HYPERMETHYLATOR PHENOTYPE IN HUMAN BREAST CANCER: THERAPEUTIC TARGET AND MECHANISM OF DNMT3B REGULATION

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ABSTRACT

RUPNINDER SANDHU: Hypermethylator Phenotype in Human Breast Cancer: Therapeutic Target and Mechanism of DNMT3b Regulation
(Under the direction of William B Coleman, Ph.D.)

A subset of primary breast cancers and breast cancer cell lines express a hypermethylation defect characterized by DNMT hyperactivity and DNMT3b overexpression. The objectives of this project were (i) to determine if targeting the methylome enhances the sensitivity of breast cancer cells to chemotherapy, and (ii) to elucidate the molecular mechanism governing the DNMT3b-mediated hypermethylation defect in breast cancer. To address the first objective, hypermethylator breast cancer cell lines were treated with demethylating agent (5-aza-2'-deoxycytidine) and/or were subjected to RNAi-mediated DNMT3b knockdown (KD), and then tested for sensitivity to doxorubicin hydrochloride, paclitaxel, and 5-fluorouracil. The results show that pharmacologic demethylating pretreatment sensitizes hypermethylator breast cancer cells to cell killing by cytotoxic drugs, and provide proof-of-concept that direct targeting of DNMT3b also improves cell kill by these drugs. These findings suggest that targeting the methylome improves chemotherapeutic efficacy of cytotoxic drugs against hypermethylator breast cancer cells as a function of dose and duration of exposure to demethylating treatment. To address the second objective, the expression of microRNAs (miRs) that regulate or are predicted to regulate *DNMT3b* were examined in hypermethylator or non-hypermethylator breast cancer cell lines and in primary breast cancers. Hypermethylator cell lines express

diminished levels of regulatory miRs compared to non-hypermethylator cell lines. Mechanistic studies were conducted to establish the role of miR expression in the hypermethylation defect. Antagomir-mediated knockdown of regulatory miRs in non-hypermethylator cell lines resulted in increased *DNMT3b* mRNA and forced re-expression of regulatory miRs reduced *DNMT3b* mRNA in hypermethylator cell lines. In primary breast cancers, miR expression patterns revealed two distinct subsets among the basal-like subtype. Most hypermethylator basal-like cancers exhibit diminished expression of regulatory miRs. These findings strongly suggest that diminished expression of multiple regulatory miRs contributes to *DNMT3b* overexpression. Together, these results support the conclusion that the molecular mechanism governing the DNMT3b-mediated hypermethylation defect in breast cancer cells is the loss of post-transcriptional regulation of *DNMT3b* by regulatory miRs, and that combined epigenetic and cytotoxic treatment will improve the efficacy of breast cancer chemotherapy.

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LIST OF ABBREVIATIONS

$\Delta\Delta C_T$	Comparative method for real-time PCR analysis
5-aza	5-aza-2'-deoxycytidine
5-FU	5-Flurouracil
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
bp	Base pairs
BM1	Body mass index
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BRIP1	BRCA1 interacting protein C-terminal helicase 1
CDH1	E-cadherin
cDNA	Complementary DNA
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CHEK2	CHK2 checkpoint homolog
CIMP	CpG island methylator phenotype
CK5/6	Cytokeratin 5/6
CK18	Cytokeratin 18
CST6	Cystatin E/M
DCIS	Ductal carcinoma *in situ*
DFS	Disease-free survival
DGCR8	DiGeorge syndrome critical region gene 8
DNMT	DNA methyltransferase

DNMT1	DNA methyltransferase 1
DNMT2	DNA methyltransferase 2
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
DOX	Doxorubicin
EGCG	Epigallocatechin-3-gallate
ESR1	Estrogen receptor 1
ER	Estrogen receptor
EGFR	Epidermal growth factor receptor
FFPE	Formalin-Fixed, paraffin-embedded
FGFR2	Fibroblast growth factor receptor 2
GFP	Green fluorescent protein
GNA11	Guanine nucleotide binding protein, alpha 11
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HMGA2	High mobility group AT-hook 2
hnRNP-E2	Heterogeneous nuclear ribonucleoprotein E2
IC_{50}	Concentration of a drug required for 50% inhibition *in vitro*
KD	Knockdown
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein
LKB1	Serine/threonine kinase 11
LSP1	Lymphocyte-specific protein 1
MAP3K1	Mitogen-activated protein kinase kinase kinase 1

miR	MicroRNA
μg	Microgram
μM	Micormolar
ml	Milliliter
mM	Millimolar
MRE11	Meiotic recombination 11
mRNA	Messenger RNA
MSP	Methylation-sensitive PCR
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MUC1	Mucin 1
MYB	v-myb myeloblastosis viral oncogene homolog
ng	Nanogram
nM	Nanomolar
NPV	Negative predictive value
NSAID	Nonsteroidal anti-inflammatory drug
OS	Overall survival
PALB2	Partner and localizer of BRCA2
PAX	Paclitaxel
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGR	Progesterone receptor
PPV	Positive predictive value
PR	Progesterone receptor

PRDM2	PR domain containing 2, with ZNF domain
PTEN	Phosphatase and tensin homolog
PTK9	Protein tyrosine kinase 9
PVDF	Polyvinylidene difluoride
RAD51L1	RAD51-like 1
RARα	Retinoic acid receptor alpha
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
RB	Retinoblastoma-associated protein
RISC	RNA-induced Silencing Complex
RNAi	RNA-interference
RT-PCR	Reverse transcription polymerase chain reaction
SCNN1A	Sodium channel nonvoltage-gated 1 alpha
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis,
SEER	Surveillance, Epidemiology, and End Results
S.E.M.	Standard error of the mean
STK11	Serine/threonine kinase 11
TBST	Tris-Buffered Saline Tween-20
TFAP2A	Transcription factor AP-2 alpha
TFF3	Trefoil factor 3
TOX3	TOX high mobility group box family member 3
TP53	Tumor protein 53
TRBP	Transactivating response RNA-binding protein
UTR	Untranslated region

INTRODUCTION

Breast Cancer Statistics

Breast cancer is a disease that is diverse in natural history, response to treatment, and patient outcomes. It remains the most common non-cutaneous female malignancy with an estimated 209,060 new cases in 2010 in the United States [1]. Breast cancer-associated mortality is second only to lung cancer in the United States among women, with an estimated 40,230 deaths in 2010 [1]. Breast cancer is also the most commonly diagnosed female malignancy worldwide. According to the *American Cancer Society* (www.cancer.org), approximately 1.2 million women worldwide are diagnosed with breast cancer each year. The lifetime probability of developing breast cancer in developed countries is 1 in 8 women [1]. The incidence of breast cancer as well as the associated mortality rate increases with age **(Figure 1.1)**. Women above age 40 accounted for 95% of new cases and 97% of deaths associated with breast cancer from 2002-2006 (www.cancer.org). The highest incidence rate of 441.9 cases per 100,000 women is observed in women between 75-79 years of age and the lowest incidence of 1.4 cases per 100,000 women is observed in women from 20-24 years-old (www.cancer.org). Reduction in incidence rates observed among women over 80 years of age may be an indication of lower rates of screening, cancers detected before 80 years (by mammography), and incomplete detection (www.cancer.org). From 2003-2007, the age-adjusted death rate associated with breast cancer was 24.0 per 100,000 women per year and the median age at death due to breast cancer was 68 years of age (www.cancer.org). 1% of

these deaths occurred in women below the age of 34 years. There was an increase in deaths with age from age 35 onwards; women between 35 and 44 years of age accounted for 6% of the breast cancer deaths, women between 45 and 54 years of age accounted for 15% of the deaths and the 20.8% of breast cancer associated deaths were seen in women with ages between 55 and 64. Likewise, women with ages between 65 and 74 accounted for 19.7% of the deaths, women with ages between 75 and 84 accounted for 22.6%; and women above 85 years of age accounted for 15.1% of the breast cancer associated deaths (www.cancer.org).

Race/ethnicity plays a major role in the development of breast cancer. In the United States, the incidence rate of breast cancer is highest among Caucasian women above the age of 45 years. Below age 45, African-American women have the highest incidence of breast cancer. **Figure 1.2** Incidence of breast cancer among other racial/ethnic groups is lower than the incidence among Caucasian and African-American women. The overall age-adjusted incidence rate based on 2004-2008 SEER data was 124 per 100,000 women per year **(Table 1.1)**. African-American women also have the highest mortality rate associated with breast cancer at every age in comparison with Caucasian women and women of other races/ethnicities **(Table 1.2)**.

Since the late 1980s, there has been significant reduction in deaths related to breast cancer. This decline occurred partly due to the advancements in the therapy and partly due to improved screening and early diagnosis. Widely implemented screening programs (including self-examination and screening mammography) have not only affected the survival rates but have also shifted the cancer profile characteristics detected today increasingly towards smaller tumors. These gains have resulted in a current overall 5-year survival rate of 89% (www.cancer.org). The five-year relative survival by race is 90% for Caucasian women and

77% for African-American women (www.cancer.org). As with other cancers, detection at early stage is associated with better prognosis in breast cancer **(Table 1.3)**. Paradoxically, improved screening technology (such as digital mammography) and its increasingly widespread use may partially account for the rise in breast cancer rates over the last 25 years, although the precise cause for the increasing incidence remains unknown. In the United States, breast cancer incidence rates increased sharply in the 1980s and continued to rise, although less rapidly, in the 1990s. Since 1999, incidence rates have declined by approximately 2% per year. In addition to the contribution of screening mammography to effective resulting in increased detection of breast cancers too small to be detected by palpation, the increase in incidence is also attributed to changes in reproductive patterns, like having fewer children and delayed age of childbearing, which are well known risk factors of breast cancer. Increase in breast cancer incidence in the late 1990s could also be related to increases in obesity and post-menopausal hormonal replacement therapy. The recent decline in breast cancer has been attributed to decreased utilization of hormone replacement therapy, as well as decrease in mammography prevalence [2-6].

Risk Factors for Breast Cancer

A number of etiological factors contribute to the risk of breast cancer. Established risk factors for breast cancer include (i) reproductive/hormonal factors like early menarche, late menopause, nulliparity, and late first full-term pregnancy, (ii) lifestyle factors like obesity and alcohol consumption, (iii) genetic factors like family history, mutations in *BRCA1/2* genes, previous history of breast cancer, and (iv) high mammographic breast density [7-14]. Factors that may contribute to breast cancer risk include (a)

reproductive/hormonal factors like hormonal replacement therapy, and recent use of oral contraceptives, (b) lifestyle factors like smoking, diet, physical activity, breast feeding, and NSAID use, and (c) other factors like exposure to ionizing radiation. [14]. Although age and sex are considered the chief risk factors for breast cancer development, it is important to note that it is the combination of numerous factors that drives the initiation, development, and progression of breast cancers. Most of these risk factors increase the likelihood that a woman will develop breast cancer. However, having one or more of these risk factors does not confer 100% chance that breast cancer will ever develop.

Reproductive Factors

Based on epidemiologic studies, a number of factors determining life-time exposure to estrogen have been established as risk factors for breast cancer. These factors are associated with reproductive history and include ages at menarche, parity, first birth, and menopause, as well as factors like infertility and nulliparity [7, 15]. The first recognition of the importance of life-time estrogen exposure included the observation of relatively higher incidence rates of breast cancer among nuns compared to the women who bore children [16]. The fact that more than two-thirds of breast cancers are stimulated by estrogen at some point during the course of cancer progression makes the association between estrogen exposure and breast cancer risk very important [7].

Obesity

Obesity is an independent risk factor for breast cancer development in post-menopausal women [7, 14, 17, 18]. A pooled analysis showed an inverse relationship

between the baseline weight and body mass index (BMI) and breast cancer in pre-menopausal women and a positive relationship in post-menopausal women [17]. The effects of weight gain during particular periods of lifetime on breast cancer risk have been investigated in numerous studies. Different studies have shown that weight gain during reproductive years increases the risk of postmenopausal breast cancer [19-21]. The effects of weight gain/excess weight during childhood on breast cancer risk are not very clear. A study by Berkey *et al* showed that higher childhood BMI was associated with reduced risk of pre-menopausal breast cancer, but increasing BMI between 10 and 20 years of age did not reduce the risk of pre-menopausal or post-menopausal breast cancer [7, 22]. Other studies have shown a protective effect of excess weight in early years on risk of breast cancer [7, 23]. This protective effect was more pronounced and consistent in women who remained overweight in adult life ruling out the protective role of subsequent weight loss. The proposed mechanisms for this linkage include association of a longer period of anovulatory cycles after menarche in women with lasting obesity and the increased intake of substances that are 'protective' against the early events of breast carcinogenesis. Some studies found an independent association between the risk of breast cancer and presence of central adiposity, independent of BMI [24, 25]. However, other studies found no such association [26]. It has been suggested that adult obesity increases the risk of breast cancer by increasing the circulating levels of estrogen. Increased aromatase activity in adipose tissue and decrease in concentration of sex hormone binding globulin leads to an increase in the bioavailable estrogen, leading to increased breast cancer risk [7]. Obesity affects not only the risk factors for breast cancer, but also the disease prognosis through numerous pathways, including associated adverse disease features and morbidities that can hinder the treatment [17].

Breast Density

Dense breast tissue seen on mammography and characterized by increase in stromal fibrosis and epithelial proliferation increases the risk for breast cancer [7]. After adjusting for known risk factors for breast cancer, case-control and cohort studies have shown an increased risk associated with high density mammographic parenchymal patterns compared with low density patterns [27-29]. The biological basis underlying this association is unclear. However, the attributable risk for breast cancer was estimated to be 30% for women with 50% or greater breast density [27].

Diet and Physical Activity

In developed countries, factors in addition to reproductive factors and breast density may contribute to the high incidence of breast cancer. These include a high fat diet [30], low levels of physical activity, and obesity [31, 32]. Other factors like smoking [33] and alcohol consumption [34] may also play a role in determining the risk for breast cancer. High intake of fruits and vegetables has been linked to low incidence of cancer overall, but the association for decreasing the risk of breast cancer remains inconclusive [35]. Contradictory reports exist regarding the relationship between cigarette smoking and breast cancer [33, 36]. Studies that followed the immigration of Asian women (typically with low incidence of breast cancer) to Western countries provided evidence for the role of a Western diet in risk for breast cancer development. The first generation women of Asian-descent born in Western countries have breast cancer rates similar to those of Caucasians [37]. The Asian-American women born in the West have a 60% increase in breast cancer risk compared to Asian-

Americans born in the East [37]. In another study, it was observed that Chinese women who ate a more traditional Eastern diet (high in vegetables) had half the risk of developing breast cancer compared to Chinese women who ate a more Westernized diet (high in meat, white bread, milk, etc.) [38].

Genetic Factors

It is now well established that genetic factors play a strong role in the development of breast cancer. An affected first-degree relative (such as a mother, sister, or daughter) confers a two-fold to four-fold increased risk of developing breast cancer [7]. Genetic variations linked with increased breast cancer risk are classified as high-penetrance mutations, moderate penetrance variants, and low-penetrance polymorphisms [39]. High-penetrance mutations are associated with very high risk (relative risk with presence of these mutations – 5 to >20 fold) but are rare in the population, account for a relatively small percentage (about 20-25%) of the familial risk [39, 40]. The studies in 1990s discovering the association of *BRCA1* and *BRCA2* with breast cancer significantly advanced the field of genetic susceptibility to breast cancer development. These linkage studies led to the seminal discovery that mutations in tumor suppressor genes, *BRCA1* and *BRCA2*, confer a high risk of breast cancer. Breast cancer was linked to *BRCA1* in 52% and to *BRCA2* in 32% of the families with multiple cases of breast cancer. Likewise, in families with breast and ovarian cancers, linkage was established to *BRCA1* in 84% and to *BRCA2* in 14% of families [41, 42]. In addition to *BRCA1* and/or *BRCA2,* other high-penetrance mutations have been identified, mostly as part of heritable cancer syndromes, including *PTEN* mutations in Cowden syndrome [43], *TP53* mutations

found in Li-Fraumeni cancer syndrome [44, 45], and *STK11/LKB1* mutations in Peutz-Jegher syndrome [46].

The moderate penetrance variants include the genes associated with moderate risk, and because of the relative low frequency of this class of genetic variants, their familial risk is estimated to be less than 3% [39]. These variants mainly affect the genes that are involved in DNA repair mechanisms including *CHEK2* [47], *PALB2* [48], *BRIP1* [49], *ATM* [50], and *MRE11* [51]. The low-penetrance polymorphisms are common and are associated with small increases in risk (relative risk <1.5 fold). It is believed that most otherwise unexplained familial risk maybe due to a polygenic mechanism involving multiple low-penetrance polymorphisms [39, 52]. Candidate gene studies and genome wide association studies have identified numerous breast cancer susceptibility loci. In some cases, these loci contain or are proximal to known genes like *FGFR2, TOX3, MAP3K1, LSP1* [53] and *RAD51L1* [54], in other cases these loci map to regions lacking gene density like 8q24 [53] and 2q35 [55, 56]. Using a statistical model, the low-penetrance polymorphisms detected so far are estimated to account for ~10% of familial risk, suggesting that many other variants remains to be detected [39].

