ASIP OUTSTANDING INVESTIGATOR AWARD LECTURE

Dysregulation of the Epigenome in Human Breast Cancer

Contributions of Gene-Specific DNA Hypermethylation to Breast Cancer Pathobiology and Targeting the Breast Cancer Methylome for Improved Therapy

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Triple-negative breast cancers (including basal-like and claudin-low molecular subtypes) represent 20% to 25% of all breast cancers, but disproportionately contribute to breast cancer–associated death. We have identified a novel fundamental biological property of triple-negative breast cancers: most triple-negative breast cancers express aberrant DNA hypermethylation due to overexpression of DNA methyltransferase 3b (and hyperactivity of the DNA methyltransferase enzymes). DNA methyltransferase 3b overexpression occurs secondary to loss of miRNA-mediated post-transcriptional regulation. The resulting hyperactivity of DNA methyltransferase 3b produces concurrent DNA methylation-dependent silencing of numerous critical gene targets (including tumor suppressors and pro-apoptotic genes) and resistance to cytotoxic chemotherapy. This observation presents new opportunities for development of innovative treatment strategies on the basis of the epigenome as a novel therapeutic target in triple-negative breast cancers. Epigenetic therapy represents a new principle in cancer treatment in which restoration of critical molecular pathways occurs secondary to reexpression of silenced genes that encode negative mediators of cancer cell growth. (*Am J Pathol* 2015, 185: 282–292; <http://dx.doi.org/10.1016/j.ajpath.2014.12.003>)

Breast cancer is not a single disease, but is a collection of diseases with differing molecular and cellular characteristics, and variable clinical behaviors and outcomes. Nonetheless, all forms of breast cancer share in common certain biological properties of the cells that compose the tumors, including unregulated (clonal) cell growth, impaired cellular differentiation, invasiveness, and metastatic potential. It is now well recognized that breast cancer in its simplest form is a genetic disease, and more precisely a disease of abnormal gene expression. The molecular mechanisms governing dysregulated cellular functions in breast cancer involve loss, mutation, or dysregulation of genes that positively and negatively regulate cell proliferation, migration, and differentiation. Breast carcinogenesis is the multistep process through which breast cancer develops in response to changes in gene expression driven by chromosomal

alterations, gene mutations, and epigenetic alterations of DNA.^{1–3} In breast cancer cells, epigenetic alterations occur within the larger context of extensive changes to chromatin structure related to altered patterns of histone modification, and

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methylation gains and losses on CpG dinucleotides within DNA sequences.⁴

DNA methylation (chemical alteration of cytosine bases) is the most studied and best-understood mechanism of epigenetic regulation^{3,5,6} and is regarded as the hallmark of epigenetic modification. DNA methylation occurs almost exclusively on cytosines within CpG dinucleotides, which are found in the genome at approximately 20% of the predicted frequency, and most (>70%) of CpG dinucleotides are typically methylated.⁷ However, regions of CpG density, termed CpG islands,^{8,9} occur in the promoter sequences of many genes, proximal to their transcription start site.¹⁰ CpG islands are conventionally defined as ≥ 200 bp with $\geq 50\%$ G + C and ≥ 0.6 CpG observed/CpG expected,¹¹ although a more rigorous definition (≥ 200 bp with $\geq 60\%$ G + C and ≥ 0.7 CpG observed/CpG expected) has been proposed.⁹ Gene promoters and other regulatory regions of housekeeping genes are largely unmethylated in most cell types, and tissue-specific genes are unmethylated only in the cell types in which these genes are transcriptionally active.¹² Most promoter CpG islands are unmethylated in normal tissues, but may become methylated in human cancers leading to transcriptional silencing of tumor-suppressor genes (and other negative mediators of neoplastic growth), resulting in selective growth advantages for emergent neoplastic cells.

Neoplastic transformation is associated with alterations in DNA methylation, including both global hypomethylation and gene-specific hypermethylation.^{13–15} DNA hypomethylation in cancer was first recognized in rat models of hepatocellular carcinoma,¹⁶ and subsequently was reported in human cancers.^{17–19} Hypomethylation of cancer cell genomes reflects loss of methylation in CpG-depleted regions where most CpG dinucleotides would be expected to be methylated.^{17,19,20} The loss of methylation in these regions of the genome may be associated with aberrant or inappropriate expression of some genes that could contribute to neoplastic transformation, tumorigenesis, or cancer progression.¹⁸ In addition, genome-wide demethylation can contribute to chromosomal instability by destabilizing pericentromeric regions of certain chromosomes.^{21–23} Gains in DNA methylation in cancer cells typically reflect hypermethylation of CpG islands or other regions of CpG density in gene promoter regions, which can lead to gene silencing.¹³ Methylation-dependent gene silencing is a normal mechanism for regulation of gene expression.²⁴ In cancer cells, methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism for inactivation of tumor-suppressor genes.²⁵ A significant number of cancer-related genes have been identified that are subject to methylation-dependent silencing,^{7,26} and many of these genes contribute to the hallmarks of cancer.^{27,28}

