK*, which are highly expressed in BCSCs [149, 150, 178]. These oncogenes further support the formation and maintenance of BCSCs *via* self-renewal and maintenance of undifferentiated status of BCSCs [178]. In BCSCs, miR-30 is downregulated 30 fold, leading to increased expression of its targets: ubiquitin conjugating enzyme 9 (*Ubc9*) and integrin β 3 (*ITBG3*), and promoting the self-renewal ability of BCSCs. Specific knockdown of miR-30 induced differentiation of BCSCs, suggesting that miR-30 regulates self-renewal and tumorigenicity of breast cancer [179]. Furthermore, three families of miRNAs, namely, miR-200c-141, miR-200b-200a-429, and miR-183-96-182 are known to be downregulated in human BCSCs, normal human and murine mammary stem/progenitor cells, and embryonal carcinoma cells. MiR-200c affects breast cancer proliferation by modulating the expression of *BMI1*, which regulates the self-renewal of stem cells. Furthermore, miR-200c has been shown to inhibit the development of normal mammary stem cells into mammary ducts as well as the ability of human BCSCs to form tumors *in vivo* [180].

4.2.6. MiRNAs resulting in breast cancer therapy resistance

Several miRNAs have been described as controlling genomic stability of breast cancer cells. DNA double-strand breaks are lesions induced by ionizing radiation (IR) and can be efficiently repaired by DNA homologous recombination, a system that requires RAD51 recombinase. Overexpression of miR-155 in human breast cancer cells reduces the level of *RAD51* and affects the cellular response to IR. Consequently, tumors overexpressing miR-155 are sensitive to radiation therapy. Furthermore, high miR-155 levels are associated with lower *RAD51* expression and with better overall survival of patients in a large series of triple-negative breast cancers [181]. Other miRNAs have also been shown to sensitize breast cancer cells to chemo/radio sensitivity. The tumor suppressor p53 whose expression is affected by DNA damage and oncogenic stress, is the direct inducer of miR-34a [182]. It has been observed that elevated expression of miR-34a in a HER-2 positive breast cancer cell line (UACC-812) contributes to increased resistance to ionizing radiation as opposed to MDA-MB-231 expressing low levels of miR-34a. Thus, while the mechanism is unknown, in p53-mutant breast cancers, inhibition of miRNA-34a enhances radio sensitivity [183]. Also,

miRNA-182 controls DNA repair of breast cancer cells by targeting *BRCA1*, and inhibition of miRNA-182 leads to resistance to PARP inhibitors (poly ADP ribose polymerase) [184]. Because the BRCA pathway controls mammary stem cell fate, it is possible that overexpression of miR-182 in breast cancer stem cells would sensitize this radio-resistant cell population to radiation therapy. Anti-estrogen therapies are given to the patients with ER-positive breast tumors. Despite initial response, 25% of primary tumors and almost all metastatic tumors will develop resistance. MiRNA-221/222 are key regulators of hormonal resistance of breast cancer stem cells. MiRNA-221/222 act through diverse mechanisms by targeting ER α , by upregulating β -catenin and the TGF- β pathway [185] or by targeting the cell-cycle inhibitor *p27* [186]. Finally, 15–20% of breast tumors display an overexpression of the ERBB2 oncoprotein. ERBB2 overexpression promotes the expansion of the breast cancer stem cells through the activation of a PI3K/AKT/GSK3 β /WNT signaling. ERBB2-positive tumors can be treated with several targeted therapeutics. MiRNA-21 plays a role in the resistance displayed by ERBB2-positive tumors to trastuzumab, by targeting the PTEN tumor suppressor. The authors further show that knockdown of miR-21 could restore PTEN levels thus sensitizing the cells to anti-HER-2 therapy [187]. MiRNA-205 regulates the ERBB3 receptor [188]. ERBB3 transactivates ERBB2, and both receptors trigger the PI3K/AKT signaling pathway. ERBB2/ERBB3 interaction could lead to anti-ERBB2 resistance of breast tumors. However, miR-205 is downregulated in breast cancer. Thus, restoration of miRNA-205 in breast tumors could help overcome resistance to anti-ERBB2 therapy.