Breast Cancer: A Heterogeneous Entity (Molecular Subtypes)

Breast cancer is not a single disease. Rather, breast cancer represents a diverse spectrum of diseases that includes several distinct biological entities and subtypes. These subtypes are associated with specific morphological characteristics and different clinical outcomes [57-65]. The molecular signatures of these breast cancer subtypes reflect not only the distinct biological features of these malignant neoplasms, but also predict their clinical

behavior and responses to chemotherapy [66-69], with certain subtypes having better outcomes than others. To some extent, the observed variation in disease outcome among breast cancer patients reflects the successful identification of therapeutic targets for some subtypes and the development of effective targeted therapies. The diverse spectrum of breast cancer includes a number of morphologic subtypes. Invasive ductal carcinoma is the most common morphological subtype, representing 80% of the invasive breast cancers. Invasive lobular carcinoma is the next most common subtype, representing approximately 10% of invasive breast cancers. The less common subtypes of the invasive breast cancers include mucinous, cribriform, micropapillary, papillary, tubular, medullary, metaplastic, and inflammatory carcinomas. Representative examples of invasive ductal carcinomas are shown in **Figure 1.3**.

Routine subclassification of invasive ductal carcinomas is accomplished by immunostaining tumor tissues for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptors (HER1 and HER2), and various cytokeratins. The differential expression of ER, PR, and HER2 in different subtypes of breast cancer based upon immunohistochemical staining is shown in **Figure 1.3**. The differential expression of these protein biomarkers is used as an immunohistochemical surrogate for gene expression analysis to determine molecular subtype. Approximately 70-75% of invasive breast cancers express the estrogen receptor (ER+). Collectively, the ER+ malignant neoplasms are classified as luminal cancers. These cancers are further subclassified into luminal A and luminal B subtypes based on their HER2 status and proliferation rate. The majority of ER+ tumors also express PR. The presence of normal PR levels suggests an intact ER signal transduction pathway in the breast cancer cells, and PR expression typically follows the ER

expression pattern. The ER- breast cancers are subclassified as HER2+ or basal-like based on the HER2 overexpression/gene amplification, basal cytokeratin expression, and EGFR (HER1) expression. An immunohistochemical staining proxy based on 5 biomarkers classifies breast cancers into the major subtypes (shown schematically in **Figure 1.4)**: (i) Estrogen receptor positive cancers (ER+) are subclassified into luminal A (ER+, PR+, HER2-) and luminal B (ER+, PR+, HER2+), (ii) Estrogen receptor-negative cancers (ER-) are subclassified into triple-negative breast cancer (ER-, PR-, HER2-), and human epidermal growth factor receptor 2-positive (ER-, PR-, HER2+), and (iii) unclassified cancers (negative for all 5 markers) [58-60, 70]. Basal-like breast cancers are distinguished from other triple-negative breast cancers (ER-, PR-, HER2-) by expression of cytokeratin 5/6 and/or EGFR. There is no international consensus on the criteria used to define cancers as basal-like in formalin-fixed, paraffin-embedded tissues. Therefore, the term basal-like is not yet routinely used in clinical practice. Rather, the basal-like breast cancers are contained in the triple-negative classification.

Breast cancers, like most epithelial cancers, are associated with better treatment and survival outcomes when diagnosed at an early stage. However, outcomes of early stage breast cancers differ depending upon the molecular subtype **(Figure 1.5)**. In general, with stage matched breast cancers, the ER+ breast cancer subtypes (luminal A and luminal B) exhibit a good prognosis and excellent long-term survival (approximately 80-85% 5-year survival), while the ER- subtypes (HER2-positive and basal-like) are difficult to treat and associated with poor prognosis (approximately 50-60% 5-year survival). The ability of patients with ER+ breast cancers to survive their disease reflects the availability of effective targeted therapy in the form of anti-estrogen treatment (e.g., tamoxifen). However, among

the ER+ breast cancers, the luminal B neoplasms are associated with a significantly worse prognosis than luminal A subtype [60] **(Figure 1.5)**. This difference in outcome is partly due to variations in response of ER+ subtypes (luminal A and luminal B) to anti-estrogenic treatment [71]. Targeted therapy of HER2 overexpressing breast cancers, [luminal B or HER2-positive (ER-) neoplasms] with trastuzumab (Herceptin), either concurrent or sequential with adjuvant chemotherapy, has improved survival for these breast cancer subtypes [72].

Basal-like breast cancers are characterized by autonomy of growth that is independent of expression of hormone receptors. Since these cancers lack the appropriate targets for the drugs like tamoxifen (targeting ER) and trastuzumab (targeting HER2), patients with these cancers do not derive benefit from these drugs. Basal-like breast cancers are associated with overall poor prognosis and shorter long-term survival. The poor clinical outcomes associated with basal-like breast cancer reflect the fact that these cancers are refractory to chemotherapy or recur following therapy. Lack of identification of 'druggable' targets in basal-like breast cancers and poor prognosis makes the identification of molecular signatures and therapeutic targets in these cancers to be of utmost significance. No widely available targeted therapies for this breast cancer subtype have been developed to date, although phase II studies of PARP inhibitors have shown promising results [73].

DNA Methylation Machinery Abnormalities in Breast Cancer

With the emergence of evidence demonstrating that genome modifications that do not alter DNA sequence make a substantial contribution to the regulation of gene expression, epigenetics has emerged as important mechanism contributing to the process of

carcinogenesis [74-76]. In contrast to genetic changes like deletions, translocations, and amplifications, epigenetic changes related to DNA methylation, genomic imprinting, and histone modifications are frequent, as well as reversible [77]. DNA methylation of CpG dinucleotides represents an epigenetic event of major importance in cancer induction and progression. A number of genes contain CpG-rich regions, known as CpG islands (defined as ≥200 bp with ≥50% G+C content and ≥0.6 CpGs observed/CpGs expected) [78], in their promoter sequences proximal to the transcriptional start sites. CpG islands in regulatory regions of expressed genes are typically unmethylated, while transcriptionally silent genes are often associated with methylated CpG islands [76]. Alterations in methylation, both global hypomethylation and gene-specific hypermethylation are associated with neoplastic transformation [79-82]. Genome-wide demethylation may be associated with aberrant expression of some genes that could contribute to neoplastic transformation, tumorigenesis, or cancer progression [83, 84]. Also, demethylation can contribute to chromosomal instability by destabilizing pericentromeric regions of certain chromosomes [84-86]. Methylation-dependent gene silencing is a normal mechanism for regulation of gene expression [87]. However, in cancer cells methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism of inactivation of tumor suppressor genes [88], genes associated with DNA repair or apoptosis. Recently, it was shown that genes lacking typical CpG islands are also susceptible to methylation-dependent silencing, indicating that methylation-dependent gene silencing is not limited to methylation events at cytosines within CpG islands [89]. This relationship between DNA methylation and gene silencing suggests that changes in normal methylation patterns can result in altered gene expression. A significant number of genes that are involved in the hallmarks of cancer [90]

are subject to methylation-dependent silencing [91]. Epigenetic gene inactivation being at least as frequent, if not more so, than mutation in carcinogenesis [92-94], represents a fundamental aspect of cancer and plays a key role in neoplastic transformation and progression.

DNA methylation results from covalent addition of a methyl group from an S-adenosyl-methionine donor to 5-position of cytosine in a CpG dinucleotide. This transfer is catalyzed by DNA methyltransferases with somewhat distinctive roles. To date, four human DNA methyltransferases have been identified: DNMT1, DNMT2, DNMT3a, and DNMT3b. However, DNMT2 was identified by sequence similarity alone and does not possess any recognized methyltransferase activity [95]. Functionally, two types of DNA methyltransferases are known to occur *in vivo*: (i) *de novo* methyltransferases that initially establish the methylation pattern of a given DNA segment, (ii) maintenance methyltransferases that ensure that the methylation patterns are faithfully copied to daughter strands. Typically, *de novo* methylation function is carried out by DNMT3a and DNMT3b, which are highly expressed during embryogenesis, but at lower levels in normal adult tissues [96]. Maintenance methylation is usually carried out by DNMT1 which is constitutively expressed in proliferating cells and has a significant preference for hemi-methylated substrates [97]. However, recent findings suggest that the roles of individual DNMTs are not clearly delineated and there is evidence of interplay and partial redundancy among these enzymes [97, 98]. For example, DNMT1 has been found to express *de novo* activity on unmethylated substrates which surpasses that of DNMT3a and DNMT3b [97]. Some studies suggest that DNMT3a and DNMT3b or DNMT1 and DNMT3b are jointly necessary for maintenance methylation of specific sequences [99-101].

Numerous DNMT abnormalities have been associated with process of neoplastic transformation, carcinogenesis, and tumor progression. Owing to the tissue-specific nature of findings related to DNMTs, many questions regarding the roles of individual DNMTs remain unanswered. Cancer cells, in general have higher methylation capabilities than normal cells, but the range of DNMT overexpression is quite wide, reflecting highly variable expression. The range of DNMT overexpression was shown to vary from 4-3000 fold in one study [102]. For example, in leiomyomas, DNMT3a and DNMT3b are decreased in 75% of the cases, in contrast to increased expression of DNMT1 in 50% of the cases [103]. Another study showed that ovarian cancer cell lines exhibit DNMT1 expression levels at three times normal, along with increased DNMT3b levels [104]. However, not all DNMTs are overexpressed in all cancer types. In another study comprised of colorectal, bladder, renal, and pancreatic cancers, a significant increase in the expression of DNMT3b was observed, but there was no overexpression of DNMT1 or DNMT3a [105]. This suggests that the type and extent of aberrant expression of different DNMTs in cancer cells is significantly determined by the tissue type.

Various studies based on the modulation of DNMT levels have been performed to dissect the roles of different DNMTs in the aberrant methylation seen frequently in different types of human cancer. Several studies found that inhibiting DNMT1 was sufficient to cause re-expression of methylation-silenced genes in bladder [106], lung [107], breast [107, 108], and colon [109] cancer cells. Other studies found that knocking down DNMT1 alone is not sufficient, and inhibiting DNMT3b in combination with DNMT1 is required for re-expression of methylation-silenced tumor suppressor genes [110-112]. In a study in colorectal cancer cells, concomitant loss of both DNMT1 and DNMT3b, but not individual

loss, inhibited almost all methyltransferase activity [110], indicating that these enzymes act cooperatively. Additionally, DNMT3b was found to be overexpressed in a greater percentage of breast cancers than DNMT1 or DNMT3a, and was significantly related to more aggressive cancers and poorer prognosis in patients receiving adjuvant hormone therapy [113]. DNMT3b-overexpressing cancers demonstrated increased proliferation and were more likely to be ER-negative suggesting that overexpression of certain DNMTs may result in important differences in cancer biology. Overexpression of DNMT3b has been suggested to be involved in multistage carcinogenesis not only by affecting the expression of specific genes but also by inducing chromosomal instability [114]. The results of these studies largely depend on the cell type studied, method used to inhibit DNMT, methods used to detect methylation changes, and on the target genes examined for detecting methylation changes [115, 116].

A number of different genes have been shown to be inactivated in breast cancer through methylation-dependent gene silencing, suggesting that epigenetic mechanisms play a major role in breast carcinogenesis [74]. These genes include but are not limited to cell cycle control genes (*APC, RASSF1, RB, TFAP2A*), tumor suppressor genes (*CST6, BRCA1, PRDM2*), metastasis-associated genes (*CDH1, CEACAM6, LGALS3BP*), steroid receptor genes (*ESR1, PGR, RARα*), and others [117-119]. For example, estrogen receptor (*ESR1*) is a steroid hormone receptor which activates transcription of cell growth genes. Loss of *ESR1* expression is associated with poor differentiation, insensitivity to hormonal therapy, and poor clinical outcome, and is an important prognostic factor in breast cancer [120]. A considerable percentage of breast cancers lack expression of the estrogen receptor (and other steroid receptors), but the loss of *ESR1* expression is not always caused by deletion or mutation

[121]. Methylation-dependent silencing of the *ESR1* gene is seen in as many as 46% of breast tumors [122, 123]. Adenomatous polyposis coli (*APC*) that exercises cell cycle control by regulating β-catenin-induced proliferation has been found to be methylated in 36-49% of primary breast tumors [124, 125] and the frequency of methylation seems to increase with tumor size and stage [126]. Therefore, *APC* methylation represents an independent marker of poor prognosis in breast cancer patients [127]. In addition, *APC* is often methylated concurrently with other biologically important genes such as *RASSF1A* [128]. Cystatin M (*CST6*) encodes a putative breast cancer suppressor gene and is silenced in many breast cancer cell lines and primary tumors through promoter hypermethylation [89, 129]. It has been shown that treatment with demethylating agents like 5-aza results in the re-expression of *CST6* in breast cancer cell lines that normally lack expression of this gene [130]. E-cadherin (*CDH1*) plays a vital role in the maintenance of cell-to-cell adhesion and suppression of metastasis [131]. The frequency of methylation for *CDH1* is estimated to be between 53-72% for primary breast tumors and upto 90% for lymph node metastases making it one of the most commonly methylated genes in breast cancer [125, 132]. Methylation of *CDH1* is an important marker for aggressive breast tumors as it is associated with a higher incidence of lymph node metastasis, poor differentiation, and decreased patient survival [133]. The vast number of genes reported to be silenced by methylation in breast cancer in association with the cellular activities in which they participate shows that methylation is likely to have a significant impact on clinical behavior in breast cancer.

Hypermethylation Defect and Association with Basal-like Breast Cancer

Some cancers exhibit aberrant concurrent hypermethylation of numerous genes, a phenomenon known as the CpG island methylator phenotype (CIMP). CIMP was first identified in colorectal carcinomas where it affects a distinct subset of tumors with high rates of concordant methylation of specific genes [134]. More recently, CIMP has been identified in other human cancers, including solid tumors like cancers of the ovary [135], bladder [136], prostate [136], lung [137, 138], stomach [139-142], liver [143], pancreas [144], esophagus [145], and kidney [146], neuroblastomas [147], as well as hematopoietic malignancies like leukemias and lymphomas [148, 149]. Despite the fact that many epigenetically-regulated genes are known to be directly silenced by DNA methylation in breast cancer, definitive evidence for a hypermethylation defect (similar to CIMP) among human breast cancers did not emerge until recently [150]. Previously, some investigators suggested that such a hypermethylation defect does not occur in breast cancer [151]. To characterize aberrant DNA methylation in human breast cancer, Roll *et al* examined the gene expression status of 64 epigenetically-regulated genes in a panel of 16 breast cancer cell lines (BT-20, BT-549, HCC1937, HS578T, MCF-7, MDA-MB-231, MDA-MB-415, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MDA-MB-468, SK-BR-3, SUM102, SUM149, SUM185, and ZR-75-1) and the normal mammary epithelial cell line MCF12A. The genes were selected by including the genes aberrantly expressed in breast cancer and the genes known to be predictive of CIMP in other tumor systems. Unsupervised cluster analysis of gene expression patterns revealed two distinct groups of breast cancer cell lines that possess distinct methylation signatures: (i) hypermethylator cell lines, and (ii) non-hypermethylator cell lines [150]. The hypermethylator cell lines include BT-549, HCC1937, HS578T, MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-453 SUM102, SUM149, and SUM185 and the non-

hypermethylator cell lines include BT-20, MCF-7, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1 [150]. The hypermethylation defect observed is associated with a characteristic gene expression signature that reflects methylation-dependent loss of expression of a panel of epigenetic biomarker genes (including *CDH1, CEACAM6, CST6, ESR1, GNA11, MUC1, MYB, SCNN1A,* and *TFF3*) [150]. Hypermethylator cell lines also exhibit higher total DNMT activity levels than that of non-hypermethylator cell lines and non-neoplastic MCF12A cells. Quantitation of the relative DNMT1, DNMT3a, and DNMT3b protein levels between hypermethylator and non-hypermethylator cell lines revealed that average DNMT1 and DNMT3a protein levels for the hypermethylator cell lines and the non-hypermethylator cell lines were indistinguishable from those of MCF12A. In striking contrast to DNMT1 and DNMT3a, the average DNMT3b protein levels for the hypermethylator cell lines were much higher than those of the non-hypermethylator cell lines. In addition, a strong association between DNMT activity and DNMT3b protein levels was also observed. This hypermethylation defect that characterizes a subset of breast cancer cell lines reflects concurrent epigenetic silencing of methylation-sensitive genes secondary to overexpression of DNMT3b and DNA methyltransferase hyperactivity [150].