This review discusses five sets of experimental studies related to aberrant DNA hypermethylation in breast cancer. In the first set of studies, we identified genes that are

sensitive to methylation-dependent silencing in breast cancer through a discovery-based experimental design,²⁹ and characterized these genes extensively for CpG features and methylation state in breast cancer.^{29–31} In the second set, the methylation-sensitive genes discovered were used to classify breast cancer cell lines for aberrant DNA hypermethylation.³⁰ In the third set, we identified overexpression of DNA methyltransferase (DNMT) activity and specific overexpression of DNMT3b as the mechanism behind aberrant DNA hypermethylation in breast cancer cell lines³² and showed that the loss of regulatory miRNAs leads to overexpression of DNMT3b.^{33,34} In the fourth set, we probed data from microarray-based gene expression studies to determine whether aberrant DNA hypermethylation occurs in primary breast cancers and to determine the affected molecular subsets of breast cancer,^{32,34} and linked the loss of regulatory miRNAs to overexpression of DNMT3b.³³ In the final set of experiments, we demonstrated that the normalization of aberrant DNA hypermethylation (through inhibition of DNMT activity) sensitizes breast cancer cells to cytotoxic chemotherapy.³⁵

Identification and Characterization of Methylation-Sensitive Genes in Breast Cancer

Although numerous methylation-sensitive genes have been identified over time in various cell lines and cancer model systems, we set out to identify epigenetically regulated genes in breast cancer using an unbiased and innovative cell line-based discovery approach.²⁹ We hypothesized that the genes of greatest interest from the perspective of epigenetic regulation in breast cancer would display certain characteristics: i) express at low or undetectable levels in control cells (reflecting a silenced condition), ii) display robust expression after demethylating treatment (reflecting activation or enhancement of expression on release from silencing), and iii) exhibit loss or reduction of expression after removal of demethylating treatment (reflecting gene silencing on remethylation of the promoter sequences).²⁹ In addition, we reasoned that low-dose treatment of cells with demethylating agents over a longer exposure period would eliminate concerns over acute toxicity associated with high-dose treatment of cells with these drugs. Therefore, to identify genes that are epigenetically regulated in human breast cancer, we treated MCF7 breast cancer cells with low-dose 5-aza-2'-deoxycytidine (5-aza) or low-dose 5-aza combined with low-dose trichostatin A over a period of 3 weeks, followed by a 5-week recovery period after treatment withdrawal.²⁹

Microarray-Based Gene Expression Profile

Microarray-based gene expression profiles identified twofold or greater increased expression of 79 and 107 genes in MCF7 cells exposed to 5-aza alone or 5-aza + trichostatin A, respectively.²⁹ To enrich for genes that are epigenetically

regulated through promoter methylation, we analyzed the microarray data to identify genes that showed increased expression levels in response to demethylating treatment (presumably related to a promoter demethylation event), but returned to control expression levels after treatment withdrawal (presumably related to a promoter remethylation event). This analysis identified 37 and 70 genes in MCF7 cells exposed to 5-aza alone or 5-aza + trichostatin A, respectively.²⁹ Comparison of these gene lists identified 20 genes (*BF*, *C8orf4*, *CEACAM5*, *CEACAM6*, *CRIP1*, *CST6*, *CYP11B1*, *FLJ10134*, *GIP2*, *GIP3*, *IFI27*, *IGFBP5*, *ISGF3G*, *KRTHB1*, *LCN2*, *LGALS3BP*, *PSG6*, *SAT*, *SCNN1A*, and *ZC3HDC1*) in common between treatment groups.²⁹

Gene Silencing by Promoter DNA Methylation

It is well known that DNA methylation affecting gene promoter sequences and downstream proximal sequences can result in gene silencing, but that DNA methylation elsewhere in a gene will not hinder transcription.^{36,37} Therefore, we expected (and predicted) that many or most epigenetically regulated genes identified in MCF7 cells would contain CpG islands within their promoter and/or downstream proximal sequences. However, we found that only 45% of epigenetically regulated genes identified contain typical CpG islands¹¹ in their promoter and/or exon 1 sequences. This observation is consistent with other studies reporting that genes lacking CpG islands are frequently induced by demethylating drugs.^{38–42}