4.2.7. Potential prognostic value of miRNAs

Several studies have evaluated the role of specific miRNAs in breast cancer spread and survival. A screen identified five upregulated miRNAs (miR-30b, miR-148a, miR-150, miR-450a and miR-155) and six downregulated miRNAs (miR-24, miR-99a, miR-99b, miR-125b, miR-130b and miR-205) in primary breast cancer tumors versus corresponding lymph nodes [189]. Further, miR-373 was identified as being overexpressed in lymph-node metastases as compared to primary tumors [158], indicating the prognostic value of these miRNAs. Other miRNAs such as miR-187 [190], miR-27b and miR-103/107 [191] have also been found to have a prognostic value in breast cancer. Moreover, in ER-positive lymph node-negative (LNN) breast cancer patients, 12 miRNAs have been identified with early relapse versus late relapse (miR-205, miR-22, miR-516-3p, miR-7, miR-34b, miR-151, miR-210, miR-193b, miR-489, miR-449, miR-145 and miR-128a). Indeed, four of these 12 miRNAs (miR-7, miR-128a, miR-210 and miR-516-3p) have been positively linked to breast cancer aggressiveness while miR-210 has also been associated with metastatic ability of TNBC [192].

4.2.8. MiRNAs as breast cancer biomarkers

4.2.8.1. MiRNA expression from tissue biopsies

MiRNAs can serve as biomarkers for breast cancer based on their expression profile from RNA sequencing or tissue microarray assays. This can be achieved by mapping the global mRNA and miRNA expression from tumor tissues using high-throughput platforms, such as microarray chips and deep sequencing. Also, other techniques such as in-situ hybridization

(ISH) can be used to detect mRNAs and miRNAs from fresh frozen or archived paraffin-embedded (FFPE) tumor tissue samples and protein expression can be evaluated using immunohistochemistry (IHC) [193]. The use of miRNA biomarkers has several advantages over protein coding genes: (1) miRNAs are more stable than mRNA and thus enable easier and reliable detection in FFPE samples (2) the presence of mere 1000 miRNAs makes the human miRNome much easier to screen and evaluate with less demanding bioinformatic analysis than the mRNA transcriptome [194]. The expression of a number of miRNAs closely correlates with the ER, PR and HER2 status in breast cancer, highlighting their use as biomarkers of disease progression and treatment response [141, 195]. MiR-210 has been validated as a prognostic biomarker in breast cancer since elevated miR-210 levels have been associated with poor outcome both in ER-positive and ER-negative cases [196]. Moreover, miR-210 has been developed to predict outcome in ER-positive cases that received adjuvant tamoxifen treatment for 5 years [197]. Other miRNA biomarkers include miR-205, which is used as a prognostic marker for the triple negative (TN) subtype since a positive correlation has been observed between miR-205 expression and favorable clinical outcome in TN cases [198].

4.2.8.2. Circulating miRNAs as breast cancer biomarkers

Circulating miRNAs are ideal for clinical use, since they are highly stable and can be detected by a noninvasive manner in a blood sample. Serum or plasma miRNAs have been shown to be resistant to RNases and DNases thus are more stable than their cellular counter parts as well as mRNAs. Serum and plasma miRNAs can be easily isolated and quantified by RT-qPCR analysis. Moreover, specific miRNAs have also been demonstrated as being indicative of the breast cancer stage and/or ER/PR status. Numerous studies have documented the presence and quantified serum miRNAs from breast cancer patient samples. Asaga et al. assayed circulating miR-21 of 102 breast cancer patients and 20 healthy controls and found higher concentrations in these patients, especially in metastatic cases [199]. A study that quantitatively profiled the expression of seven miRNAs by real-time PCR, in tissue and blood samples of patients with breast cancer at different clinical stages and age-matched healthy individuals found that, while the expression of two miRNAs, miR-195 and let-7a was significantly higher in blood samples of breast cancer patients in comparison to control subjects, their circulating levels remarkably decreased after surgical resection in a subset of 29 cases, reaching levels comparable with control subjects [200, 201]. 26 circulating miRNAs with two-fold differential expression have been identified from the plasma of early stage breast cancer patients as compared to healthy controls [202].