To determine if a similar hypermethylation defect could be detected in primary sporadic invasive breast cancers, microarray-based expression data from the UNC Microarray Database was examined. An unsupervised cluster analysis of 90 tumors in one dataset from UNC revealed three major clusters. One of these clusters was composed of 21 tumors (23%) that expressed a hypermethylation signature identified by low expression of seven or more genes out of the nine genes analyzed. Within this cluster, 100% (21/21) of the tumors were of the basal-like subtype and included 88% (21/24) of all basal-like tumors

within the dataset. The second cluster consisted of 51 tumors and was composed primarily of luminal A and luminal B breast cancers (65% and 29%), with one basal-like and two Her2+ cancers within this cluster. The third cluster consisted of 19 cancers with 84% (16/19) of these classified as Her2+ along with one luminal B and two basal-like cancers. These results suggest that expression of the hypermethylation defect may be associated with the basal-like subtype of breast cancer. In order to validate this suggestion, multiple datasets were analyzed, consisting of an expanded dataset from UNC containing 272 cancers **(Figure 1.6)**, and datasets obtained from Hess *et al* [152] consisting of 133 primary breast cancers, Wang *et al* consisting of 295 primary breast cancers [153], and van de Vijver *et al*. composed of 246 primary breast cancers [63]. The details of these analyses are summarized in **Table 1.4** and all these results were in concordance with the initial results from UNC dataset. In total, 946 primary breast cancers were examined to explore the possibility that the hypermethylator signature is expressed by primary breast cancers *in vivo*. Among the tumors analyzed, 23% (220/946) exhibited the hypermethylation signature based on the rule (defined as having seven or more target genes with expression levels below the median). Among these hypermethylator cancers, 79% (174/220) were basal-like and out of all the basal-like cancers examined, 63% (174/277) were also hypermethylators. These findings suggest significant correspondence between expression of the hypermethylator defect and the basal-like subtype of breast cancers.

Basal-like Breast Cancer

Discovery of Basal–like Breast Cancers

The basal breast cancer subtype was first described in studies based on immunohistochemistry [154-157]. These cancers are designated basal-like because they exhibit some cellular characteristics associated with the basal myoepithelial cell layer, such as expression of cytokeratins 5/6, 14, or 17, vimentin, and laminin, but these tumors are not derived only from myoepithelial cells [158-160]. The basal-like breast cancer subtype was rediscovered following the application of microarray-based gene expression profiling to breast cancer classification [57-61, 63, 64]. That the basal-like breast cancers were identified independently by two different methodologies indicates strongly that these cancers represent a distinct biological entity. Basal-like breast cancers are best classified through gene expression profiling [57-61, 63, 64]. However, in routine clinical practice, immunohistochemistry has become the surrogate for the gene expression analysis for diagnosis of basal-like breast cancers **(Figure 1.3)**. Correctly classifying these cancers significantly impacts clinical decisions and research efforts. In the clinic, there is a need to correctly identify breast cancer subtypes for prognostication purposes in relation to individual patients and for decision-making related to appropriate treatment course. On the other hand, in the research environment, correct breast cancer subclassification is essential to ensure that investigations expand our understanding of the biological basis for the behavior and characteristics of these cancers.

Association with Risk Factors

The development of basal-like breast cancer is associated with distinct risk factors, including early-onset menarche, younger age at first full-term pregnancy, high parity combined with lack of breast feeding, and abdominal adiposity (based upon waist-hip ratio)

[161]. These breast cancers are over-represented among patients of certain age and ethnic groups, and are frequently associated with certain genetic mutations. Specifically, basal-like breast cancer is over-represented among premenopausal African-American women [66]. However, these cancers can and do affect women of every age and ethnic group [161]. The differences in distribution of basal-like breast cancer by age and race can be partially attributed to variations in the distribution of the risk factors described and to other risk factors (e.g., use of lactation suppressants and overexpression of leptin receptor) [161]. In addition, basal-like breast cancer occurs more frequently among hereditary breast cancer patients that harbor germ-line *BRCA1* mutation [162]. Foulkes *et al.* showed that 17/72 triple-negative breast cancers harbored a *BRCA1* mutation and 88% (15/17) of these expressed the basal-like phenotype [163]. Likewise, Sorlie *et al.* observed that 100% (18/18) of breast cancers from patients carrying *BRCA1* mutations clustered within the basal-like subgroup [60]. However, the other molecular subtypes of breast cancer can be associated with *BRCA1* mutations as well.

Morphological Features

Morphologically, basal-like breast cancers are characterized by the presence of central necrotic zones, pushing borders, and conspicuous lymphocytic infiltrate [164-168]. The presence of metaplastic elements [59, 166-168] and medullary⁄atypical medullary features [167-169] are more prevalent in basal-like breast carcinomas than in other types of breast cancer. Recent studies have shown that more than 90% of metaplastic breast carcinomas [59], as well as the majority of medullary carcinomas [169, 170], exhibit a basal-like phenotype. Basal-like breast cancers are aggressive, with the high rates of cellular

proliferation, high histological grade, and extremely poor clinical outcomes [59, 60]. These factors combine to account for the disproportionate contribution of basal-like breast cancer to breast cancer mortality. It has been suggested that the high level of cellular proliferation observed in these neoplasms might account for the over-representation of basal-like breast cancers among the so-called interval breast cancers (the cancers arising between annual mammograms).

Clinical Behavior of Basal-Like Breast Cancers

Currently, there is no consensus on the immunohistochemical criteria for the diagnostic classification of basal-like breast cancers. Studies have shown that the profile constructed using ER-/PR-, HER2-, CK5/6+, and/or EGFR+ is 100% specific but only 55% to 76% sensitive [171]. Breast cancers that are ER-/PR-/HER2- are broadly classified as triple-negative neoplasms **(Figure 1.3)**. The triple-negative breast cancers include most (or all) basal-like breast cancers [172]. Interpreting the percentage of positive cells and intensity of immunohistochemical staining is subjective. Variability in immunostaining techniques and procedures is a concern as well. Hence, standardization and/or automation of immunostaining procedures and interpretation to remove technical and subjective variation will benefit this analysis in the clinical laboratory. The low sensitivity associated with classification of basal-like breast cancers using immunohistochemical staining may indicate that these cancers are much more heterogeneous than previously thought. Gene expression profiling-based molecular classification of breast cancers predicts the general clinical behavior of breast cancers corresponding to the different molecular subtypes. Microarray studies show that the basal-like breast cancers express a common gene expression signature

and these cancers are associated with an extremely bad prognosis [59]. Among the patient cohort examined in the initial study of this type, 100% of the patients with basal-like subtype succumbed to their disease within four years of diagnosis [59]. Basal-like breast cancers respond to preoperative (neoadjuvant) chemotherapy [173, 174]. However, despite the observation of pathologic complete response in many patients, these individuals exhibit poor long-term survival. The poor survival outcomes among these patients despite response to chemotherapy may be related to a higher likelihood of relapse in individuals where pathologic complete response was not achieved [174].

The malignant neoplasms that constitute the basal-like breast cancer subtype are not biologically homogeneous. For example, in one study, unsupervised hierarchical clustering within 43 cytokeratin-14 positive (basal-like phenotype) tumors revealed four clusters, and one of these displayed a worse prognosis than other three, strongly suggesting intra-subtype heterogeneity [175]. Variable prognosis within the basal-like subtype has also been reported by other groups [176, 177]. Rakha *et al*. divided the basal-like breast cancers into those with a "dominant basal pattern" (>50% of cells are positive for cytokeratin 5/6 and 14) and the remaining "basal" cancers (<50% of cells are positive for cytokeratin 5/6 and 14) [176]. These subsets of basal-like breast cancers demonstrated differences in grade, presence of pushing margins, local spread, and long-term outcomes (disease-free survival and overall survival) [176]. Likewise, Laakso *et al.* distinguished "basal" (uniformly positive for cytokeratins 5 and 14) and "basoluminal" (heterogeneously positive for cytokeratin expression) subtypes of basal-like breast cancers, and showed that these subsets of basal-like cancers differ with respect to tumor size, proliferation index, expression of other markers (like vimentin), and recurrence-free survival [177]. These observations underscore the

necessity to further define biological subsets of basal-like breast cancer (particularly in terms of clinical behavior). The pattern of metastatic spread among basal-like breast cancers has been suggested to be different compared to other breast cancer subtypes. The basal-like breast cancers have a tendency to disseminate through hematogenous routes, involving brain (resulting in higher rate of cerebral metastasis) and lung, and are less likely to spread to lymph nodes, liver, or bones [164, 178-180]. In general, cancer prognosis is linked to various clinical parameters, including tumor size, tumor grade, lymph node status, and the presence of distant metastasis. However, among basal-like breast cancers, prognosis has been shown to be less dependent on tumor size, tumor grade, and lymph node status reflecting the deviant nature of these cancers. Expression of basal markers like cytokeratins 5/6 was associated with poor outcome and proved to be a prognostic factor independent of the usual clinical parameters [181]. These observations highlight the requirement to examine further the transcriptome of basal-like breast cancers in order to uncover the molecular basis for the biological behavior of subsets of these cancers [181].

Prognosis

Neoplasms representing different subsets of basal-like breast cancers may have the same clinical stage based on traditional classification criteria and may be histologically and morphologically similar, yet their biological (clinical) behavior may be remarkably different. Survival rates associated with basal-like breast cancers are dismal. Numerous studies have shown that patients with basal-like breast cancer exhibit significantly shorter overall survival (OS) and disease-free survival (DFS), and have high rates of tumor recurrence, highlighting the aggressive course of these cancers. A retrospective study of 49 basal-like and 49 grade

and age-matched non basal-like tumors [182] showed that patients with basal-like breast cancers were associated with significantly shorter disease-free and overall survival, and a higher recurrence rate. Another study based upon a cohort of 930 breast cancer patients showed that expression of basal cytokeratins (indicative of basal-like phenotype) was associated with poor progression-free survival and poor overall survival [171]. Analysis of 496 primary breast cancers from the Carolina Breast Cancer Study showed that progression-free survival differed by breast cancer subtype and survival time is significantly shortened in basal-like and HER2+ subtypes [66]. These studies are consistent with similar observations of poor prognosis in basal-like cancers made by numerous other groups before and after these aforementioned studies [59-61, 69, 181, 183, 184]. In the absence of molecular targets (like ER or HER2), options for basal-like breast cancer therapy are limited to aggressive cytotoxic chemotherapy. However, cytotoxic chemotherapy (whether neoadjuvant or adjuvant) has proven largely ineffective in the treatment of basal-like breast cancer based on OS among these patients. The general failure of chemotherapy in the treatment of basal-like breast cancer may be related to the lack of targeted approaches and/or our current inability to stratify patients according to their likelihood of response to specific drugs or treatment modalities.

Post-transcriptional Regulation of *DNMT3b* by MicroRNAs

DNMT3b is constitutively expressed by all mammalian cell types, but is often overexpressed in cancer [105, 113, 185, 186]. However, unlike other genes that are overexpressed in cancer, the mechanisms accounting for increased *DNMT3b* levels infrequently involves gene mutation and/or gene amplification [187]. Likewise, increased

DNMT3b transcription due to increased *trans*-activation does not commonly occur in cancer [187]. Rather, it is now recognized that *DNMT3b* is subject to post-transcriptional regulation by microRNAs (miRs), which are small endogenous non-coding RNAs (19-25 nucleotide long) that have emerged as key players in regulation of gene expression [188]. miRs were discovered approximately 20 years ago when investigators determined that the traditionally non-functional areas of the genome had gene regulatory capabilities and were later termed microRNAs [189-191]. The post-transcriptional regulation by miRs occurs through sequence-specific targeting of mRNAs as a result of recognition of complementary sites, most often in the 3' untranslated region (UTR) of the target mRNA, producing either translational repression or degradation of the target mRNA [189, 192-195]. Less frequently, miRs have also been documented to target 5'UTRs and coding regions of mRNAs [196-198]. Recently, it has been shown that miRs can also target proteins where a novel function of miRs called 'decoy activity' was reported. It was shown that miR-328 regulates RNA-binding protein by interacting with a ribonucleoprotein, hnRNP-E2 [199]. miRs are expressed in a tissue-specific manner and have been implicated in the regulation of several biological processes, including cellular proliferation, differentiation, apoptosis, and development [200-203]. More than 30% of the human transcriptome is estimated to be targeted by microRNAs [191]. A single miR can target multiple mRNAs and a single mRNA can be targeted by multiple miRs. A single miR or a group of miRs can therefore regulate pathways that are essential to biological/pathological processes like angiogenesis, survival and growth that can directly affect cancer cell behavior. Some miRs also participate in or trigger feedback or feedforward loops by cooperating with their target genes, further complicating the network of gene-regulation [204, 205]. More than 1000 miRs have been

identified in human genome, although it is predicted that many more may exist that are yet unidentified [206].

miR biogenesis is a complex system that involves multiple steps and several enzymes. Within the nucleus, a long transcript known as pri-miRNA is first transcribed by RNA polymerase II [188, 207]. Pri-miRNA is cleaved by RNAse III endonuclease Drosha to a smaller hair pin, precursor miRNA (pre-miRNA) approximately 70 nucleotides long [208, 209]. RNAse Drosha belongs to a family of double stranded RNA specific ribonucleases and acts in combination with its partner, DGCR8, forming the processing complex called the Microprocessor [210, 211]. The pre-miRNA is transported out of the nucleus into the cytoplasm by the nuclear export protein, Exportin 5 [212]. Pre-miRNA in the cytoplasm is subsequently cleaved by another RNAse III enzyme Dicer that acts in conjunction with RNA-binding protein, TRBP, to yield double stranded miR molecule approximately 22 nucleotides in length [191, 213]. This double stranded molecule consists of mature miRNA/miRNA nucleotide duplex that is separated into two single-stranded molecules; the mature miR gets incorporated into the RNA-induced Silencing Complex (RISC) and the other strand undergoes degradation [188, 214]. The 'seed' region comprised of nucleotides 2-7 of the mature miR sequence specifies the target mRNA that the miR will bind to and is critical in determining the effect in terms of degradation of mRNA or inhibition of translation [188, 191].

More than half of human miR genes have been identified in fragile sites and are hence susceptible to alterations like deletion, translocation and amplification. Dysregulation of miRs has been shown to play oncogenic as well as tumor suppressor roles depending on the disease/pathway involved and the tissue affected [191]. Altered miR expression is

associated with several types of human cancer, including breast cancer [215-218]. The deregulated pattern of expression of miRs between normal and cancerous tissues in breast cancer has been extensively studied. The expression patterns of different miRs have been correlated with tumor stage, estrogen and progesterone receptor expression, proliferation index, vascular invasion, epithelial to mesenchymal transition, metastasis and neovascularization [216, 219-221]. miR-155, miR-21, miR-17-922, miR-182, miR-200, and miR-9 have been shown to be overexpressed in multiple studies in breast cancer; likewise let-7, miR-143/145, miR-10b, miR-125b, and miR-126 have been shown to be downregulated [188, 191, 205, 214, 216, 219, 222]. Circulating miRs are also being explored as noninvasive biomarkers for the purposes of diagnosis as well as surveillance of disease status in breast cancer as well as other malignancies.

Recent studies have identified miRs as both regulators of DNA methyltransferase (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 cancer [223] and acute myeloid leukemia [224]. Likewise, the miR-148 family (miR-148a and miR-148b) regulates *DNMT3b* in cell lines of multiple origin, including the MCF-7 breast cancer cell line [225]. There is evidence that miRs not only regulate epigenetic machinery but are also epigenetically regulated themselves. In human bladder cancer, miR-127 is silenced by promoter hypermethylation [226]. In similar fashion, miR-148a is epigenetically silenced in human cancer cell lines established from lymph node metastasis from colon, melanoma, and head and neck, suggesting that epigenetic loss of miR-148 is associated with progressive changes such as development of metastatic potential [217]. All of

these observations indicate direct interactions as well as cross-talk between the DNA methylation machinery and miRs.

Objective of This Dissertation Research

The objective of this project was to understand and characterize the contribution of DNMT3b to the biology and clinical outcome of basal-like breast cancers and to elucidate the role of microRNAs in dysregulation of DNMT3b expression among these breast cancers. To address our objective, we employed an experimental model of human basal-like breast cancer that is based on (i) well characterized hypermethylator cell lines that exhibit DNMT3b hyperactivity, and (ii) primary human breast cancers of known molecular classification. In this study, we analyzed the combination of epigenetic treatment and chemotherapy in three hypermethylator breast cancer cell lines. The purpose 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 (i) pharmacologic inhibition of DNA methyltransferase activity using 5-aza-2'-deoxycytidine (5-aza), and (ii) targeted inhibition of overexpressed DNMT3b using RNAi-mediated DNMT3b knockdown (KD). The results show that 5-aza pretreatment sensitizes hypermethylator breast cancer cells to cell killing by cytotoxic drugs, and that the improved chemotherapeutic efficacy is a function of dose and duration of exposure to 5-aza. We also observed an increase in the effectiveness of chemotherapeutic drugs after targeted inhibition of DNMT3b. These results demonstrate that DNMT3b is an excellent target for development of rational therapeutic approaches for hypermethylator breast cancers (such as basal-like breast cancers). We provide proof-of-concept that targeting DNMT3b in hypermethylator breast cancer cells sensitizes them to cell

killing by cytotoxic drugs and that this strategy can be exploited to improve the efficacy of breast cancer chemotherapy.