Mechanisms of Gene Silencing in Genes Lacking CpG Islands

Genes lacking CpG islands may respond to demethylating drugs as a result of indirect regulation by DNA methylation (genes regulated directly by the protein products of genes containing CpG islands). Alternatively, novel CpG targets of DNA methylation may function to confer methylation sensitivity to genes lacking CpG islands. Given that the accepted criterion for a CpG island reflects an arbitrarily defined set of sequence features,¹¹ CpG targets of biological significance may not conform.³⁷ In our study, 40% of epigenetically regulated genes contained weak CpG islands, and three genes were identified that lack features expected for epigenetically regulated genes (typical or weak CpG islands). Weak CpG islands are defined as >50 bp, but <200 bp, with $\geq 50\%$ G + C and ≥ 0.6 CpG observed/CpG expected.²⁹ Hence, we grouped the putative epigenetically regulated genes identified into three distinct classes, including the following: i) genes with typical CpG features (typical CpG islands within the promoter or exon 1), ii) genes with intermediate CpG features (weak CpG islands within the promoter or exon 1), and iii) genes with atypical CpG features (no typical or weak CpG islands) (Figure 1).

Having identified several genes that lack the CpG features expected for a methylation-sensitive gene, we set forth to

determine whether genes lacking typical CpG islands might be subject to DNA methylation-dependent silencing. Methylation analysis by bisulfite sequencing⁴³ was performed on the promoter regions of prototype genes from each proposed class of putative epigenetically regulated gene: *CST6* (typical CpG features), *C8orf4* (intermediate CpG features), and *IFI27* (atypical CpG features). This analysis produced evidence for the regulation of genes in each proposed class by CpG methylation in MCF7 breast cancer cells,²⁹ and identified CpG methylation events that are critical for gene silencing. In *CST6*, methylation of the promoter/exon 1 CpG island correlates with gene silencing, and demethylation leads to gene expression.²⁹ In *C8orf4*, methylation of the weak CpG island in exon 1 correlates with gene silencing and demethylation results in gene expression.²⁹ In *IFI27*, methylation at specific CpG dinucleotides within the promoter correlates with gene silencing and demethylation at these CpG sites leads to gene expression.²⁹

Although our studies focused on characterization of a single prototype gene from each class, we have generated preliminary data with additional genes from each class that support the conclusions presented herein (unpublished studies). It is intriguing to suggest that novel CpG target sequences may confer methylation sensitivity to these genes (and genes with similar CpG features). Direct evidence for methylation-dependent regulation of genes lacking CpG islands has emerged from a few investigations.^{44–46} Therefore, on the basis of our results and studies from the literature, we proposed expansion of the current model for DNA methylation-dependent epigenetic regulation of gene expression to include genes lacking typical CpG islands (Figure 1). The expanded model recognizes that all promoter CpG dinucleotides represent legitimate targets for methylation; sites for methylation may represent regional targets (CpG islands), local CpG density (weak CpG islands), or isolated CpGs; discrete methylation events occurring within CpG target sequences contribute to gene silencing; and CpG methylation contributes to gene silencing (or diminished expression) through different mechanisms (Figure 1). This expanded model highlights the importance of the CpG characteristics of individual gene promoters and the targets for methylation that they contain, the nature of specific methylation events, and how these factors combine to regulate gene expression and/or silencing.

Aberrant DNA Hypermethylation in Breast Cancer Cell Lines and Among Primary Breast Cancers

Unsupervised Cluster Analysis of Breast Cancer Cell Lines

Our early studies that focused on *CST6* as a prototype methylation-sensitive gene in breast cancer suggested that breast cancer cell lines vary in their state of aberrant DNA

Figure 1 An expanded model for gene silencing in response to CpG methylation events. Promoter DNA sequences are depicted as horizontal lines, with vertical lines corresponding to CpG dinucleotides. **Yellow circles** on top of vertical lines indicate CpG dinucleotide methylation; **green, purple, and blue shapes**, a generic transcription factor enzymatic complex; and **pink and orange ovals**, methylated DNA binding proteins (requiring many or few methylated CpGs for binding). **A:** Promoter sequences of epigenetically regulated (methylation-sensitive) genes may contain conventionally defined CpG islands (typical CpG features), may contain short regions of CpG density or weak CpG islands (intermediate CpG features), or may lack regions of CpG density (atypical CpG features). **B:** Transcription factors and complexes interact with specific sequences of gene promoters to bind and initiate transcription. CpG dinucleotides (or regions of CpG density) frequently impinge on these regulatory sequences. **C:** Methylation of individual CpGs or regions of CpG density can interfere directly with transcription factor or complex binding, eliminating transcriptional activity. **D:** Methylation of individual CpGs or regions of CpG density can interfere indirectly with transcription factor or complex binding or movement through a regional blockade of the promoter sequence secondary to binding of numerous methylated DNA binding proteins. **E:** Methylation of individual CpGs or regions of CpG density can interfere indirectly with transcription factor, complex binding, or movement through a barrier blockade secondary to binding of one or a few methylated DNA binding proteins. The red X marks indicate a failure of binding by the complexes depicted.