This mounting evidence generates the hypothesis for a signature of circulating miRNAs that could be a reliable biomarker for disease progression.

4.2.9. MiRNA therapeutics in breast cancer

4.2.9.1. AntagomiRs and anti-miRNA oligonucleotides targeting oncomiRs and metastamiRs

The most common miRNA therapeutic approach to inhibit the functions of miRNAs involve, targeting by using antisense miRNAs (antagomiRs) capable of knocking down these miRNAs. AntagomiRs are synthetic RNA molecules with favorable stability, resistance to RNase

and pharmacologic properties that allow *in vivo* miRNA inhibition [203]. MiRNA knockdown therapy can be used in conjunction with chemotherapy to facilitate knockdown of oncomiRs along with concomitant targeting of the proliferating cells using anticancer drugs. Knockdown of miR-10b using sequence-specific antagomiRs led to an upregulation of its target mRNA *Hoxd10*. However, the use of miR-10b antagomiRs did not reduce primary mammary tumor growth in animal model but was successful in suppressing the formation of lung metastases. Furthermore, miR-10b antagomiR does not induce toxicity in healthy mice and thus can be a safe therapeutic option [204]. Moreover, knockdown of miRNAs by anti-sense approach also sensitizes cancer cells to chemotherapeutic drugs as in the case of miR-21, the knockdown of which sensitized MCF7 cells to the chemotherapeutic agent and topoisomerase inhibitor, topotecan [205].

Another approach of ablating miRNAs function is by using anti-miRNA oligonucleotides (AMOs) with 2-O-methyl groups and AMOs based on locked nucleic acid (LNA). AMOs are stable synthetic antisense oligonucleotides that can rapidly, selectively and irreversibly bind endogenous miRNAs, sequester and make them functionally inactive [206, 207]. Targeting oncomirs *via* either antagomiRs or AMOs has been demonstrated to reduce cancer cell proliferation and metastasis [79], sensitization to chemotherapeutic agents [80], hormone therapy [52] and anti-HER2 therapy [71] in breast cancer cell lines.

In addition to knocking down miRNAs, upregulating the expression and activity of tumor suppressor miRNAs has potential in ameliorating breast cancer. Tumor suppressor miRNAs can be upregulated using miRNA mimics, which are synthetic molecules with short double-stranded synthetic oligonucleotides with sequence similarity to the particular miRNA under consideration. Overexpression of TS miRNAs using miRNA mimics has been shown to decrease cancer cell proliferation as well as induce chemosensitivity in breast cancer cell lines [208, 209].

4.2.9.2. Peptide nucleic acids (PNA)

Peptide nucleic acid (PNA) is an artificially synthesized oligonucleotide similar to DNA and RNA with a backbone consisting of repeats of 2-aminoethylglycine units [210]. The absence of phosphate groups renders a neutral charge to the PNA resulting in stronger and specific bonds between complementary PNA/DNA and PNA/RNA as compared to DNA/DNA or RNA/RNA. Owing to its synthetic nature, PNA is resistant to degradation by DNases and proteases leading to increased intracellular stability. Inactivation of miR-221 with PNA has been successful in aggressive breast cancer cell lines where miR-221 is overexpressed [211]. An anti-miR-221 PNA (R8-PNA-a221) conjugated with polyarginine-peptide (R8) could inactivate miR-221 and upregulate its target mRNA, *p27/Kip1*. R8-PNA-a221 displayed efficient uptake within target cells without using transfection reagents. To assess the potential of PNA-anti-miR-221 on inhibition of breast cancer *in vivo*, MCF-7 cells treated with PNA-anti-221 or control PNAs were injected in nude mice [212]. Tumor formation was observed only in 60% of mice treated with anti-miR-221 therapy as compared to control. These studies highlight the potential of PNAs as anti-tumor therapeutics.