We analyzed breast cancer cell lines for differential expression of regulatory miRs to determine if loss of miR-mediated post-transcriptional regulation of *DNMT3b* represents the molecular mechanism that governs the overexpression of DNMT3b that drives the hypermethylation defect in breast cancer. The results show that multiple miRs (miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203) post-transcriptionally regulate DNMT3b in combination and loss of expression of these regulatory miRs contributes to *DNMT3b* overexpression in hypermethylator cell lines. We observed that enforced expression of regulatory miRs results in reduced *DNMT3b* mRNA levels in hypermethylator breast cancer cell lines, and that down-regulation of regulatory miRs results in increased *DNMT3b* mRNA levels in non-hypermethylator breast cancer cell lines. These observations combine to suggest that the loss of multiple regulatory miRs that post-transcriptionally regulate DNMT3b levels represents the molecular mechanism governing the DNMT3b-mediated hypermethylation defect in breast cancer cell lines. In addition, we examined the expression of microRNAs (miRs) that regulate DNMT3b in primary breast cancers and normal mammoplasty tissues to determine the mechanism governing DNMT3b overexpression in the hypermethylation defect. Examination of methylation-sensitive gene expression in primary breast cancers showed that majority of basal-like tumors express the hypermethylation defect as compared to other molecular subtypes of breast cancer. Significantly reduced expression of miR-29c distinguished basal-like cancers from other subtypes. miR expression patterns revealed two groups among the basal-like breast cancers corresponding to diminished expression and normal levels of expression. Our results suggest

strongly that (i) reduced expression of miR-29c is characteristic of basal-like breast cancers, (ii) two subgroups of basal-like breast cancers can be identified based on miR expression and methylation-sensitive gene expression, and (iii) the subgroup of basal-like breast cancers with reduced expression of multiple regulatory miRs express the hypermethylation defect. We provide proof-of-concept that targeting DNMT3b in hypermethylator breast cancer cells sensitizes them to cell killing by cytotoxic drugs and that this strategy can be exploited to improve the efficacy of breast cancer chemotherapy. Our results strongly support the suggestion that loss of miR expression may account for the DNMT3b-mediated hypermethylation defect among basal-like breast cancers and this significant molecular alteration present during the process of breast carcinogenesis can be targeted to significantly alter the prognosis of breast cancer.

Table 1.1. *Breast Cancer Incidence for American Women, By Ethnicity (Age-Adjusted Incidence Rate From 2004-2008).* These data were adapted from the SEER Cancer Statistics Review (www.seer.cancer.gov).

Race/Ethnicity	**Incidence (per 100,000 women)**
All Ethnicities	124.0
Caucasian/White	127.3
African-American	119.9
Asian/Pacific Islander	93.7
American Indian/Alaskan Native	77.9
Hispanic	78.1

Table 1.2. *Breast Cancer Mortality for American Women, By Ethnicity (Age-Adjusted Mortality Rate From 2003-2007).* These data were adapted from the SEER Cancer Statistics Review (www.seer.cancer.gov).

Race/Ethnicity	**Mortality (per 100,000 women)**
All Ethnicities	24.0
Caucasian/White	23.4
African-American	32.4
Asian/Pacific Islander	12.2
American Indian/Alaskan Native	17.6
Hispanic	15.3

Table 1.3. *Stage Distribution and 5-year Relative Survival By Stage at Diagnosis for Females, All Races, 2001-2007.* These data were adapted from the SEER Cancer Statistics Review (www.seer.cancer.gov).

Stage at Diagnosis	Stage Distribution (%)	5-year Relative Survival (%)
Localized (confined to primary site)	60	98.6
Regional (spread to regional lymph nodes)	33	83.8
Distant (cancer has metastasized)	5	23.4
Unknown (unstaged)	2	52.4

Table 1.4. *Association of Hypermethylation Defect with Basal-like Breast Cancer.*

Dataset	Number of Cancers	Hypermethylator Cancers (%)	Molecular Subtypes Among Hypermethylator Cancers	% of Basal-like that are Hypermethylator
Expanded UNC[1]	272	80/272 (29%)	**65/80 (81%) Basal** 1/80 (1%) LB 1/80 (1%) HER2+ 13/80 (16%) CL	**65/103 (63%)**
Hess et al. [152]	133	33/133 (25%)	**26/33 (79%) Basal** 4/33 (12%) HER2+ 2/33 (6%) LA/B 1/33 (3%) NL	**26/32 (81%)**
Wang et al. [153]	295	59/295 (20%)	**44/59 (75%) Basal** 12/59 (20%) LA/B 3/59 (5%) HER2+	**44/76 (58%)**
Van de Vijver et al. [63]	246	48/246 (20%)	**39/48 (81%) Basal** 7/48 (15%) LA/B 2/48 (4%) HER2+	**39/66 (59%)**
Total	**946**	220/946 (23%)	**174/220(79%) Basal** 22/220 (10%) LA/B 6/220 (3%) HER2+ 14/220 (6%) other	**174/277 (63%)**

Subtypes abbreviated as follows: luminal A (LA), luminal B (LB), claudin-low (CL), normal-like (NL), HER2 overexpressing (HER2+), and Basal-like (Basal)

[1] **https://genome.unc.edu/**

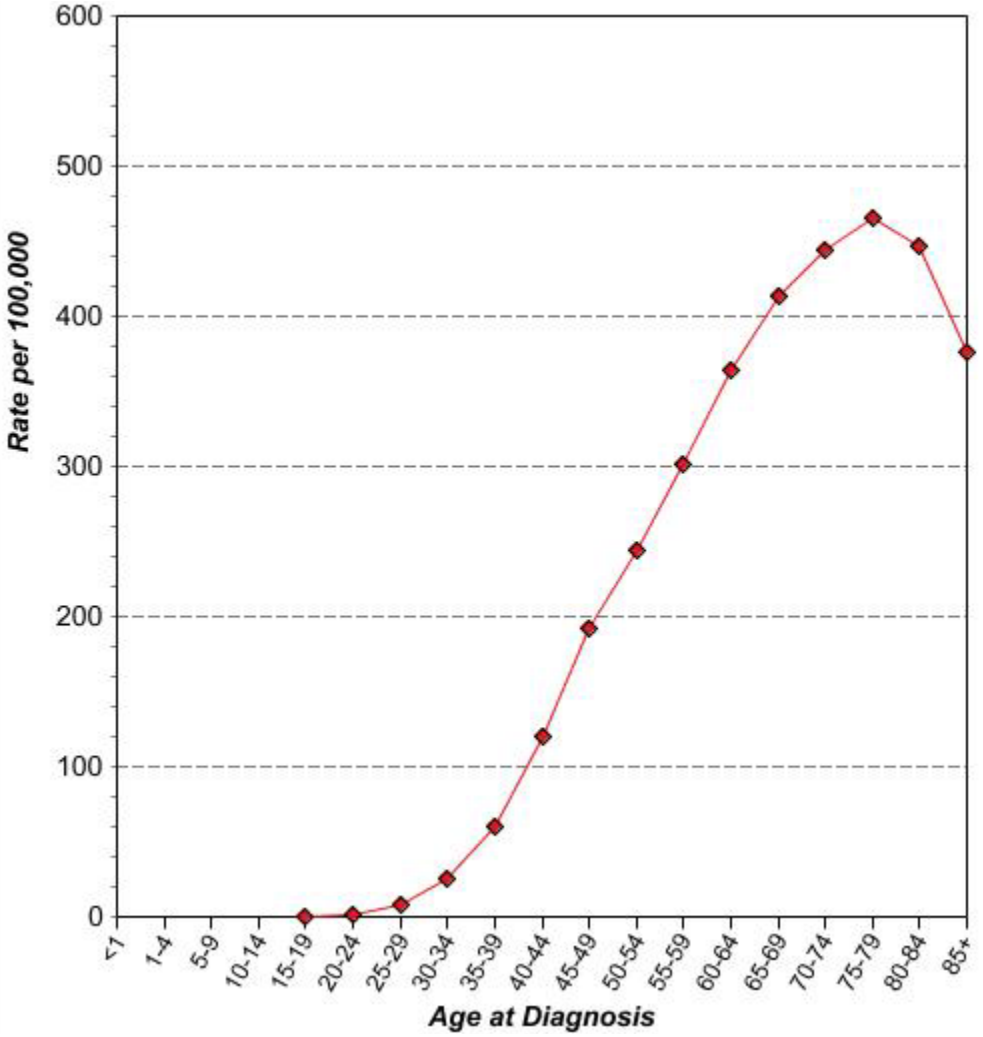

600
500
400
300
200
100
0
Rate per 100,000
<1
1-4
5-9
10-14
15-19
20-24
25-29
30-34
35-39
40-44
45-49
50-54
55-59
60-64
65-69
70-74
75-79
80-84
85+
Age at Diagnosis

Figure 1.1 *Age-Specific SEER Incidence Rates by Cancer Site All Ages, All Races Female; 1992-2008***.** The graph showing incidence rate per 100,000 women at different age intervals below 1 year to 85+ years of age from 1992-2008. These observations were made based on SEER Cancer Statistics Review (www.seer.cancer.gov).

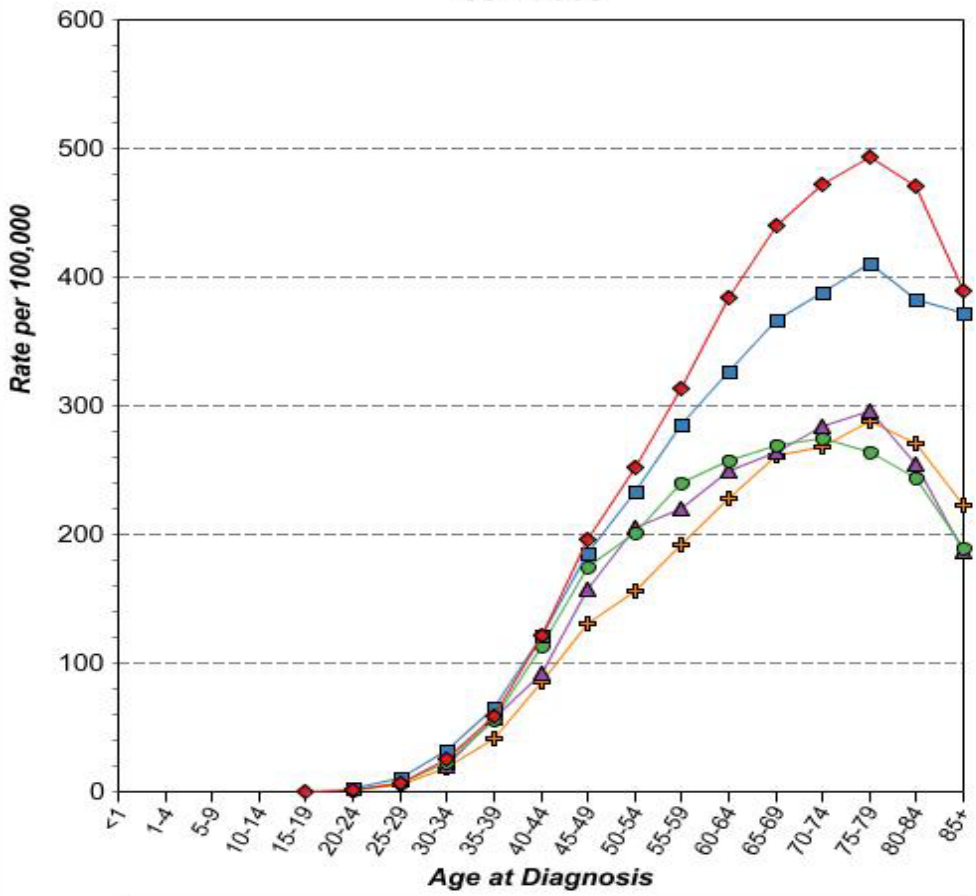

600
500
400
300
200
100
0
Rate per 100,000
<1
1-4
5-9
10-14
15-19
20-24
25-29
30-34
35-39
40-44
45-49
50-54
55-59
60-64
65-69
70-74
75-79
80-84
85+
Age at Diagnosis

Figure 1.2. *Age-Specific SEER Incidence Rates by Race and Sex (Female), All Ages, 1992-2008*. The graph shows age-specific incidence rate per 100,000 women of different races from 1992-2008. These data were adopted from the SEER Cancer Statistics Review (www.seer.cancer.gov). The red line indicates the incidence in Caucasian females, the blue line indicates the incidence in African-American females, the green line indicates the incidence in Asian/Pacific Islander females, the purple line indicates the incidence in American Indian/AK Native females, and the yellow line indicates the incidence in Hispanic females.

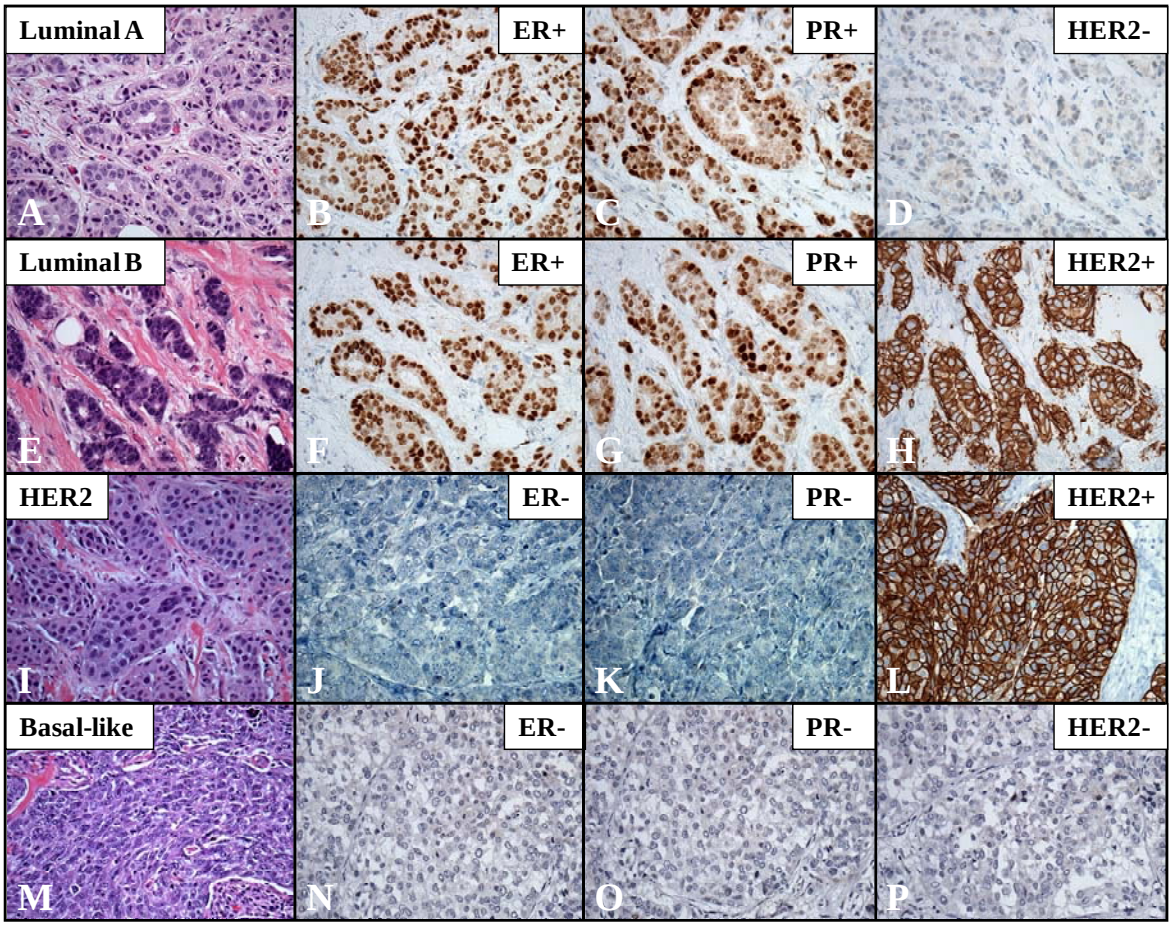

Luminal A
ER+
PR+
HER2-
A
B
C
D
Luminal B
ER+
PR+
HER2+
E
F
G
H
HER2
ER-
PR-
HER2+
I
J
K
L
Basal-like
ER-
PR-
HER2-
M
N
O
P

Figure 1.3. *Differential Expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Receptor 2, (HER2) Among Different Subtypes of Breast Cancer.* Breast cancer is classified into various subtypes based on differential immunohistochemical staining for ER (Panels B, F, J, and N show immunostaining for ER and the results, ER+ or ER-, are indicated), PR (Panels C, G, K, and O show immunostaining for PR and the results, PR+ or PR-, are indicated), HER2 (Panels D, H, L, and P show immunostaining for HER2 and the results, HER2+ indicative of HER2 amplification or HER2-, are indicated), HER1 (not shown), and cytokeratins (not shown). Panels A-D, luminal A breast cancer; Panels E-H, luminal B breast cancer; Panels I-L, HER2+ breast cancer; Panels M-P, basal-like breast cancer. Panels A, E, I, and M show H&E staining for each breast cancer subtype.