hypermethylation.³⁰ Therefore, to determine the state of aberrant DNA hypermethylation among breast cancer cell lines, we evaluated the expression of 64 methylation-sensitive genes among 12 breast cancer cell lines.³² This experimental approach uses methylation-sensitive gene expression status as a predictor of promoter methylation state. Unsupervised cluster analysis of gene expression patterns revealed two distinct groups of breast cancer cell lines, one with marked loss of expression of multiple methylation-sensitive genes and the other without loss of expression of methylation-sensitive genes.³² Subsequent methylation analyses confirmed that the groups of breast cancer cell lines identified had either aberrant DNA hypermethylation or low-frequency hypermethylation.³²

The cell lines with aberrant DNA hypermethylation are characterized by high rates of concurrent methylation of six

genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *LCN2*, and *SCNN1A*), whereas the cell lines with low-frequency hypermethylation typically lack methylation of these genes.³² Subsequently, a nine-gene signature was used to classify cell lines for aberrant DNA hypermethylation status (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNAI1*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*) with similar results.³⁴ Methylation analyses (including methylation-specific PCR, bisulfite sequencing, and response to 5-aza treatment) provide evidence that loss of methylation-sensitive gene expression reflects gene silencing secondary to promoter hypermethylation events.³² These observations strongly suggest the existence of a distinct subset of human breast cancer cell lines that possess novel biological properties related to dysregulation of the methylation machinery, resulting in the acquisition of aberrant DNA hypermethylation.

Microarray Gene Expression Data Mining in Breast Cancer Cell Lines

The detection of aberrant DNA hypermethylation among breast cancer cell lines constituted a first step toward determining if this phenomenon occurs in primary breast neoplasms *in vivo*. To test this idea, we conducted a preliminary microarray gene expression data mining study using methylation-sensitive gene expression status (using six methylation-sensitive genes: *CEACAM6*, *CDH1*, *CST6*, *ESR1*, *LCN2*, and *SCNN1A*) as a predictor of promoter methylation state (as was done in breast cancer cell lines), among 88 primary breast cancers (from the UNC Microarray Database).³² These studies were performed using primary breast cancers in patients without any known genetic predisposition or familial cancer gene mutations. Hence, these observations are limited to sporadic breast cancers and may differ from results in hereditary breast cancers.

Unsupervised Cluster Analysis of Breast Cancers

Unsupervised cluster analysis of the methylation-sensitive gene expression data identified a cluster composed of 18 breast cancers that exhibit a gene expression signature characterized by lack or low expression of the six genes analyzed, consistent with concurrent methylation-dependent silencing of these genes. Strikingly, 100% (18/18) of these breast cancers with putative aberrant DNA hypermethylation were found to be of the basal-like molecular subtype, and this cluster contains 75% (18/24) of all basal-like breast cancers in the data set. This observation suggests that the aberrant

DNA hypermethylation observed in breast cancer cell lines also occurs in primary breast cancers and represents a biological property of basal-like breast cancers. Basal-like breast cancers make up approximately 20% to 25% of all breast cancers, but contribute disproportionately to breast cancer–associated death because these cancers tend to display more aggressive clinical characteristics, such as large size, rapid growth, high rates of metastasis, frequent relapse, and lower overall patient survival.^{47–49}

Microarray Gene Expression Data Mining in Breast Cancers

In a subsequent investigation,³⁴ 946 primary breast cancers (from publicly available databases)^{50–56} were classified for aberrant DNA hypermethylation status on the basis of microarray-based gene expression patterns for nine methylation-sensitive genes (*CDH1*, *CEACAM6*, *CST6*, *ESR1*, *GNA11*, *MUC1*, *MYB*, *SCNN1A*, and *TFF3*). Of these 946 breast cancers, 220 (23%) exhibit the aberrant DNA hypermethylation-associated gene expression signature (Figure 2). Breast cancers expressing this gene expression signature [174/220 (79%)] are of the basal-like molecular subtype, and 174 (63%) of 277 basal-like breast cancers express the aberrant DNA hypermethylation-associated gene expression signature (Figure 2). In addition, 13 (6%) of 220 breast cancers expressing this gene expression signature are of claudin-low molecular subtype and 13 (87%) of 15 claudin-low breast cancers express the aberrant DNA hypermethylation-associated gene expression signature (Figure 2). Hence, this analysis identified a strong association between aberrant DNA hypermethylation and triple-