4.3. Long noncoding RNAs in breast cancer

Long noncoding RNAs are endogenous RNA molecules with a mature length of more than 200 bases that do not code for functional proteins [213]. LncRNAs are epigenetic regulators, and they control gene expression at both the transcriptional and posttranscriptional levels. LncRNAs utilize a variety of mechanisms to regulate gene expression. They can recruit chromatin modifiers to impair access to targeted genes, they can act as scaffolds to assemble complexes that do not have interacting domains, they can interact with transcription factors to directly regulate gene expression, and they can serve as 'miRNA sponges' to trap miRNAs and regulate translation. Moreover, lncRNAs can be involved in the regulation of the expression of either their neighboring genes in cis or more distant genes in trans. LncRNAs act as coactivators, binding to transcription factors and enhancing their transcriptional activity [214].

4.3.1. Oncogenic LncRNAs in breast cancer

H19 is among the first discovered lncRNAs and displays elevated expression in breast cancer [215]. This upregulation of expression is on account of increased binding of the transcription factor *E2F1* factor to H19 promoter. H19, in turn, promotes cell proliferation in MDA-MB-231 cells *in vitro* [216] and also accelerates tumor growth *in vivo* in animal model [217], possibly by repressing tumor suppressor genes such as *caveolin-1* [218].

HOTAIR is remarkably overexpressed in metastatic breast cancer. Upregulated HOTAIR in breast cancer cells provides a scaffold for *PRC2* and *LSD1-CoREST* (lysine-specific demethylase-1 with its corepressor protein CoREST (RE1 silencing transcription factor/neural-restrictive silencing factor)). *PRC2* binds to the 5' region of HOTAIR while *LSD1-CoREST* binds to its 3' region. This complex regulates the histone modifications H3K27me3 and H3K4me2 at the promoters of metastasis suppressing genes such as *PCDH10*, *PCDHB5* and *JAM2*. As a result, these metastasis suppressor genes are silenced; thereby contributing to HOTAIR-induced breast cancer metastasis. Indeed, overexpression of HOTAIR in breast cancer cell lines has been observed to increase their invasiveness both *in vitro* and *in vivo*. Furthermore, knock-down of HOTAIR has been shown to attenuate *EZH2*-induced invasion, in benign immortalized breast cells overexpressing *EZH2* [219]. Upregulated HOTAIR levels in primary breast tumor are an indicator of metastasis thus identifying HOTAIR as an important prognostic factor for breast cancer [220].

Urothelial cancer-associated 1 (UCA1) has been identified as an oncogene in breast cancer. Huang et al. demonstrated the oncogenic role of UCA1 in breast cancer, in part through suppression of *p27*. UCA1 competes with *p27* mRNA to form a ribonucleoprotein complex with hnRNP I (heterogeneous nuclear ribonucleoprotein I) thereby increasing UCA1 stability and decreasing *p27* protein levels, hence leading to increased proliferation of breast cancer cells [221]. Further, UCA1 has been shown to bind to and sequester miR-143 thus decreasing its expression in invasive breast cancer cell lines. MiR-143 is known to target and regulate the expression of ERBB3. Hence, it is possible that UCA1 increases breast cancer cell proliferation by deregulating miR-143-based ERBB3 repression [222].

MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) or NEAT2 is a conserved nuclear noncoding RNA. The role of MALAT1 in breast cancer was controversial with reports indicating an oncogenic role by promoting cell proliferation, migration and invasion during breast cancer development [223] while a loss of MALAT1 was shown to promote EMT *via* phosphatidylinositol-3 kinase-AKT pathways on MALAT1 [224]. Recently, Arun et al. demonstrated an oncogenic role of MALAT1 in breast cancer where knockdown of MALAT1 using antisense oligonucleotides (ASOs) in a mouse model resulted in slower tumor growth and a reduction in metastasis. Thus, knockdown of MALAT1 using ASOs represents a viable therapeutic option in breast cancer [225].