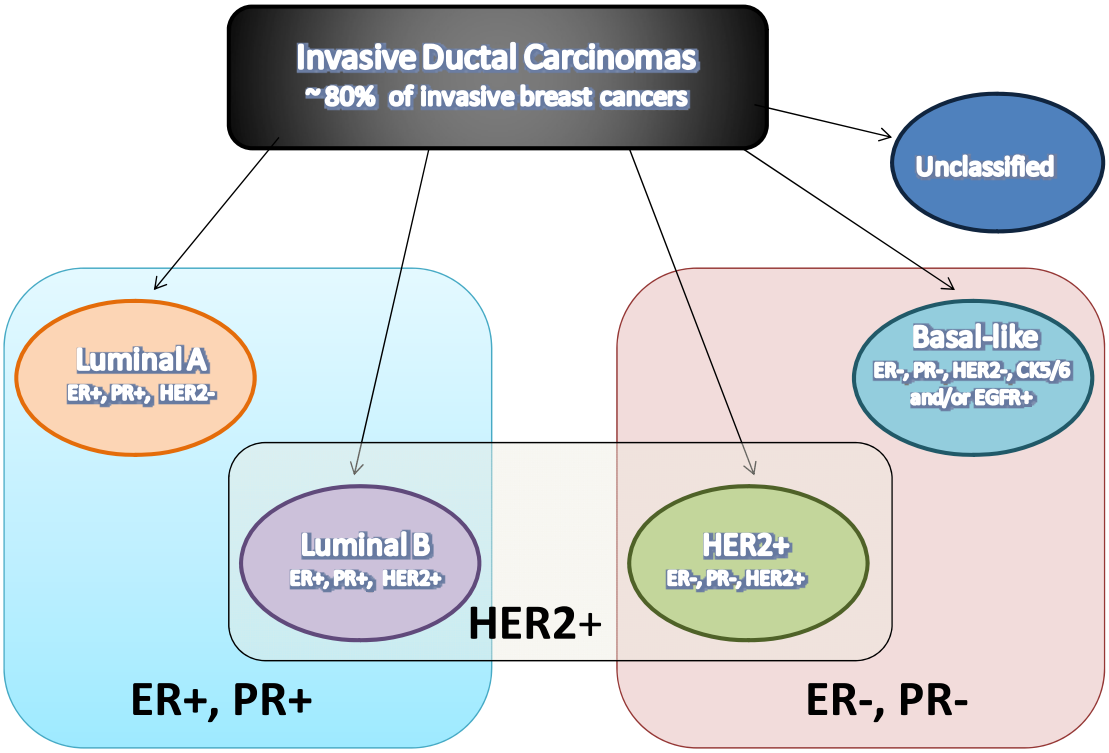

Invasive Ductal Carcinomas
~80% of invasive breast cancers
Unclassified
Luminal A
ER+, PR+, HER2-
Basal-like
ER-, PR-, HER2-, CK5/6 and/or EGFR+
Luminal B
ER+, PR+, HER2+
HER2+
ER-, PR-, HER2+
HER2+
ER+, PR+
ER-, PR-

Figure 1.4. *Molecular Subtypes of Breast Cancer.* The blue and pink rectangles group the subtypes based on the expression of ER/PR, positive in the blue (Luminal A and Luminal B) and negative (HER2+ and Basal-like) in the pink. The central grey rectangle (with black outline) indicates the presence of HER2 amplification in Luminal B and HER2+ subtypes.

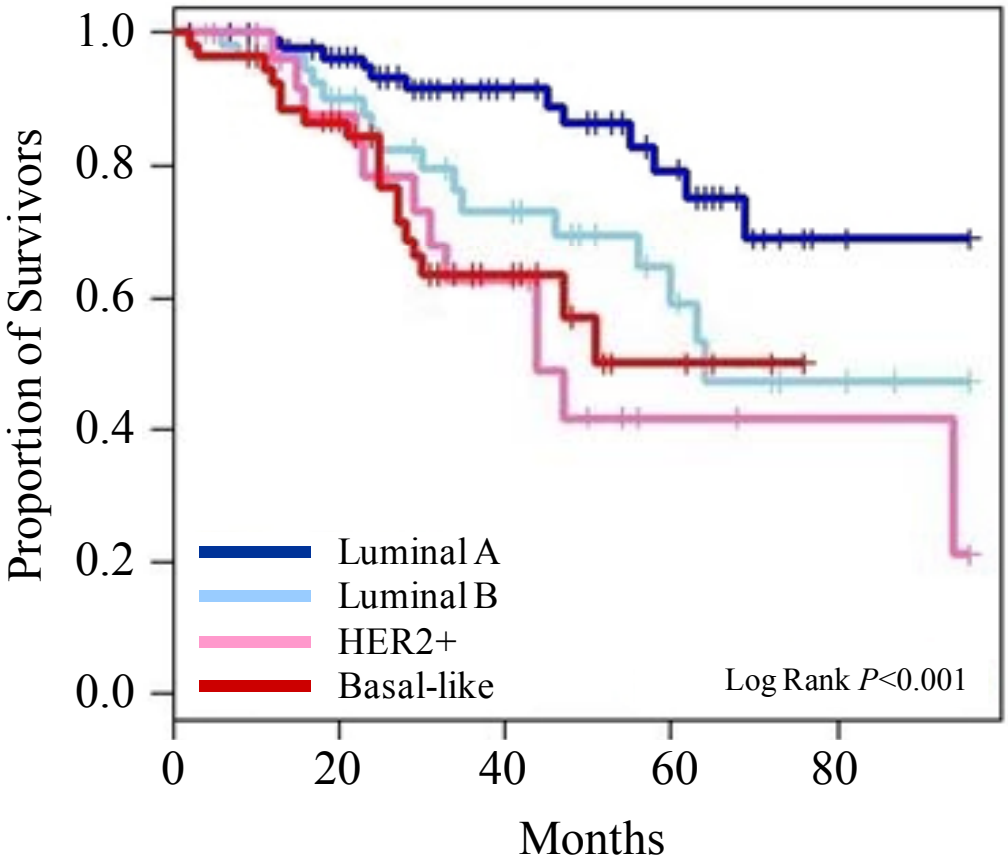

1.0
0.8
0.6
0.4
0.2
0.0
Proportion of Survivors
0
20
40
60
80
Months
Luminal A
Luminal B
HER2+
Basal-like
Log Rank P<0.001

Figure 1.5. *Survival Plot of 294 Breast Cancer Patients.* A Kaplan-Meier survival plot of overall survival corresponding to 294 breast cancers from the publicly available UNC database is shown grouped by molecular subtype. The plot was generated by Dr. Joel S. Parker (UNC Lineberger Cancer Center, UNC Chapel Hill). The *p*-value was calculated using the Log-rank test. Details of these 294 samples along with clinical annotation can be found at https://genome.unc.edu/pubsup/breastGEO/.

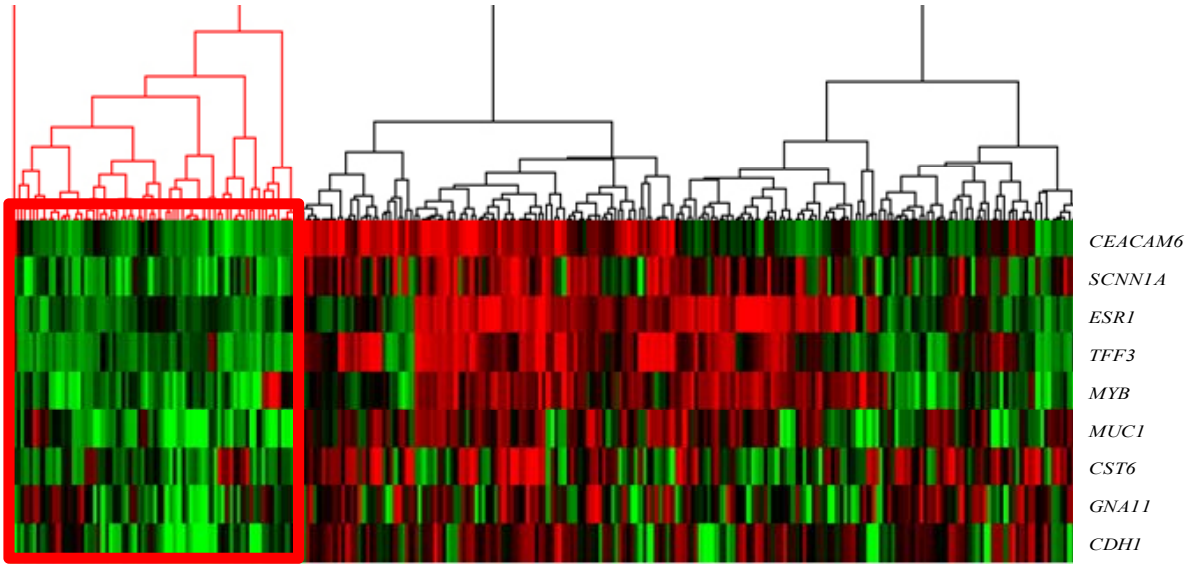

CEACAM6
SCNN1A
ESR1
TFF3
MYB
MUC1
CST6
GNA11
CDH1

Figure 1.6. *Microarray Analysis of 272 Primary Breast Tumors from Expanded UNC Database Suggests a Linkage Between Basal Breast Tumors and the Hypermethylator Phenotype.* Supervised analysis of microarray expression data from the Expanded UNC microarray database. Gene expression patterns for individual tumors were analyzed to determine the expression of hypermethylation defect. Cancers with ≥7 genes below the median expression level were classified as hypermethylators. Microarray data mining analysis was performed by Dr. Wendell Jones (Expression Analysis, Durham, NC). Red indicates high level expression, green indicates low level expression, and black indicates normal expression levels for the genes of interest. The hypermethylator cluster is highlighted with a red rectangle. This cluster demonstrates concurrent downregulation of genes indicative of the hypermethylation defect and is predominately (81%) composed of basal-like cancers.

MATERIALS AND METHODS

Methods Related to Chemotherapy Experiments

Hypermethylator Breast Cancer Cell Lines and Cell Culture Conditions

Human breast cancer cell lines BT549 (ATCC# HTB122), Hs578T (HTN126), and MDA-MB-453 (HTB131) were obtained from the Tissue Culture Core Facility of the UNC Lineberger Comprehensive Cancer Center (Chapel Hill, NC). These cell lines exhibit high levels of total DNA methyltransferase (DNMT) activity, overexpression of DNMT3b, and concurrent silencing of multiple methylation-sensitive genes (including *CEACAM6, CST6, ESR1* and *SCNN1A*) [150]. Breast cancer cell lines were grown in medium recommended by the ATCC (http://www.atcc.org/). Hs578T and MDA-MB-453 cells (and derivative cell lines) were cultured in Dulbecco's modified Eagle's medium, supplemented with 4 mM L-glutamine, 10 μg/ml insulin (Gibco/Invitrogen Life Technologies Grand Island NY), 10% fetal bovine serum (Hyclone, Logan, UT), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). BT549 cells (and derivative cell lines) were cultured in RPMI-1640 medium (Gibco/Invitrogen Life Technologies) containing 10% fetal bovine serum (Hyclone), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). Growth medium was refreshed three times weekly unless otherwise specified for demethylating and/or cytotoxicity assays. Cells were maintained at 37ºC and 5% CO_2.

Generation of DNMT3b KD and Scrambled Control Cell Lines

Expression vectors for *DNMT3b* shRNA and scrambled control were used to target the overexpressed DNMT3b protein associated with the hypermethylation defect in human breast cancer cell lines [150]. These expression vectors were a kind gift from the laboratory of Dr. P.P. Jagodzinski (Poznan University of Medical Sciences, Poznan, Poland) and their construction has been described [227]. Briefly, the expression vectors were constructed in pLVTHM transfer plasmids with a *DNMT3b*-specific oligonucleotide (5'-CGC GTC CCC AGA TGA CGG ATG CCT AGA Att caa gag aCT CTA GGC ATC CGT CAT CTT TTT TGG AAA T-3') or a scrambled control oligonucleotide (5'-GAT CCC GGA CAA GGG TCC TGA TCG TTt tca aga gaA ACG ATC AGG ACC CTT GTC CTT TTT TGG-3') [227]. The resulting pLVTHM^{DNMT3b} and pLVTHM^{SCRAM} vectors express the targeting or control oligonucleotides from the H1 promoter, and each vector contains the green fluorescence protein (GFP) gene enabling the monitoring of transfection efficiency and plasmid retention in transfected cells. Breast cancer cells were grown in a six-well polystyrene plate to 60-70% confluency in 2.5 ml of complete growth medium for pLVTHM^{DNMT3b} and pLVTHM^{SCRAM} transfection with 2.5μg plasmid DNA using TransIT-LT1 Transfection reagent (Mirus, Madison, WI) according to manufacturer's protocol. After 48 hours the presence of GFP+ cells was assessed in the knockdown and scrambled control transfected cells. Selection of positively transfected cells was accomplished using expression of GFP as a selectable marker. Transfected cell lines (*DNMT3b KD* and scrambled control) were subjected to flow sorting under sterile conditions at the UNC Flow Cytometry Core Facility (Chapel Hill, NC) to select for pure populations of GFP+ cells. Post-sort analysis of isolated cell populations indicated that 95-98% of cells were strongly GFP+. GFP expression

in the post-sort populations was monitored weekly by fluorescence microscopy to ensure the continual presence of the transfected plasmids.

Demethylating Treatment of Breast Cancer Cell Lines with 5-Aza-2'-Deoxycytidine

5-aza-2'-deoxycytidine (5-aza, Cat # A3656, Sigma-Aldrich, St Louis, MO) was employed as a demethylating agent. Stock solutions of 5-aza (5 mM) were prepared in dimethyl sulfoxide (Cat # D2650, Sigma Chemical Company). Founder populations of breast cancer cells were propagated in freshly made growth medium (described above) containing 250 nM, or 500 nM 5-aza for 3 days or 7 days, with re-feeding of fresh medium containing the drug each day. Re-feeding of cultures every day eliminates concerns about the half-life of 5-aza in culture medium, and insures a constant exposure to the drug. Control populations of cells (no drug exposure) were maintained in parallel to treated cultures. Low concentrations of 5-aza (≤500nM) were used to avoid cytotoxicity, which can be observed at higher doses [89]. No evidence of 5-aza-related cytotoxicity was noted in this study. At the conclusion of the demethylating pretreatment, cells were trypsinized, counted, and plated in 24-well plates at a concentration of 50,000 cells per well for subsequent exposure to cytotoxic drugs.

Chemotherapeutic Drugs

Chemosensitivity assays were carried out using three chemotherapeutic drugs: Doxorubicin (Adriamycin; CAS 23214-92-8), Paclitaxel (Taxol; CAS 33069-62-4), and 5-Fluorouracil (Adrucil; CAS 51-21-8). The drugs were obtained from Sigma-Aldrich (Doxorubicin, Cat # 44583; Paclitaxel, Cat # T7191; 5-Fluorouracil, Cat # F6627). The stock

solution of Doxorubicin (DOX; 20 μM) was prepared in sterile water, and stock solutions of Paclitaxel (PAX; 11.71 mM) and 5-Fluorouracil (5-FU; 100 mM) were made in dimethyl sulfoxide. Stock solutions were stored as recommended by the manufacturer.

Cytotoxicity Assays

MDA-MB-453, BT549, and Hs578T cells, their derivative cell lines (transfected with pLVTHM^{DNMT3b} or pLVTHM^{SCRAM}) and cells after demethylating treatment were analyzed for sensitivity to DOX, PAX, and 5-FU. Cells were trypsinized, counted, and plated in 24-well plates at a concentration of 50,000 cells per well. After 24 hours, cells were exposed to fresh medium containing a range of doses of chemotherapeutic drugs for 72 hours (DOX, 0-1 μM; PAX, 0-5 nM; 5-FU, 0-6 mM). At the end of the treatment period, the MTT assay was used to quantitate residual viable cells. The stock solution (5 mg/ml) of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Cat # M5655, Sigma-Aldrich) was made in Dulbecco's Phosphate Buffered Saline (Cat # 14190, Gibco/Invitrogen Life Technologies). The drug containing medium was replaced with a 10% solution of MTT in complete growth medium and cells were incubated at 37º C with 5% CO_2 for four hours. The resulting pigment was solubilized with 0.1 N HCl in isopropanol and absorbance readings were taken at 570 nm (minus background absorbance at 690 nm). All the assays were performed in triplicate. The result of each treatment was expressed as a percentage of viable cells remaining relative to untreated control cells.