Figure 2 Distribution of breast cancers exhibiting aberrant DNA hypermethylation. **A:** Proportion of all breast cancers that exhibit aberrant DNA hypermethylation. **B:** Breakdown of molecular subtypes among breast cancers that exhibit aberrant DNA hypermethylation. **C:** Proportion of triple-negative breast cancers that exhibit aberrant DNA hypermethylation.

negative breast cancer subtypes (basal-like and claudin-low breast cancers on the basis of intrinsic gene expression patterns).³⁴

Sequence-Based Methylation Analysis of Primary Breast Cancers

Methylation analysis of primary breast cancers using methylation-specific PCR and bisulfite sequencing verified extensive promoter hypermethylation of methylation-sensitive genes among triple-negative breast cancers, compared to other breast cancer subtypes in which promoter hypermethylation events were less frequent.³⁴ These observations combine to strongly suggest that expression of aberrant DNA hypermethylation is a common characteristic of triple-negative breast cancers and may represent a fundamental biological property of most basal-like and claudin-low breast cancers. Subsequently, 779 primary breast cancers from the UNC Microarray Database were classified for aberrant DNA hypermethylation status and subjected to Kaplan-Meier analysis of recurrence-free survival, which revealed a disadvantage for patients with breast cancers that exhibit aberrant DNA hypermethylation.³⁴ The identification of this distinguishing trait may form the basis for development of new rational therapies that target the epigenome in patients with basal-like and claudin-low breast cancers (and other triple-negative breast cancers).

Mechanisms Governing Aberrant DNA Methylation among Breast Cancer Cell Lines

With the identification of breast cancer cell lines that differentially display aberrant DNA hypermethylation, the mechanistic basis for this characteristic was addressable. Hence, we began by investigating DNMT enzymes and their activity among breast cancer cell lines with and without aberrant DNA hypermethylation.³² The results suggest that elevated DNMT activity secondary to overexpression of DNMT3b accounts for aberrant hypermethylation phenotype.³²

DNMT3b protein is significantly elevated in cell lines that exhibit aberrant DNA hypermethylation, and these cells exhibit aberrantly increased DNMT enzymatic activity and correspondingly high rates of methylation-dependent gene silencing compared to both breast cancer cell lines with low-frequency methylation and nonneoplastic breast epithelial cells.³² These results are consistent with studies in which aberrant DNMT3b overexpression is implicated in the methylation abnormalities of breast cancers⁵⁷ and other cancers.⁵⁸ Breast cancers that overexpress DNMT3b are more likely to be *ESR1* negative, to display increased proliferation, and to be associated with poor patient prognosis,⁵⁷ consistent with our findings.^{32,34} Thus, it seems reasonable to expect that aberrant expression of DNMT3b protein and associated changes to the methylome may

produce significant differences in the biology of breast cancers that exhibit aberrant DNA hypermethylation.

DNMT3b is constitutively expressed by all mammalian cell types, but is frequently overexpressed in cancer.^{57,59–61} However, unlike other genes that are overexpressed in cancer, the mechanisms accounting for increased *DNMT3b* levels infrequently involve gene mutations and/or gene amplification.⁶² Likewise, increased *DNMT3b* transcription due to increased transactivation does not commonly occur in cancer.⁶² Rather, it is now recognized that *DNMT3b* is subject to post-transcriptional regulation by miRNAs that regulate gene expression through sequence-specific targeting of mRNAs, producing either translational repression or degradation of the target mRNA^{63,64} (Figure 3A).

Altered miRNA expression is associated with several types of human cancer,^{65,66} including breast cancer.⁶⁷ The dysregulated pattern of expression of miRNAs between normal and cancerous tissues in breast cancer correlates with tumor stage, estrogen and progesterone receptor expression, proliferation index, vascular invasion, epithelial-to-mesenchymal transition, metastasis, and neovascularization. miRNAs are now well recognized as both regulators of DNMT expression and targets of aberrant DNA methylation in various tissue types.