SRA (steroid receptor RNA activator protein) gene generates both a coding as well as noncoding form of SRA RNA [226]. The noncoding (lnc) SRA RNA is significantly upregulated in breast cancer tumors [226], especially in PR-positive tumors [227] and in aggressive and invasive breast cancer cell lines (MDA-MB-231 and MDA-MB-468) [228]. LncSRA acts as a scaffold in assembling coregulator complexes by interacting with and coactivating several nuclear receptors (steroid and nonsteroid) and other transcription factors. This hints to a possibility of lncSRA aiding in the transcription of key oncogenes in breast cancer. Thus, it was no surprise that knockdown of SRA in MDA-MB-231 cells reduced cell invasion along with a downregulation of the genes associated with this phenotype. Moreover, depletion of lncSRA in MCF7 cells exhibited a similar response with a decrease in the genes responsible for invasion and metastasis [229].

Long stress-induced noncoding transcripts (LSINCTs) are a group of lncRNAs upregulated in breast cancer tumor tissues and cell lines. LSINCT5 has been shown to mediate cellular proliferation and is aided by lncNEAT-1 and *PSPC1* (Paraspeckle Component 1) in breast cancer [230, 231].

4.3.2. Tumor suppressor lncRNAs in breast cancer

The lncRNAs that are downregulated in cancer and whose enforced expression is associated with the suppression of cell proliferation or cell death are termed as tumor suppressor lncRNAs.

Maternally expressed gene 3 (MEG3) is a tumor suppressor lncRNA with a decrease in expression in breast cancer, especially in the most aggressive TNBC subtype [232, 233]. MEG3 forms a RNA-DNA triplex structure to regulate the TGF- β pathway genes in breast cancer cells [234]. Since TGF- β is an inducer of EMT and invasiveness in breast cancer, inhibition of this pathway *via* MEG3 could present a therapeutic opportunity to control breast cancer. MEG3 is also known to reduce breast cancer proliferation and invasion by indirectly modulating p53 activity. MEG3 regulates MDM2 (mouse double minute 2 homolog) leading to accumulation of p53 levels in breast cancer cell lines. This p53 could in turn bind to the promoters of its target genes and metastasis suppressors *p21*, *MSPIN* and *KAI1* inhibiting migration and invasion of MCF-7 breast cancer cells [232].

GAS5 (growth arrest specific 5), in breast cancer, the expression level of GAS5 has been shown to be significantly reduced in tumor samples as compared to surrounding normal breast epithelia [235]. This decrease in GAS5 expression was observed in grade I and II breast

cancer patients, indicating that the GAS5 downregulation is an early event in breast cancer progression. Further, this observation also indicates that GAS5 expression may be used as a biomarker to predict cancer stage. GAS5 has additional roles in drug resistance and will be discussed in the next part.

NKILA (NF- κ B interacting lncRNA) binds to the NF- κ B/I κ B complex masking the phosphorylation site on I κ B. Thus, IKK is unable to phosphorylate I κ B resulting in I κ B remaining bound to NF- κ B, rendering NF- κ B inactive. Expression of NKILA was observed to increase apoptosis and reduce invasion in MDA-MB-231 cells. Moreover, ectopic expression of NKILA decreases invasion and metastasis in breast cancer mouse models. Also, low NKILA expression is associated with poor patient prognosis [236]. Thus, inhibiting NF- κ B through NKILA may be a mechanism to suppress breast cancer metastasis.

4.3.3. *LncRNAs and breast cancer stem cells*

A number of lncRNAs have been implicated in maintaining stemness of breast cancer stem cells, thus promoting the spread of the cancer. The lncRNA HOTAIR has been shown to downregulate miRNA-7 associated with EMT and STAT3 activity [237]. The stemness factor SOX2 is upregulated by lncRNAs such as SOX2OT [238] and linc00617 [239]. Further, the self-renewal hedgehog (HH) pathway is activated by lncRNAs including lncRNA-Hh, which promotes CSCs maintenance through the activation of the HH-GLI1-SOX2 axis [240].

4.3.4. *LncRNAs and drug resistance in breast cancer*

The lncRNA BCAR4 (breast cancer antiestrogen resistance 4) was identified from a screen designed to find mechanisms of estrogen resistance in breast cancer. Ectopic expression of BCAR4 in tamoxifen-sensitive ZR-75-1 breast cancer cells inhibited the cancer cell death mediated by tamoxifen, thereby making BCAR4 an important biomarker for tamoxifen resistant breast cancer. Since BCAR4 expression has only been detected in human placenta apart from breast cancer epithelia, silencing of BCAR4 in breast cancer patients could be a potential anticancer therapy due to the limited number of side effects of diminishing BCAR4 expression in other healthy tissues [241].