Determination of Chemotherapeutic Drug Efficacy

The half maximal inhibitory concentration (IC_{50}) for each drug was estimated using GraphPad Prism Version 5.00 (GraphPad Software, San Diego, CA) [228]. Data points were fitted to sigmoidal dose-response curve with variable slope using Y= 100/[1+10^{(LogIC50-X)*HillSlope)}] where X is the logarithm of concentration and Y is the response (GraphPad Prism Version 5.00). All IC_{50} values expressed in this study reflect 72 hrs of drug treatment.

DNMT3b Protein Analysis by Western Blotting

Cultured cells (corresponding to MDA-MB-453, Hs578T or BT549 breast cancer cells) and their derivative cell lines (transfected with $pLVTHM^{DNMT3b}$ or $pLVTHM^{SCRAM}$) were lysed in 1x phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4) containing 0.1 mM phenylmethanesulphonylfluoride (PMSF), 1 µg/ml pepstain A, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and 0.1% Triton X-100. Cell lysates were utilized for western analysis using standard methods. Protein concentrations were determined using the Bradford assay (BIO-RAD Quick Start Bradford, Cat. # 500-0205). Protein lysates (20 – 40 µg) were resolved on 8% SDS-PAGE gels, followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Cat. #162-0184, BIO-RAD Sequi-Blot PVDF, 0.2 µM pore size, Millipore; Billerica, MA). PVDF membranes were blocked for 30 minutes in TBST (10 mM Tris-Cl, pH 7.6, 150 nM NaCl, 1% Tween-20) containing 5% milk, and then incubated with either anti-DNMT3b mouse monoclonal antibody (Cat # IMG-184A Imgenex, San Diego, CA) diluted 1:5000 or anti-actin rabbit polyclonal antibody diluted 1:10,000 (Cat # sc-1616 Santa Cruz Biotechnology, Santa Cruz, CA) overnight in TBST containing 1% milk. Subsequently, membranes were washed with TBST 3 times for 5 minutes, and then incubated

with a sheep anti-mouse (1:5000, Cat # NA931 GE Healthcare; Piscataway, NJ) or donkey anti-rabbit (1:10,000, Cat # NA934 GE Healthcare; Piscataway, NJ) horseradish peroxidase-conjugated secondary antibody in TBST containing 1% milk for 1 hour at room temperature. The membranes were washed with TBST 3 times for 10 minutes each, and bound primary antibody was detected using ECL-Plus substrate (GE Healthcare; Piscataway, NJ).Western blots were quantitated using the image analysis tools contained in Adobe Photoshop 6.0. The absolute intensity of individual protein bands was calculated from measures of total pixel count and mean pixel density. Levels of DNMT3b protein were expressed relative to actin.

Expression Analysis of Methylation-Sensitive Genes

RNA was prepared from breast cancer cell lines, their transfected derivative cell lines (carrying pLVTHM^{DNMT3b} or pLVTHM^{SCRAM}), and cells following demethylating treatment (7 days treatment with 250 nM or 500 nM 5-aza). Total RNA was isolated utilizing the method of Chomczynski and Sacchi [229], modified to utilize TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. Nucleic acid samples were DNAse (Cat # M610A; Promega, Madison, WI) treated (0.02 U/µl at 37ºC for 30 minutes), and purified using the Qiagen RNeasy mini-kit (Cat # 74104; Qiagen, Valencia, CA). Isolated RNA was quantified after extraction using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Gene expression analysis was accomplished by real-time PCR utilizing an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Total RNA samples (2 µg) were reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Part # 4368814 Applied Biosystems) according to the manufacturer's protocol. Real-time primers

and probes for *CEACAM6* (Hs00366002_m1), *CST6* (Hs00154599_m1), *ESR1* (Hs00174860_m1), *SCNN1A* (Hs00168906_m1), and *β-actin* (Hs99999903_m1) were purchased from Applied Biosystems. All real-time PCR reactions were performed in triplicate using TaqMan Universal PCR Master Mix (Cat # 4324018, Applied Biosystems) in 20 µl volume (10 µl Taqman Universal PCR Master Mix, 1.0 µl TaqMan Real-time primers and probes, and 9 µl cDNA and nuclease-free water) and the following amplification conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were normalized using *β-actin* for each cell line and differences in gene expression were determined using the comparative Ct method described in the ABI Prism 7700 User Bulletin #2 (Applied Biosystems).

Methylation-Specific PCR Analysis

Genomic DNA from $2x10^6$ cultured cells was isolated using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, PA). Bisulfite modification of genomic DNA was performed using a procedure adapted from Grunau *et al* [230]. Genomic DNA (3 µg) was digested with one unit of Xho I (New England Biolabs, Beverly, MA) overnight in 12 µl total volume and heat inactivated at 65°C for 20 minutes; 5 µl of digest was subjected to bisulfite modification. Briefly, approximately 1 µg of DNA in 45 µl of distilled water was denatured by adding 5 µl 3 M NaOH and incubating for 20 minutes at 42°C, followed by addition of 450 µl of sodium bisulfite solution (saturated sodium bisulfite, 10 mM hydroquinone, pH 5.0) and incubation at 55°C for 4 hours. Bisulfite-modified DNA (500 µl) was purified using the Wizard DNA Clean-Up kit (Promega, Madison, WI), reconstituted with 50 µl of 1 mM Tris-Cl (pH 8.0) and desulfonated by addition of 5.5 µl 3 M NaOH and

incubation at 37°C for 20 minutes. The solution was neutralized by adding 40 µl 7.5 M ammonium acetate and precipitated with 100% ethanol at -20°C for at least 30 minutes. The DNA pellet was washed with 70% ethanol, dried briefly, and resuspended in 20 µl 1 mM Tris-Cl (pH 8.0). Bisulfite-converted DNA was used to conduct methylation-specific PCR (MSP) using primers that were previously described or designed using Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and directed to specific segments of the promoter regions and exon 1 of *CST6* [129, 150]*, ESR1* [150, 231], and *SCNNIA*. The MSP primer sequences and thermocycling conditions for *CST6, ESR1,* and *SCNNIA* genes are given in **Table 2.1**. PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis

The values for the mean and standard error of the mean (S.E.M.) were calculated using the statistical function of Microsoft Excel 2007. Statistical significance was determined using an unpaired t-test. Error bars depicted in bar graphs and/or indicated in tables represent S.E.M. of three independent experiments.

Methods Related to MicroRNA Experiments in Breast Cancer Cell Lines and Primary Breast Cancers

Cell Lines and Growth Conditions

Human breast cancer cell lines BT20 (ATCC# HTB19), BT549 (HTB122), Hs578T (HTB126), MCF7 (HTB22), MDA-MB-231 (HTB26), MDA-MB-415 (HTB128), MDA-MB-435S (HTB129), MDA-MB-436 (HTB130), MDA-MB-453 (HTB131), MDA-MB-468 (HTB132), SKBR3 (HTB30), and ZR-75-1 (CRL-1500) were obtained from

the Tissue Culture Core Facility of the University of North Carolina Lineberger Comprehensive Cancer Center (Chapel Hill, NC). Human breast cancer cell lines SUM102, SUM149, and SUM185 were a gift from the laboratories of Dr. Carolyn I. Sartor (Department of Radiation Oncology, UNC School of Medicine, Chapel Hill, NC) and Dr. Stephen Ethier (Department of Pathology, Wayne State University School of Medicine, Detroit, MI). Human breast cancer cell line HCC1937 **(**CRL-2336**)** was a gift from the laboratory of Dr. William K. Kaufmann (Department of Pathology and Laboratory Medicine, UNC School of Medicine, Chapel Hill, NC). The normal breast epithelial cell line MCF12A (CRL-10782) was obtained from the ATCC (American Type Culture Collection, (http://www.atcc.org/). Cell lines were propagated in growth medium recommended by the ATCC, except for SUM102, SUM149, and SUM185 cells which were cultured in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12, Gibco/Invitrogen Life Technologies, Carlsbad, CA) medium supplemented with 10% horse serum (Gibco/Invitrogen Life Technologies), and 1% Antibiotic-Antimycotic (Gibco/Invitrogen Life Technologies). Growth medium was refreshed three times weekly unless otherwise specified for antagomir and pre-miR transfections. Cells were maintained at 37°C and 5% CO_2 (except for MDA-MB-468 which was propagated in 100% atmospheric air).

Human Breast Tissue: **Primary Breast Cancers and Normal Mammoplasty Tissue**

70 paraffin-embedded human primary breast tumors and 18 normal mammoplasty tissues were obtained from the paraffin archives of the UNC Lineberger Comprehensive Cancer Center at the University of North Carolina (Chapel Hill, NC) with the assistance of

Dr. Chad A. Livasy (Carolinas Medical Center, Charlotte, NC). The primary breast cancers included 36 luminal A, 13 luminal B, 5 HER2+, and 16 basal-like tumors. Determination of molecular subtype was accomplished by immunohistochemistry for ER, PR, HER2, CK5/6, and EGFR. Protection of patient privacy and handling of specimens followed strict policies of the Institutional Review Board of the University of North Carolina.

RNA Extraction from Cell Lines for Gene Expression Analysis

Total RNA for gene expression analysis and miR expression analysis was isolated from breast cancer cell lines, MCF12A (normal mammary epithelial cell line), and transfected cell lines (antagomir or pre-miR transfected) utilizing the method of Chomczynski and Sacchi [229] modified for TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. Nucleic acid samples were DNAse (Cat # M610A; Promega, Madison, WI) treated (0.02U/µl at 37ºC for 30 minutes), and purified using the Qiagen RNeasy mini-kit (Cat # 74104; Qiagen, Valencia, CA). Isolated RNA was quantified after extraction using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RNA Isolation for Real Time Analysis from Tumors and Normal Mammoplasty Sections

The paraffin blocks were blinded (in terms of molecular subtypes) before selection for analysis and up to 35 mg of unsectioned core samples were obtained from the Translational Pathology Laboratory Core Facility at Department of Pathology and Laboratory Medicine, UNC School of Medicine, Chapel Hill. H&E slides from all the paraffin blocks

were initially analyzed to select the areas of the blocks to be cored. This selection for the cancer blocks ensured that the cores consisted of cancer tissue, similarly for normal breast tissue blocks, it ensured that the cores consisted of normal breast epithelium (and not stroma/fat). Total RNA was isolated from breast cancers and normal breast epithelium using Recover All™ Total Nucleic Acid Isolation Kit for FFPE according to the manufacturer's instructions (Part # 1975, Applied Biosystems). The cores were crushed and ground in liquid nitrogen, and then deparaffinized using a series of washes with slide brite (instead of xylene) and ethanol. Pulverized tissues were suspended in 1ml of slide brite, vortexed briefly to mix and heated for 3 min at 50°C to melt the paraffin. The samples were centrifuged to pellet the tissue; slide brite was removed without disturbing the pellet and the pellet was subjected to two washes with 100% ethanol. After discarding the ethanol after second wash, the samples were subjected to additional centrifugation to get rid of any additional ethanol. The pellet was air-dried before digesting it in digestion buffer and protease at 50°C for three hours. Isolation additive was added to the samples before vortexing and mixing with 100% ethanol. The samples were loaded on to filter cartridges placed in collection tubes and centrifuged to pass the mixture through the filter. The flow through was discarded. Wash 1 and wash 2/3 provided in the kit were used to wash through the filter. The filter assembly was centrifuged for additional 30 seconds to remove residual fluid from the filter. DNAse mix (10X DNAse buffer, DNAse, and nuclease-free water) was added to the center of each filter cartridge and incubated for 30 min at room temperature followed by one wash with wash 1 and two washes with wash 2/3. The filter assembly was centrifuged at 10,000 g for an additional minute to remove residual fluid from the filter. The elution solution was pre-heated to 95°C and added to the center of the filter. After letting it sit for at least one minute, the RNA was eluted in

fresh collection tube by centrifuging at maximum speed for one minute. The elution steps were repeated one more time to maximize the yield. Nucleic acid samples were purified using the Qiagen RNeasy mini-kit (Cat # 74104; Qiagen, Valencia, CA). Isolated RNA was quantified after extraction using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

MicroRNA Expression Analysis

We identified candidate miRs as potential regulators of *DNMT3b* using the computational tools of target prediction programs and resources from publicly available databases, including Miranda (http://www.microRNA.org/), TargetScan (http://www.targetscan.org/vert_42/), miRGen (http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html), PicTar (http://pictar.mdc-berlin.de/), and miRBase (http://microrna.sanger.ac.uk/sequences/) computing for target predictions based on searches using Gene symbol DNMT3b (Entrez Gene ID 1789 and Ensembl Gene ID ENSG00000088305). Based on high stringency *in-silico* selection criteria that included PicTar score (indicative of HMM maximum likelihood fit), highly conserved miRs, and good mirSVR scores (indicative of seed-site pairing, site context, free-energy, and conservation), we identified 25 additional miRs that potentially target DNMT3b **(Figure 2.1**). We prioritized the candidate miRs based on the available literature and/or their recognition as potential candidates by multiple target prediction programs **(Figure 2.1)**. miRs that were differentially expressed among breast cancer cells in primary tumors [216] and cell lines [232] were considered for further analysis. Based upon this computational analysis, we

selected nine miRs for examination: miR-29a, miR-29b, miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, miR-203, and miR-222 **(Figure 2.1)**.

miR expression analysis was accomplished by real-time PCR utilizing an ABI 7500 Real Time PCR System (Applied Biosystems) according to TaqMan miRNA assay protocol (Applied Biosystems). Total RNA samples (10 ng) were reverse transcribed using the TaqMan MiRNA Reverse Transcription Kit (Part # 4366596 Applied Biosystems) and TaqMan miRNA specific primers (Applied Biosystems) according to the manufacturer's protocol. Real-time primers and probes for miR-29a (Assay ID 000412), miR-29b (Assay ID 000413), miR-29c (Assay ID 000415), miR-148a (Assay ID 000470), miR-148b (Assay ID 000471), miR-26a (Assay ID 000405), miR-26b (Assay ID 000407), miR-203 (Assay ID 000507), miR-222 (Assay ID 002276), and RNU66 (Assay ID 001002) were purchased from Applied Biosystems. These assays specifically detect mature miRNAs (not pre-miRNAs). All real-time PCR reactions were performed in triplicate using TaqMan Universal PCR Master Mix (Cat # 4324018, Applied Biosystems) in 20 µl volume containing 10 µl TaqMan Universal PCR Master Mix, 1 µl of primers and probe mix of the miR-specific TaqMan MicroRNA Assay (Applied Biosystems), 1.33 µl of RT product, and 7.67 µl of nuclease free water and the following amplification conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression levels for each miR were calculated based upon the expression of RNU66 and differences in gene expression were determined relative to MCF-12A using the comparative Ct method described in the ABI Prism 7700 User Bulletin #2 (Applied Biosystems).

Gene Expression Analysis

Gene expression analysis was accomplished by real-time PCR utilizing an ABI 7500 Real Time PCR System (Applied Biosystems). Total RNA samples (2 µg) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Part # 4368814 Applied Biosystems) according to the manufacturer's protocol. Real-time primers and probes for *CEACAM6* (Hs00366002_m1), *CDH1* (Hs00170423_m1), *CST6* (Hs00154599_m1), *DNMT3b (*Hs00171876_m1), *ESR1* (Hs00174860_m1), *GNA11* (Hs01588833_m1), *MUC1* (Hs00159357_m1), *MYB* (Hs00920554_m1), *SCNN1A* (Hs00168906_m1), *TFF3* (Hs00173625_m1), and *β-actin* (Hs99999903_m1) were purchased from Applied Biosystems. All real-time PCR reactions were performed in triplicate using TaqMan Universal PCR Master Mix (Cat # 4324018, Applied Biosystems) in 20 µl volume (10 µl TaqMan Universal PCR Master Mix, 1.0 µl TaqMan Real-time primers and probes, and 9 µl cDNA and nuclease-free water) and the following amplification conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression levels for each gene were calculated based upon the expression of *β-actin* for each cell line and differences in gene expression were determined relative to MCF-12A in the breast cancer cell lines and to normal breast tissue from reduction mammoplasties for primary tumors using the comparative Ct method described in the ABI Prism 7700 User Bulletin #2 (Applied Biosystems).

DNMT3b Protein Expression in Breast Cancer Cell Lines

Cultured breast cancer cell lines, MCF12A (normal mammary epithelial cell line), and transfected cell lines (antagomir or pre-miR transfected) were lysed in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4)

containing 0.1 mM phenylmethanesulphonylfluoride, 1 µg/ml pepstain A, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, and 0.1% Triton X-100. Cell lysates were utilized for western analysis using standard methods as described above in the methods related to chemotherapy experiments.