The miR-29 family (miR-29a, miR-29b, and miR-29c) directly targets *DNMT3a* and *DNMT3b* in lung cancer⁶⁸ and acute myeloid leukemia.⁶⁹ Likewise, the miR-148 family (miR-148a and miR-148b) regulates *DNMT3b* in cell lines of multiple origin, including the MCF7 breast cancer cell line.⁷⁰ Loss/reduced levels of these miRNAs have been documented in various forms of cancer, supporting the suggestion that these miRNAs possess tumor suppressor–like function. miR-29a and miR-29b are down-regulated in chronic lymphocytic leukemia, acute myeloid leukemia, lung cancers, cholangiocarcinoma, and prostate cancer.^{68,69,71–75} Diminished expression of miR-26a occurs in hepatocellular carcinoma, oral squamous cell carcinoma, bladder cancer, thyroid anaplastic carcinoma, Burkitt lymphoma, acute myeloid leukemia, papillary carcinoma, prostate cancer, and breast cancer.^{76–78} miR-26b expression is decreased in Hodgkin lymphoma, oral squamous cell carcinoma, and prostate cancers.⁷⁸ miR-29c expression is depressed in nasopharyngeal carcinomas, bladder cancers, chronic lymphocytic leukemia, acute myeloid leukemia, lung cancers, cholangiocarcinoma, esophageal squamous cell carcinoma, and pancreatic ductal adenocarcinoma.^{72,76–79} miR-148a is down-regulated in breast cancers, papillary thyroid carcinoma, pancreatic ductal adenocarcinoma, prostate cancer, and colorectal adenocarcinoma.^{77,78} miR-148b is expressed at diminished levels in oral squamous cell carcinoma, papillary thyroid carcinoma, prostate cancer, colorectal adenocarcinoma, and pancreatic ductal adenocarcinoma.⁷⁸

We analyzed breast cancer cell lines for differential expression of regulatory miRNAs to determine whether loss of miRNA-mediated post-transcriptional regulation of *DNMT3b*

Figure 3 Mechanisms governing post-transcriptional regulation of DNA methyltransferase 3b (DNMT3b). **A:** The *DNMT3b* gene is transcribed and then regulated through post-transcriptional mechanisms involving various miRNAs, including miR29a-c, miR-148ab, and miR-26ab. Binding of DNMT3b mRNA by these miRNAs results in RNA degradation and/or inhibition of translation, resulting in normal steady-state levels of DNMT3b protein (and associated enzymatic activity). **B:** In many triple-negative breast cancers, there is a loss of expression of regulatory miRNAs and elimination of post-transcriptional regulation of DNMT3b mRNA, leading to elevated DNMT3b protein (and enzymatic hyperactivity). **C:** Restoration of normal levels of regulatory miRNAs (through transfection of pre-miRNAs) in breast cancer cell lines results in normalized DNMT3b mRNA levels.

represents the molecular mechanism that governs the overexpression of DNMT3b, which drives aberrant DNA hypermethylation in breast cancer.⁸⁰ Our results show that multiple miRNAs (miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203) function together to post-transcriptionally regulate *DNMT3b*, and loss of expression of these regulatory miRNAs contributes to DNMT3b overexpression in breast cancer cell lines that exhibit aberrant DNA hypermethylation⁸⁰ (Figure 3B). We also observed that enforced expression of specific regulatory miRNAs (miR-26b, miR-29c, or miR-148b) results in reduced *DNMT3b* mRNA levels in breast cancer cell lines that exhibit aberrant DNA hypermethylation (Figure 3C), and that down-regulation of regulatory miRNAs results in increased *DNMT3b* mRNA levels in breast cancer cell lines that lack aberrant DNA hypermethylation.⁸⁰ These observations combine to suggest that the loss of multiple regulatory miRNAs that post-transcriptionally regulate DNMT3b levels is involved in the molecular mechanism governing the DNMT3b-mediated aberrant DNA hypermethylation in breast cancer cell lines.