Trastuzumab resistance is a major impediment in the clinical management of HER2-positive breast cancer. LncRNA GAS5 is downregulated in trastuzumab-treated breast cancer patient specimens, breast tumors in animal model *in vivo* and trastuzumab resistant breast cancer cell line, SKBR-3/Tr *in vitro*. GAS5 targets miR-21, resulting in a restoration of the levels of the miR-21 target, PTEN. Since PTEN is a tumor suppressor affecting cell proliferation, reactivation of this gene results in cell cycle arrest in breast cancer cells [242]. This identifies GAS5 as a novel prognostic marker and potential therapeutic target for HER-2 positive breast cancer. Also, GAS5 levels are significantly downregulated in TNBC cell line MDA-MB-231. Restoration of GAS5 levels in MDA-MB-231 sensitizes these cells to UV-C irradiation induced cell death. PI3K and mTOR inhibition could restore GAS5 levels [243]. Thus, reactivation of GAS5 using PI3K/mTOR inhibitors in TNBC may be a therapeutic option to sensitize this aggressive cancer to chemotherapy.

4.3.5. *LncRNAs for breast cancer prognosis, diagnosis and therapy*

LncRNAs are being evaluated to have potential as breast cancer biomarkers, for breast cancer subtype classification and developing diagnostics and therapies, owing to their cell-type specific expression and correlation with patient response to chemotherapy. In a recent study, more than 1300 lncRNAs and 2800 mRNAs were found to be enriched in HER-2-enriched subtype breast cancer as compared to normal tissue. *AFAP1-AS1* was identified as the most dysregulated lncRNA, whereas lncRNA *LOC100288637* displayed the highest positive correlation with HER-2 expression indicating the potential use of these lncRNAs as breast cancer biomarkers [244]. Furthermore, a transcriptomic analysis of triple negative (TN) breast cancer samples as compared to control identified a unique mRNA-lncRNA signature. The authors demonstrated that *HIF1A-AS2* and *AK124454* promoted cell proliferation and invasion in TNBC cells and contributed to paclitaxel resistance [245]. Such studies and many more in the future will help identify novel lncRNA biomarkers for breast cancer classification and disease progression.

Similar to miRNAs, circulating lncRNAs have been detected in plasma of cancer patients [246]. Recently, increased expression of lncRNA *RP11-445H22.4* was detected in the plasma of breast cancer patients as compared to healthy individuals [247]. Further, *HOTAIR* DNA has been established as a potential biomarker for breast cancer as these patients displayed an upregulated expression of *HOTAIR* DNA as compared to healthy individuals. Moreover, the expression level of *HOTAIR* DNA correlated with the progress of the cancer [248].

In conclusion, noncoding RNAs including miRNAs and lncRNAs represent a significant resource of novel cancer biomarkers including noninvasive circulating noncoding RNAs, prognostic aids and potential therapeutic targets to be used in conjunction with chemotherapy and adjuvant therapy. However, significant research is required, especially in the lncRNA field, to take these RNA molecules from the bench to bedside.

5. Conclusion

In summary, due to the advances in sequencing techniques and novel methods to study chromatin organization, the repertoire of information about the significant role played by the chromatin architecture, and its dysregulation in cancer cells is slowly being uncovered. The knowledge that the epigenetic landscape shapes the underlying genetic information is revolutionizing the field of cancer biology, the organized chaos in the genome of cancer cells now can be attributed at least in part to the aberrant regulation of chromatin modifiers and remodelers. The way in which cell-signaling pathways interact with epigenetic elements in the genome appears to be wide spread and complex. Integrating both networks is important not only for the comprehension of complex processes such as development, cell differentiation, cell regulation and cell plasticity but also toward the study of the relationship between signal transduction pathways and its targeted effect over diverse epigenetic processes. The therapeutic implication of targeting the epigenetic regulators has been discussed in detail and is the focus of many ongoing clinical trials as well as research. An integrative research platform will help in curating the information and translating the current epigenetic discoveries into useful diagnostic and therapeutic tools.