Breast Cancer Cell Line Transfection with Pre-miRs

Hypermethylator cell lines Hs578T, HCC1937, and SUM185 were selected for pre-miR transfection with miR-148b, miR-26b, and miR-29c. These cell lines exhibit DNMT hyperactivity, express DNMT3b at high levels [150], and have negligible levels of expression of miR-26b, miR-29c, and miR-148b. All pre-miR transfections were performed in triplicate. Pre-miR miRNA precursors (miR-148b, PM10264; miR-26b, PM12899; and miR-29c, PM10518) and standard control oligomers were obtained from Applied Biosystems. For optimization purposes, the Pre-miR miRNA Precursor Starter Kit (Applied Biosystems) was utilized for the reverse transfection procedure according to manufacturer's protocol using siPORT *NeoFX* Transfection Agent (Part # AM4510, Applied Biosystems). Four concentrations of transfection reagent (9µl, 12 µl, 15 µl, and 18 µl) were tested to obtain optimum conditions for pre-miR transfections for each cell line. Transfection reagent was diluted to 300 µl with opti-MEM (Gibco/Invitrogen Life Technologies), incubated for 10 minutes at room temperature, 24 µl of 6.25 nM of Pre-miR hsa-miR-1 miRNA precursor or Pre-miR negative control #1 was diluted to 300 µl with opti-MEM for final concentration of 50 nM and gently mixed with diluted transfection agent before incubating for 10 minutes at room temperature. The transfection complexes were dispensed into 6-well culture plates, and nontransfected controls were set up in parallel. 2.4 x 10^5 cells were transferred in 2.4 ml of

growth medium per well and incubated at recommended growth conditions. After 24 hrs, the culture medium was replaced with fresh normal growth medium. Two days after transfection, total RNA was extracted from transfected and control cells. The expression level of *PTK9* mRNA (target of Pre-miR miR-1 miRNA precursor) was assessed by real-time PCR (Hs00702289_s1, Applied Biosystems) according to the manufacturer's instructions. Optimal transfection was observed with 12 µl transfection reagent in each cell line, producing 75-90% reduction of *PTK9* mRNA after transfection with Pre-miR miR-1. Hs578T, SUM185, and HCC1937 cells were transfected with pre-miR precursors for miR-148b, miR-26b, and miR-29c employing the optimized conditions. After 48 hours, total RNA was harvested for real-time PCR analysis for miR and gene expression analyses. In addition, transfected and control cells were lysed for western blot analysis (as described above).

Breast Cancer Cell Line Transfection with Antagomirs

Non-hypermethylator cell lines BT20, MDA-MB-415, and MDA-MB-468 were selected for antagomir transfection with miR-148b, miR-26b, and miR-29c. These cell lines have lower DNMT activity, express DNMT3b at low levels [150], and normal levels of expression of miR-26b, miR-29c, and miR-148b. All antagomir transfections were performed in triplicate. Antagomirs (miR-148b, AM10264; miR-26b, AM12899; and miR-29c, AM10518) and standard control oligomers were obtained from Applied Biosystems. For optimization of transfection conditions, the reverse transfection procedure was performed using four concentrations of transfection reagent (9µl, 12 µl, 15 µl, and 18 µl), as described for pre-miR transfections. Transfection reagent was diluted to 300 µl with opti-MEM (Gibco/Invitrogen Life Technologies), incubated for 10 minutes at room temperature, 24 µl

of 6.25 nM of Anti-miR let-7c miRNA inhibitor positive control or Anti-miR negative control #1 was diluted to 300 µl with opti-MEM for final concentration of 50 nM and gently mixed with diluted transfection agent before incubating for 10 minutes at room temperature. Levels of *HMGA2* mRNA (target of Anti-miR let-7c miRNA inhibitor positive control) were assessed by real-time PCR (Hs00171569_m1, Applied Biosystems) after RNA extraction. Optimal transfection was observed with 12 µl transfection reagent in each cell line, producing 1.8-fold to 2.4-fold increases in *HMGA2* mRNA after transfection with Anti-miR let-7c miRNA inhibitor. BT20, MDA-MB-415, and MDA-MB-468 cells were transfected with antagomirs for miR-148b, miR-26b, and miR-29c and after 48 hours, total RNA was harvested for real-time PCR analysis for miR and gene expression analyses. In addition, transfected and control cells were lysed for western blot analysis (as described above).

Statistical Analysis

The values for the mean and standard error of the mean (S.E.M.) were calculated using the statistical function of Microsoft Excel 2007. Statistical significance was determined using an unpaired t-test (two-tailed). Error bars depicted in bar graphs represent S.E.M. of 3-6 independent experiments.

Table 2.1. *Methylation-Specific PCR Primer Sequences and Thermocycling Conditions For CST6, ESR1 and SCNNIA Genes*

Gene	Methylated	Unmethylated	Product Size	PCR Conditions
CST6	F: TCGAGTTTCGTTTTAGTTTTAGGTC R: CATAACCGTCAATACCGTCG	F :TGAGTTTTGTTTTAGTTTTAGGTT R: CCATAACCATCAATACCATCAA	135	U:TM = 55º 38 cycles M:TM = 60º 38 cycles
ESR1	F: GATACGGTTTGTATTTTGTTCGC R: CGAACGATTCAAAAACTCCAACT	F : GGATATGGTTTGTATTTGGTTTGT R: ACAAACAATTCAAAAACTCCAACT	123	TM = 58º 35 cycles
SCNN1A	F: TTTTTTAGTTTTTTTGTTTGTTTGC R: CTCACTATCGCGAAAACGAC	F : TTAGTTTTTTTGTTTGTTTGTGT R: AAAATCAAAACCAAAAATTTTCCA	127	TM = 55º 38 cycles

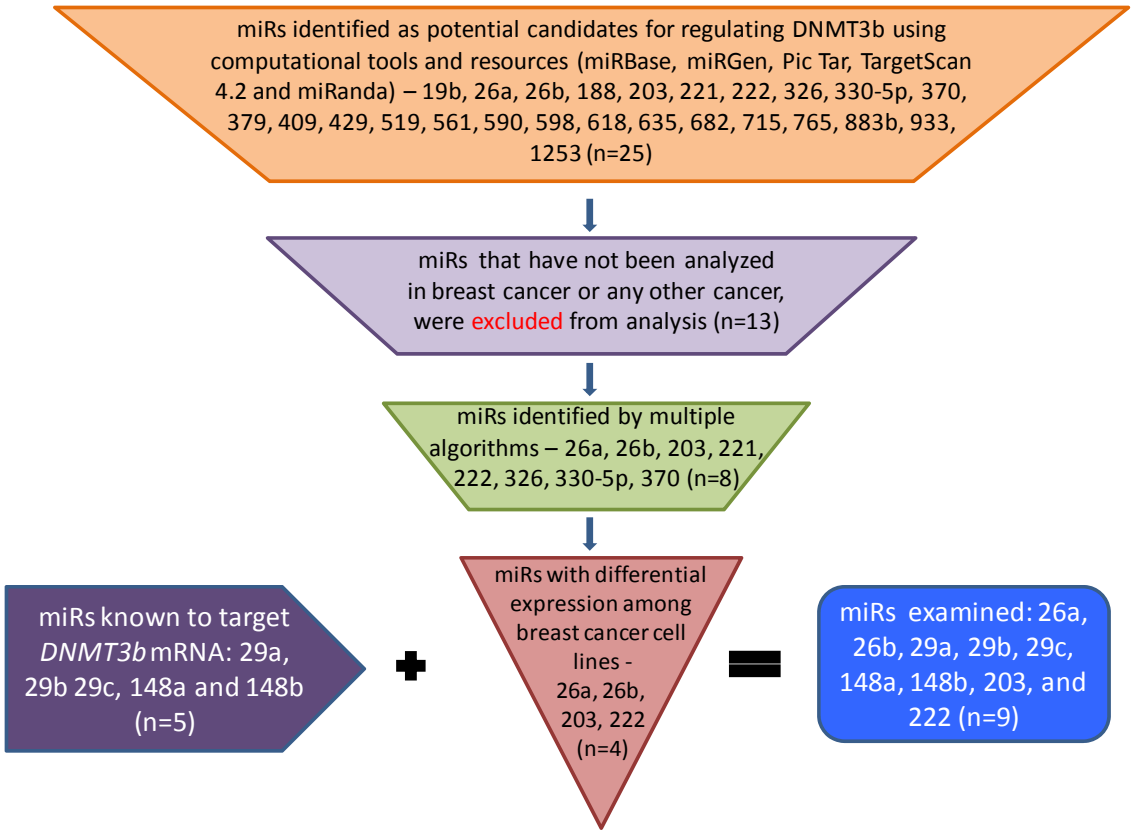

miRs identified as potential candidates for regulating DNMT3b using computational tools and resources (miRBase, miRGen, Pic Tar, TargetScan 4.2 and miRanda) – 19b, 26a, 26b, 188, 203, 221, 222, 326, 330-5p, 370, 379, 409, 429, 519, 561, 590, 598, 618, 635, 682, 715, 765, 883b, 933, 1253 (n=25)
miRs that have not been analyzed in breast cancer or any other cancer, were excluded from analysis (n=13)
miRs identified by multiple algorithms – 26a, 26b, 203, 221, 222, 326, 330-5p, 370 (n=8)
miRs known to target DNMT3b mRNA: 29a, 29b 29c, 148a and 148b (n=5)
miRs with differential expression among breast cancer cell lines - 26a, 26b, 203, 222 (n=4)
miRs examined: 26a, 26b, 29a, 29b, 29c, 148a, 148b, 203, and 222 (n=9)

Figure 2.1. *miR Regulators of DNMT3b mRNA*. Schematic illustrating the selection and prioritization of miR regulators of *DNMT3b* for analysis. Several target prediction programs were utilized to predict miR interactions with *DNMT3b*. Criteria for filtering potential candidates are described in the schematic. In addition to selection of candidate miR regulators, known regulators of *DNMT3b* were identified from the literature. This selection strategy yielded nine miRs for examination: miR-29a, miR-29b, miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, miR-203, and miR-222.

RESULTS

Results Related to Chemotherapy Experiments

Chemosensitivity of Wild-Type Breast Cancer Cell Lines

The IC_{50} for DOX, PAX, and 5-FU have been reported for the breast cancer cell lines used in this study [233-235]. However, these IC_{50} values vary widely from laboratory to laboratory and with specific experimental conditions. Hence, we ascertained the IC_{50} for the drugs of interest and in the selected cell lines for the employed experimental conditions to establish a solid baseline for comparison. We found the MDA-MB-453 cells to be most sensitive to these chemotherapeutic drugs and the Hs578T cells to be most resistant **(Table 3.1)**. In response to DOX, MDA-MB-453 and BT549 cells exhibited similar levels of sensitivity, whereas Hs578T cells were relatively more resistant, displaying >2-fold higher IC_{50} compared to the other cell lines (2.7-fold greater than MDA-MB-453, 2.3-fold greater than BT549). Hs578T and BT549 were more resistant to PAX than MDA-MB-453 cells (IC_{50} 5.2-fold and 4-fold higher than MDA-MB-453). Hs578T cells displayed a 1.3-fold higher IC_{50} in response to PAX compared to BT549 cells. In response to 5-FU, BT549 and Hs578T cells display similar levels of resistance with 1.4-fold and 1.5-fold higher IC_{50} compared to MDA-MB-453 cells, respectively.

Demethylating Treatment Sensitizes Hypermethylator Breast Cancer Cells to Chemotherapeutic Drugs

Hypermethylator breast cancer cell lines are characterized by high rates of concurrent methylation-dependent silencing of multiple methylation-sensitive genes (including *CEACAM6, CST6, ESR1,* and *SCNN1A*) [150]. Expression of epigenetically-regulated genes following demethylating treatment with 5-aza was monitored to ensure that the conditions employed produced a biological effect. We observed re-expression of *CEACAM6, CST6, ESR1,* and *SCNN1A* in all three cell lines following 250 nM and 500 nM 5-aza exposure for 7 days. The re-expression of these methylation-sensitive genes was robust and statistically significant for all four genes in all cell lines with 500 nM 5-aza exposure. In contrast, 250 nM 5-aza exposure resulted in a more modest, yet statistically significant re-expression of all genes in Hs578T cells and statistically significant re-expression of all genes except *CEACAM6* in MDA-MB-453 and BT549 cells. Following 250 nM 5-aza exposure, the MDA-MB-453 cells showed the greatest increase in *CST6* expression (8-fold) followed by *ESR1* (7.9-fold), *SCNNIA* (2.9-fold), and *CEACAM6* (2.2-fold) **(Figure 3.1a)**. In BT549 cells, we observed the highest increase in *SCNN1A* expression (139-fold), followed by *CEACAM6* (23-fold), *CST6* (16-fold), and *ESR1* (4-fold) **(Figure 3.1a)**. Hs578T cells showed the greatest increase in expression of *SCNN1A* (118-fold) followed by *ESR1* (70-fold), *CST6* (54-fold), and *CEACAM6* (23-fold) **(Figure 3.1a)**. Following 500 nM 5-aza exposure, the MDA-MB-453 cells demonstrated highest increase in *CST6* expression (1610-fold) followed by *CEACAM6* (210-fold), *SCNNIA* (186-fold), and *ESR1* (168-fold) **(Figure 3.1b)**. In BT549 cells, we observed the highest increase in *SCNN1A* expression (1370-fold) followed by *CEACAM6* (559-fold), *CST6* (77-fold), and *ESR1* (34-fold) **(Figure 3.1b).** Hs578T cells exhibited highest increase in expression of *CST6* (1099-fold) followed by *SCNN1A* (800-fold), *ESR1* (488-fold) and *CEACAM6* (449-fold) **(Figure 3.1b)**. These results

demonstrate that the conditions for demethylating treatment utilized produced significant alterations in gene expression profile, including re-expression of these methylation-sensitive genes in hypermethylator cells.

Following pretreatment of the breast cancer cell lines with 5-aza, we observed no discernible changes in cell morphology or cell proliferation rate compared to the untreated controls. While pretreatment with 5-aza clearly produced alterations in gene expression, no evidence of 5-aza related toxicity was observed in this study (with either 250 nM or 500 nM 5-aza). To determine if demethylating treatment sensitizes hypermethylator cells to cytotoxic chemotherapy, we compared cell kill after 5-aza pretreatment to that in control cells. The results show that 5-aza pretreatment sensitizes the hypermethylator breast cancer cells to DOX, PAX, and 5-FU, and that the improved sensitivity is a function of dose and duration of exposure to 5-aza. Pretreatment of cells with 250 nM 5-aza or 500 nM 5-aza for 3 days did not increase the sensitivity of breast cancer cells to DOX. Likewise, pretreatment with 250 nM 5-aza for 7 days did not increase sensitivity of MDA-MB-453 and Hs578T cells to any of the drugs evaluated **(Table 3.1)**. However, 7 days pretreatment with 250 nM 5-aza sensitized BT549 cells, reducing the IC_{50} for DOX by 32% **(Figure 3.2a)**, for PAX by 24%, and for 5-FU by 46% **(Table 3.1)**. In contrast, pretreatment with 500 nM 5-aza for 7 days significantly improved the sensitivity of each breast cancer cell line to DOX, PAX, and 5-FU (representative dose-response curves are shown in **Figure 3.2**). Increased sensitivity following pretreatment with 500 nM 5-aza observed with each cell line (and 250 nM 5-aza in BT549) was detected at all the employed doses of three drugs **(Table 3.1)**. MDA-MB-453 cells showed reduction in IC_{50} for DOX (0.086 μM to 0.034 μM; 60% reduction), PAX (0.497 nM to 0.311 nM; 37%), and 5-FU (0.817 mM to 0.065 mM; 90%); BT549 cells

demonstrated reduction in IC_{50} for DOX (0.099 μM to 0.052 μM; 47% reduction), PAX (1.974 nM to 1.015 nM; 48%), and 5-FU (1.183 mM to 0.472 mM; 60%); Hs578T cells also exhibited a reduction in IC_{50} for DOX (0.230 μM to 0.110 μM; 52% reduction, PAX (2.605 nM to 1.466 nM; 43%), and 5-FU (1.211 mM to 0.0.371 mM; 69%). The greatest reduction in IC_{50} (reflecting greatest improvement of sensitivity, with 5-aza treatment) was observed with 5-FU (60-92%), followed by DOX (47-60%), and PAX (37-48%) **(Table 3.1)**.

Increased drug sensitivity following pretreatment with 250 nM 5-aza was observed only in BT549 cells. This responsiveness appears to correlate with the levels of DNMT3b expressed in BT549 cells compared to MDA-MB-453 and Hs578T cells. Previous studies established that among these breast cancer cell lines, MDA-MB-453 cells exhibit the highest levels of DNMT3b protein and BT549 cells express the lowest levels [150]. These observations suggest that 250 nM 5-aza was sufficient to target the comparatively lower levels of DNMT3b seen in BT549 cells **(Figure 3.2a),** but was insufficient to exert significant effects in MDA-MB-453 and Hs578T cells with higher DNMT3b levels **(Figure 3.2b-c; Table 3.1)**.