Mechanisms Governing Aberrant DNA Methylation among Primary Breast Cancers

Our investigations show that aberrant DNA hypermethylation occurs secondary to DNMT hyperactivity and overexpression of DNMT3b, results in concurrent methylation-dependent gene silencing events, and is significantly associated with basal-like breast cancer.^{32,34} Our subsequent studies in breast cancer cell lines generated evidence that loss of post-transcriptional regulation of *DNMT3b* by miRNAs leads to DNMT3b overexpression.⁸⁰

To determine whether this mechanism accounts for DNMT3b overexpression and aberrant DNA hypermethylation in primary basal-like breast cancers, we examined levels of expression of miRNAs that are known or predicted to regulate *DNMT3b* (miR-26a, miR-26b, miR-29a, miR-29b, miR-29c, miR-148a, miR-148b, miR-203, and miR-222) among primary breast cancers.³³ We used a cohort of 70 primary human breast cancers of known clinical classification representing each of the intrinsic molecular subtypes (36 luminal A-like, 13 luminal B-like, 5 Her2-enriched, and 16 basal-like subtypes) to analyze expression of miRNAs that contribute to regulation of *DNMT3b*. The results show the following: i) significantly reduced expression of miR-29c distinguishes basal-like breast cancers from the other breast cancer clinical subtypes, ii) two distinct subsets of basal-like breast cancers can be distinguished from miRNA expression patterns (corresponding to those with diminished expression and those expressing normal levels of regulatory miRNAs), iii) loss of combinations of miR-29a, miR-29b, miR-148a, miR-148b, miR-26a, and miR-26b is associated with expression of aberrant DNA hypermethylation among primary invasive breast cancers, and iv) basal-like breast cancers that exhibit aberrant DNA hypermethylation express diminished levels of miRNAs that post-transcriptionally regulate *DNMT3b*.³³

Several studies have established that miRNAs exhibit altered expression in cancer tissues compared to normal tissues, suggesting that miRNAs have a role in defining the molecular and pathological profiles of cancers, including breast cancer.^{67,81–85} In addition, miRNAs have been established as key players in carcinogenesis, with oncogenic or tumor-suppressor-like functions.⁶² Our results suggest loss of combinations of miR-29a, miR-29b, miR-148a, miR-148b, miR-26a, and miR-26b is associated with aberrant DNA hypermethylation in primary invasive breast cancers,

consistent with the idea that these miRNAs function as negative mediators of DNMT3b-mediated aberrant DNA hypermethylation.

Epigenetic Therapy in Breast Cancer Treatment

Aberrant DNA methylation and epigenetic silencing of gene expression are now well recognized as frequent and reversible (unlike genetic mutation) hallmarks of cancer,^{14,15,86,87} leading numerous investigators to suggest that cancer should be treated with epigenetic therapy.^{87–93} In contrast to gene therapy, epigenetic therapy (demethylating treatment) alters gene expression patterns in breast cancer without complications from enhanced immune response to therapeutic DNA. The reversibility of epigenetic alterations makes them excellent targets for improving breast cancer outcomes. In addition, by targeting cancer-specific epigenetic patterns, one can concurrently target multiple genes to achieve changes in gene expression, including reexpression of silenced genes (like tumor-suppressor genes) that alter the clinical behavior of the cancer or response of the cancer cells to chemotherapy.

In the case of triple-negative breast cancers (basal-like and claudin-low breast cancers), we have shown that most of these cancers overexpress DNMT3b, leading to aberrant DNA hypermethylation and concurrent loss of numerous methylation-sensitive genes. It is likely that the methylation-dependent silencing of critical genes contributes significantly to the lack of sensitivity to standard chemotherapeutic agents by triple-negative breast cancers. The goal of epigenetic therapy in triple-negative breast cancers is to render cancer cells sensitive to chemotherapy through reexpression of methylation-sensitive genes secondary to normalization of DNA methylation patterns in response to inhibition of DNMT3b (Figure 4A). The observation that primary breast cancers can be classified for aberrant DNA hypermethylation on the basis of methylation-sensitive gene expression signatures presents the opportunity to exploit gene silencing events as biomarkers to identify which triple-negative breast cancer patients are most likely to benefit from epigenetic therapy.

Various forms of epigenetic therapy have been investigated. We explored pharmacological epigenetic therapy and targeted epigenetic therapy (Figure 4, B and C) in combination with cytotoxic chemotherapy in a cell line model that used breast cancer cell lines that exhibit aberrant DNA hypermethylation related to overexpression of DNMT3b.³⁵ The goal of these studies was to evaluate the effectiveness of targeting the DNA methylation machinery to modify the sensitivity of breast cancer cells to cytotoxic drugs. Epigenetic treatment was accomplished through pharmacological inhibition of DNA methyltransferase activity using 5-aza and targeted inhibition of overexpressed DNMT3b using RNA interference (RNAi)—mediated *DNMT3b* knockdown. The results show that 5-aza pretreatment sensitizes breast