Appendix

Abbreviation used	Full form
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
AE	Antiestrogens
AI	Aromatase inhibitor
<i>AIB1</i>	Amplified in breast cancer 1
<i>AKT1</i>	AKT8 virus oncogene cellular homolog
AMO	Anti-miRNA oligonucleotides
<i>APC</i>	Adenomatous polyposis coli
<i>ARID1A/ARID1B</i>	AT-rich interactive domain-containing protein 1 A/B
ASOs	Antisense oligonucleotides
ATP	Adenosine triphosphate
Aza	5-aza-2'-deoxycytidine
<i>BAF155/SMARCC1</i>	<i>BRG1</i> -associated Factor 155/SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily C member 1
<i>BCAP31</i>	B-cell receptor-associated protein 31
<i>BCAR4</i>	Breast cancer antiestrogen resistance 4
Bcl-2	B-Cell CLL/Lymphoma 2
<i>BMI1</i>	B lymphoma Mo-MLV insertion region 1 homolog
<i>BRCA1</i>	Breast cancer gene 1
<i>BRG1</i>	Brahma-related gene 1
<i>BRM</i>	Brahma
<i>BRMS1</i>	Breast cancer metastasis suppressor 1
CARM1	Coactivator associated arginine methyltransferase 1
CBP	Cyclic amp response element binding protein
CD44	Cluster of differentiation 44
<i>CDH1</i>	E-cadherin
cDNA	Complimentary deoxyribonucleic acid
CHD	Chromodomain helicase DNA-binding
ChIP	Chromatin Immunoprecipitation
CK5/CK14	Cytokeratin-5/14
CoREST	RE1-silencing transcription factor corepressor complex
CpG	Cytosine preceding Guanine
CSC	Cancer stem cells
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase

Abbreviation used	Full form
<i>E2F1</i>	Transcription factor activating adenovirus E2 gene
EED	Embryonic ectoderm development
EGFR	Epidermal growth factor receptor
ELF5	E74-like ETS transcription factor 5
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
<i>ERBB2</i>	Erb-B2 receptor tyrosine kinase 2
ERE	Estrogen-responsive elements
ERK1/2	Extracellular signal-regulated protein kinase 1/2
EZH2	Enhancer of zeste 2
FFPE	Formalin-fixed paraffin-embedded
<i>FOXC1</i>	Forkhead Box C1
GAS5	Growth Arrest Specific 5
GATA	Transcription factors that can bind to the DNA sequence (A/T)GATA(A/G).
GATA3	GATA binding protein 3
<i>GLI1</i>	Glioma-associated oncogene homolog 1 (Zinc Finger Protein)
GSK3B	Glycogen synthase kinase 3 Beta
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HER2	Human epidermal growth factor receptor 2
HH	Hedgehog
HMGA2	High mobility group AT-hook2
HMT	Histone methyl transferase
hnRNP I	Heterogeneous nuclear ribonucleoprotein I
<i>HOXA</i>	Homeobox A
HP1	Heterochromatin Protein 1
HRE	Hormone-responsive elements
IHC	Immunohistochemistry
IL-6	Interleukin 6
IR	Ionizing radiation
IRAK1	Interleukin 1 receptor-associated kinase 1
ISH	<i>In situ</i> hybridization
<i>ITBG3</i>	Integrin β 3
JARID1C	Jumonji, AT Rich Interactive Domain 1C
KDM/HDM	Lysine/histone demethylase
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LCOR	Ligand-dependent corepressor
LNA	Locked nucleic acid
lncRNAs	Long noncoding RNAs

Abbreviation used	Full form
LNN	Lymph node-negative
LSINCTs	Long stress-induced noncoding transcripts
<i>LZTS1</i>	Leucine zipper, putative tumor suppressor 1
MAL	MyD88-adaptor-like
<i>MALAT1</i>	Metastasis associated lung adenocarcinoma transcript 1
MAPK	Mitogen-activated protein kinases
MaSCs	Mammary stem cells
MBD2/3	Methyl-CpG binding domain protein 2/3
MeCP2	Methyl-CpG binding protein 2
MEG3	Maternally expressed gene 3
miRNA	microRNA
MMP	Matrix metalloprotease
MOF	Male absent on the first
MORF	MOZ-related factor
MOZ	Monocytic leukemic zinc finger
mRNA	Messenger ribonucleic acid
MSK1	Mitogen and stress activated protein kinase 1
MTA	Metastasis-associated proteins
mTOR	Mammalian target of rapamycin
MYST	Moz, Ybf1, Sas2, TIP60
NCOR	Nuclear receptor corepressor
NEAT-1	Nuclear Paraspeckle Assembly Transcript 1
NFκB	Nuclear factor κB
NKILA	NF-κB interacting lncRNA
NOTCH1	Notch Homolog 1, translocation-associated
NSD3L	Nuclear SET domain-containing protein 3 long isoform
NuRD	Nucleosome remodeling and histone deacetylation
OHT	Hydroxytamoxifen
ORM2	Orosomucoid 2
p27	Cyclin-dependent kinase inhibitor 1B (p27, KIP1)
PARP	Poly (ADP-ribose) polymerase
PCAF	p300/CBP-associated factor
<i>PCDH10</i>	Protocadherin 10
<i>PCDHB5</i>	Protocadherin Beta 5
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PDXP	Pyridoxal phosphate phosphatase

Abbreviation used	Full form
<i>PEBP1</i>	Phosphatidylethanolamine binding protein 1
PELP1	Proline, glutamate and leucine-rich protein 1
PI3K	Phosphoinositide 3 kinase
piRNA	piwi-interacting RNA
Piwi	P-element induced WImpy testis in <i>Drosophila</i>
PKB	Protein kinase B
PKC	Protein kinase C
PNA	Peptide Nucleic Acids
PP1	Phosphoprotein phosphatase 1
PP2A	Phosphoprotein phosphatase 2A
PR	Progesterone receptor
PRC2	Polycomb repressive complex 2
<i>pS2</i>	Gene which codes for Trefoil factor 1 (TFF1)
<i>PSPC1</i>	Paraspeckle component 1
PTEN	Phosphatase and tensin homolog
<i>RARβ</i>	Retinoic acid receptor beta
<i>RASSF1A</i>	Ras association domain family member 1
RB1	Retinoblastoma 1
REA	Repressor of estrogen receptor activity
RNA	Ribonucleic acid
RUNX3	Runt related transcription factor 3
S6K1	Ribosomal protein S6 kinase beta-1
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosyl methionine
SET domain	Suppressor of variegation 3-9 (Su(var)3-9), enhancer of zeste (E(z)), and trithorax (Trx) domain
SFRP1	Secreted frizzled-related protein 1
SHR	Steroid hormone receptors
siRNA	Small interfering RNA
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 1
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SOX-2	SRY (sex determining region Y)-box 2
SRA	Steroid receptor RNA activator protein
Src	Rous sarcoma oncogene cellular homolog
SRC	Steroid receptor coactivator
Suz12	Suppressor of zeste 12 protein homolog
SWI/SNF	Switch/sucrose nonfermentable
TET	Ten-eleven translocation
TGF- β	Transforming growth factor β

Abbreviation used	Full form
TIMP3	Tissue inhibitor of metalloproteinases 3
TIP60	TAT interactive protein 60 KDa
TNBC	Triple negative breast cancer
TNF- α	Tumor necrosis factor α
TP53	Tumor protein 53
TPM1	Tropomyosin 1
TRAF6	TNF receptor associated factor 6
TSA	Trichostatin A
TSG	Tumor suppressor gene
UBC9	Ubiquitin conjugating enzyme 9
UCA1	Urothelial cancer-associated 1
VA	Valproic acid
VEGFR2	Vascular endothelial growth factor receptor 2
Wnt	Wingless-type MMTV integration site family member
ZEB1/ZEB2	Zinc finger E-box binding homeobox 1/2

Abbreviations used in the text.

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