Figure 4 Approaches to epigenetic therapy in triple-negative breast cancer. **A:** Triple-negative breast cancers tend to overexpress DNA methyltransferase 3b (DNMT3b), resulting in aberrant DNA hypermethylation and loss of expression of methylation-sensitive genes. The goal of epigenetic therapy is to inhibit DNMT3b and reverse aberrant DNA hypermethylation. Evidence suggests that normalization of the breast cancer methylome might sensitize breast cancer cells to standard cytotoxic chemotherapy. **B:** Pharmacological epigenetic therapy involves the use of drugs that inhibit DNMT3b [like 5-aza-2'-deoxycytidine (5-aza)]. **C:** Targeted epigenetic therapy involves the use of small-molecule inhibitors to directly and specifically inhibit DNMT3b. **D:** Biological epigenetic therapy is based on the principle of restoration of normal post-transcriptional regulation of *DNMT3b* mRNA using biological agents like regulatory miRNAs.

cancer cells with aberrant DNA hypermethylation to cell killing by cytotoxic drugs, and that the improved chemotherapeutic efficacy is a function of dose and duration of exposure to 5-aza.³⁵ Treatment of breast cancer cells for 7 days with low-dose 5-aza resulted in substantial decreases in the inhibitory concentration of 50% for doxorubicin (60%), paclitaxel (37%), and 5-fluorouracil (93%).³⁵

Although we used 5-aza for these investigations, several other epigenetic drugs have been studied in a similar manner.^{88,92,94,95} In addition to DNMT inhibitors, histone deacetylase inhibitors may also prove to be useful in the epigenetic sensitization of breast cancer cells to chemotherapy, used alone or in conjunction with drugs like 5-aza. We also observed an increase in the effectiveness of chemotherapeutic drugs after targeted inhibition of DNMT3b using RNAi.³⁵ Although RNAi may not be a viable approach to the clinical management of breast cancer, RNAi *in vitro* models the use of targeted agents, like small-molecule inhibitors. Several small-molecule inhibitors of DNMT3b have been described.⁹⁶ Our results strongly suggest that

DNMT3b is an excellent target for development of rational therapeutic approaches for breast cancers that exhibit aberrant DNA hypermethylation (such as triple-negative breast cancers). Our studies in cell lines provide proof of concept that targeting DNMT3b in breast cancer cells with aberrant DNA hypermethylation sensitizes them to cell killing by cytotoxic drugs and that this strategy can be exploited to improve outcomes that involve standard therapeutic approaches. Increasing the efficacy of chemotherapy through epigenetic therapy might benefit patients in at least two different ways. First, increasing the efficacy of a certain fixed dose of drug may increase the benefits of chemotherapy without associated increases in toxic adverse effects. Second, a lower dose of chemotherapeutic drug may be used to achieve a certain fixed therapeutic effect, but with diminished adverse effects.

Another approach to epigenetic therapy has emerged from our recent elucidation of the molecular basis of aberrant DNA hypermethylation in triple-negative breast cancers.^{33,80} We have termed this approach biological epigenetic therapy. Biological epigenetic therapy is based on the concept that normal regulation of DNMT3b can be restored by replacement of lost regulatory miRNAs (Figure 4D). Our experimental studies in cell lines showed that restoration of post-transcriptional regulation of DNMT3b mRNA can be accomplished using specific pre-miRNAs as single agents.^{34,80} With the recent development of nanoparticles for delivery of drugs and other active agents specifically to cancer cells, it becomes possible that pre-miRNAs might have practical utility as therapeutic agents. We would suggest that pre-miRNAs might be used in combination to target *DNMT3b* regulation in triple-negative breast cancers to normalize the methylome of these cancers and sensitize them to standard chemotherapies.

Perspectives and Conclusions

The experimental studies reviewed herein highlight the importance of developing a better understanding of the role of aberrant DNA hypermethylation in the genesis and progression of breast cancer, as well as elucidation of the molecular mechanisms that drive aberrant DNA hypermethylation at specific gene targets in this disease. With a greater understanding of the mechanisms and consequences of aberrant DNA hypermethylation in breast cancer, new targets for therapy and novel epigenetic treatment strategies will emerge. These targets for therapy may include DNMT3b (and/or other DNMT enzymes) or other components of the DNA methylation machinery. Novel treatment strategies might include restoration of natural regulation of *DNMT3b* expression through delivery of regulatory miRNAs to breast cancer cells. The conclusion of these reviewed studies (and numerous others in the literature) is that epigenetic mechanisms contribute in a significant manner to breast cancer development, biological characteristics, and clinical behavior, and may

be particularly important for some molecular subtypes of disease. Given the mechanistic involvement of epigenetics in breast cancer development and progression, there is an opportunity to pursue new translational insights into this disease to target epigenetic mechanisms in breast cancer intervention and management.

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