DNMT3b Knockdown Sensitizes Hypermethylator Breast Cancer Cell Lines to Chemotherapeutic Drugs

DNMT3b protein levels in wild-type breast cancer cell lines and transfected cell lines (transfected with $pLVTHM^{DNMT3b}$ or $pLVTHM^{SCRAM}$) were evaluated by western blot analyses. Significant levels of DNMT3b protein were detected for each of the wild-type breast cancer cell lines **(Figure 3.3a)**. MDA-MB-453 cell lines expressed the highest levels of DNMT3b protein followed by Hs578T cells, and BT549 cells expressed lowest levels of

DNMT3b protein **(Figure 3.3b)**. Transfection with pLVTHM^{SCRAM} had no effect on DNMT3b expression levels, in contrast, each of the DNMT3b KD cell lines demonstrated significant alteration of DNMT3b protein levels, reflecting >90% knockdown **(Figure 3.3c)**. Subsequently, wild-type breast cancer cell lines, scrambled controls, and DNMT3b KD cells were examined for expression of methylation-sensitive genes (*CEACAM6, CST6, ESR,* and *SCNN1A*). Gene expression levels for each of these genes were low in the wild-type breast cancer cell lines and in scrambled-control cell lines. However, each of these methylation-sensitive genes was found to be re-expressed in DNMT3b KD cell lines **(Figure 3.4)**. The MDA-MB-453 DNMT3b KD cells had statistically significant re-expression of *SCNN1A* (6-fold), and *ESR1* (6-fold) **(Figure 3.4a)**. In BT549 DNMT3b KD cells, we observed statistically significant re-expression of *CST6* (13-fold)*, ESR1* (6-fold)*,* and *SCNN1A* (4-fold) **(Figure 3.4b)**. Hs578T DNMT3b KD cells exhibited statistically significant re-expression of *CST6* (310-fold)*, ESR1* (261-fold)*, SCNN1A* (30-fold), and *CEACAM6* (17-fold) **(Figure 3.4c)**. Methylation-specific PCR analysis showed changes in the methylation status of select methylation-sensitive genes after knocking down expression of DNMT3b. We observed that MDA-MB-453 cells contain only methylated alleles for *CST6, ESR1,* and *SCNN1A* **(Figure 3.5)**, whereas MDA-MB-453 cells after DNMT3b knockdown contain both methylated and unmethylated alleles **(Figure 3.5)**. Comparable results were seen in Hs578T cells and Hs578T cells after DNMT3b knockdown **(Figure 3.5)**. BT549 cells and BT549 cells after DNMT3b knockdown showed similar results for *CST6* **(Figure 3.5)** and *ESR1*. These observations combine to suggest that DNMT3b KD in these breast cancer cell lines partially or completely corrects the hypermethylation defect resulting in alteration of gene-specific promoter methylation and re-expression of methylation-sensitive genes.

To evaluate the effects of DNMT3b KD on the sensitivity of breast cancer cells to standard drug regimens, we compared the response to chemotherapeutics in wild-type hypermethylator breast tumor cells to the response in cells after RNAi-mediated knockdown of DNMT3b **(Figure 3.6)**. Scrambled control cell lines exhibited IC_{50} values for each of the drugs tested that were comparable to values for the wild-type breast cancer cell lines **(Table 3.1)**. In MDA-MB-453 DNMT3b KD cells, we observed 44% reduction in DOX IC_{50} (0.086 μM to 0.048 μM), 24% reduction for PAX (0.497 nM to 0.376 nM) **(Figure 3.6b)**, and 82% reduction for 5-FU (0.817 mM to 0.145 mM) **(Table 3.1)**. In BT549 DNMT3b KD cells, we observed 13% reduction in DOX IC_{50} (0.099 μM to 0.086 μM) **(Figure 3.6a)**, 16% reduction for PAX (1.974 nM to 1.660 nM), and 33% reduction for 5-FU (1.183 mM to 0.791 mM) **(Table 3.1)**. Hs578T DNMT3b KD cells exhibited 32% reduction in IC_{50} for DOX (0.230 μM to 0.155 μM), 29% reduction for PAX (2.605 nM to 1.839 nM), and 53% reduction for 5-FU (1.211 mM to 0.562 mM) **(Table 3.1, Figure 3.6c)**. Overall, the greatest reduction in IC_{50} was observed with 5-FU (33-82%), followed by DOX (13-44%), and PAX (16-29%) **(Table 3.1)**. These results demonstrate that knocking down DNMT3b in hypermethylator breast cancer cells increases the effectiveness of chemotherapeutic drugs.

5-Aza Enhances Chemosensitivity in Breast Cancer Cell Lines After DNMT3b Knockdown

To analyze the combined effects of DNMT3b KD and demethylating treatment, we subjected the DNMT3b KD cell lines to 250 nM and 500 nM 5-aza for 7 days and then evaluated cell kill after exposure to chemotherapeutic drugs **(Figure 3.7)**. We observed increased sensitivity of DNMT3b KD cells to DOX, PAX, 5-FU following 500 nM 5-aza

pretreatment for 7 days **(Table 3.1)**. However, 250 nM 5-aza pretreatment for 7 days led to increased sensitivity to chemotherapeutics only in BT549 DNMT3b KD cells **(Table 3.1, Figure 3.7a)**. MDA-MB-453 DNMT3b KD and Hs578T DNMT3b KD cells exhibited comparable IC_{50s} with/without pre-treatment with 250 nM 5-aza for 7 days. In MDA-MB-453 DNMT3b KD cells, we observed 59% reduction in DOX IC_{50} (0.086 μM to 0.035 μM), 37% reduction for PAX (0.497 nM to 0.313 nM), and 92% reduction for 5-FU (0.817 mM to 0.067 mM) **(Figure 3.7c)** post-5-aza exposure **(Table 3.1)**. In BT549 DNMT3b KD cells, we observed 32% reduction for DOX IC_{50} (0.065 μM to 0.086 μM), 23% reduction in PAX (1.974 nM to 1.527 nM), and 46% reduction for 5-FU (1.183 mM to 0.632 mM) after 250 nM 5-aza exposure for 7 days. However, with 500 nM 5-aza exposure for 7 days, BT549 DNMT3b KD cells demonstrated 45% reduction in DOX IC_{50} (0.099 μM to 0.054 μM) **(Figure 3.7a)**, 45% reduction for PAX (1.974 nM to 1.082 nM), and 60% reduction for 5-FU (1.183 mM to 0.470 mM) **(Table 3.1)**. In Hs578T DNMT3b KD cells, there was a 51% reduction in IC_{50} for DOX (0.230 μM to 0.111 μM), 53% reduction for PAX (2.605 nM to 0.573 nM) **(Figure 3.7b)**, and 70% reduction for 5-FU (1.211 mM to 0.359 mM) drugs after 500 nM 5-aza pre-treatment **(Table 3.1)**. These results demonstrate that pre-treatment of DNMT3b KD breast cancer cells with demethylating agents increases the effectiveness of chemotherapeutic drugs. Interestingly, the increased sensitivity observed after knocking down DNMT3b in these cell lines and further treating them with 500 nM 5-aza (and 250 nM 5-aza in BT549 cells) was comparable to the increased sensitivity seen in wild-type cells after demethylating treatment alone (representative examples are shown in **Figure 3.8**). This effect may reflect the targeting of other DNA methyltransferases (DNMT1 and/or DNMT3a)

by 5-aza. In addition, this effect could be related to 5-aza-mediated inhibition of residual DNMT3b activity.

Results Related to MicroRNA Experiments in Breast Cancer Cell Lines

Hypermethylator Breast Cancer Cell Lines Express Diminished Levels of Regulatory miRs

Previous investigations identified a hypermethylation defect in a subset of breast cancer cell lines [150]. Hypermethylator cell lines display DNMT hyperactivity and overexpression of DNMT3b, in contrast to non-hypermethylator cell lines [150]. In this study, we investigated possible molecular mechanisms governing DNMT3b overexpression in hypermethylator cell lines, with a focus on miR-mediated regulation of *DNMT3b*. Hence, we examined the levels of expression of select miRs 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 breast cancer cell lines that differentially express *DNMT3b*. Ten of these cell lines express the hypermethylation defect (BT-549, HS578T, HCC1937, MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-453, SUM102, SUM149, and SUM185) and six are non-hypermethylators (BT-20, MCF-7, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1) ([150, 236] and unpublished observations). Differential levels of miR expression were observed for six of the nine miRs evaluated, including miR-26a, miR-26b, miR-29c, miR-148a, miR-148b, and miR-203 **(Figure 3.9)**. While there was variability in expression among the miRs examined, in general the hypermethylator cell lines expressed diminished levels compared to the non- hypermethylator cell lines **(Figure 3.10a-f)**. miR-29a, miR-29b, and miR-222 did not display the pattern of expression observed with the majority of miRs. miR-29a and miR-29b were expressed at similar levels among breast

cancer cell lines irrespective of their methylation status. The lack of differential expression of these miRs is evident from a comparison of average levels in hypermethylator and non-hypermethylator cell lines **(Figure 3.9)**. In contrast to the pattern observed with other miRs, the average expression of miR-222 among hypermethylator cell lines was higher than in non-hypermethylator cell lines. This is consistent with the suggestion that miR-222 functions as an oncogenic miR [237, 238].

The average expression of miR-148a, miR-148b, miR-26a, and miR-26b among hypermethylator cell lines was significantly diminished compared to the average expression of these miRs among non-hypermethylator cell lines ($p<0.05$) **(Figure 3.9)**. 10/10 (100%) of hypermethylator cell lines expressed low levels of miR-148b, and 5/6 (83%) of non-hypermethylator cell lines express higher levels of miR-148b (except BT20; **Figure 3.10c**). Likewise, miR-148a is expressed at low levels in 9/10 (90%) of hypermethylator cell lines (except MDA-MB-453) and the majority of non-hypermethylator cell lines (5/6, 83%) express miR-148a at higher levels (except MCF7; **Figure 3.10b**). 8/10 (80%) of hypermethylator cell lines display low levels of miR-26a expression (except Hs578T and MDA-MB-453), whereas all non-hypermethylator cell lines (6/6, 100%) express higher levels of miR-26a **(Figure 3.10d)**. Similarly, 9/10 (90%) hypermethylator cell lines express low levels of miR-26b (except MDA-MB-453), and 5/6 (83%) of non-hypermethylator cell lines express higher levels of miR-26b (except BT20; **Figure 3.10e**). Differences in average expression of miR-29c and miR-203 in hypermethylator cell lines versus non-hypermethylator cell lines were not statistically significant **(Figure 3.9)**, although there was a distinct trend towards lower expression in the hypermethylator cell lines ($p=0.15$ and $p=0.19$). 6/10 (60%) of hypermethylator cell lines expressed low levels of miR-29c (except

MDA-MB-231, MDA-MB-436, MDA-MB-453, and BT549) and 5/6 (83%) of non-hypermethylator cell lines demonstrated higher levels of miR-29c (except MCF7; **Figure 3.10a**). The expression of miR-203 was low in both hypermethylator and non-hypermethylator cell lines, but with differential expression levels **(Figure 3.10f)**. 7/10 (70%) of hypermethylator cell lines expressed miR-203 at low or undetectable levels (except MDA-MB-453, SUM149, and HCC1937), while 5/6 (83%) of non-hypermethylator cell lines expressed miR-203 at easily detectable levels (except SK-BR-3).

Diminished Expression of miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203 Predict Hypermethylator Status Among Breast Cancer Cell Lines

We observed differential expression of miR-26a, miR-26b, miR-29c, miR-148a, miR-148b, and miR-203 among breast cancer cell lines with strong trends towards diminished expression in hypermethylators compared to non-hypermethylator cell lines **(Figure 3.9)**. To evaluate the value of individual miR expression levels in the prediction of the methylation status of a given breast cancer cell line, a Bayesian analysis was performed. Threshold values were determined for each of the differentially expressed miRs using correct assignments (CA) as a guiding principle. These threshold values are indicated in **Figure 3.10a-f**. The expression levels of five miRs emerged as excellent individual predictors of methylator status among breast cancer cell lines: miR-148b (CA=94%), miR-26b (CA=94%), miR-148a (CA= 88%), miR-26a (CA=88%), and miR-203 (CA=81%) **(Table 3.2)**. These miRs individually displayed excellent sensitivity (range: 80-100%) and specificity (range: 83-100%), as well as excellent positive predictive value (PPV range: 89-100%) and negative predictive value (NPV range: 71-100%) **(Table 3.2)**. The best threshold value for miR-29c produced

CA=69% (sensitivity=60%, specificity=83%, PPV=86%, and NPV=56%) **(Table 3.2)**. The remaining miRs displayed poor predictive value for determination of methylation status of breast cancer cell lines **(Table 3.2)**.

miR Expression Patterns and miR Scores for Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines

Six regulatory miRs were chosen for further analysis based on excellent characteristics related to prediction of methylation status (CA, sensitivity, specificity, PPV, and NPV) among hypermethylator and non-hypermethylator breast cancer cell lines, including miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203. miR scores were generated for each breast cancer cell line, reflecting the number of miRs with diminished expression. Hypermethylator breast cancer cell lines frequently express diminished levels of this panel of miRs. 9/10 (90%) of hypermethylator cell lines express $\geq$5 miRs at diminished levels **(Figure 3.11a)**, resulting in higher miR scores. The exception to this is MDA-MB-453, which expresses low levels of miR-148b only **(Figure 3.10c)**. Hence, MDA-MB-453 has a low miR score reflecting higher levels of expression of the majority of miRs examined **(Figure 3.11a)**. Three hypermethylator cell lines (MDA-MB-435s, SUM102, and SUM185) express diminished levels of all six miRs examined **(Figure 3.11a)**. In contrast to the hypermethylator cell lines, non-hypermethylator cell lines typically express the majority of this panel of miRs at higher levels. 5/6 (83%) of non-hypermethylator cell lines express $\geq$5 miRs at higher levels **(Figure 3.11b)**, resulting in lower miR scores. The exception was MCF7, which expresses low levels of miR-29c and miR-148a **(Figure 3.10a-b)**. Three non-hypermethylator cell lines (MDA-MB-415, MDA-MB-468, and ZR-75-1)

expressed higher levels of all six miRs in this panel **(Figure 3.11b)**. Hypermethylator breast cancer cell lines exhibit an average miR score of 4.9 ± 0.46, whereas, non-hypermethylator cell lines exhibit an average miR score of 0.67 ± 0.33 (p<0.0001).

miR Score Correlates With Gene Expression Score and Promoter Methylation Score

A linear correlation analysis was performed to determine if miR score significantly associates with methylation score and expression score for each breast cancer cell line. Methylation score and expression score reflect the combined relative promoter methylation status and the combined relative gene expression status for methylation-sensitive biomarker genes associated with the hypermethylation defect (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, TFF3,* and *SCNNIA*) [150]. A strong inverse correlation (r=-0.66, p=0.0056) was observed between miR score and gene expression score **(Figure 3.12a)**. Breast cancer cell lines that exhibit diminished expression of multiple regulatory miRs (high miR score) tend to express low levels of methylation-sensitive genes (gene expression score) and cell lines that express higher levels of regulatory miRs (low miR score) tend to express methylation-sensitive genes at higher levels **(Figure 3.12a)**. A strong correlation (r=0.72, p=0.002) was observed between miR score and methylation score **(Figure 3.12b)**. Breast cancer cell lines that exhibit diminished expression of multiple regulatory miRs (high miR score) exhibit higher methylation scores and cell lines that express higher levels of regulatory miRs (low miR score) tend to have lower methylation scores **(Figure 3.12b)**. Previous studies demonstrated significant relationships between overexpression of DNMT3b and gene expression scores and methylation scores for methylation-sensitive genes [150]. The current results strongly support the suggestion that loss of miR expression may account for the

DNMT3b-mediated hypermethylation defect among breast cancer cell lines that is characterized by methylation-dependent loss expression of methylation-sensitive biomarker genes.

Co-regulation of miR Expression in Breast Cancer Cell Lines

To determine if miRs that regulate DNMT3b are independently regulated or co-regulated at the level of expression, a linear correlation analysis was performed to examine patterns of miR expression among hypermethylator and non-hypermethylator breast cancer cell lines. Statistically significant linear relationships were observed between the levels of expression of several miRs **(Figure 3.13a-f)**: miR-26a and miR-26b (r=0.92, $p<0.0001$), miR-148a and miR-26a (r=0.88, $p<0.0001$), miR-148a and miR-26b (r=0.85, $p<0.0001$), miR-29c and miR-148a (r=0.81, $p=0.0002$), miR-148a and miR-148b (r=0.83, $p<0.0001$), and miR-29c and miR-148b (r=0.92, $p<0.0001$). In addition, significant linear relationships were observed for expression of miR-26a and miR-203 (r=0.71, $p=0.0019$), miR26b and miR-203 (r=0.68, $p=0.038$), miR-26a and miR-29c (r=0.60, $p=0.014$), miR-148a and miR-203 (r=0.60, $p=0.014$), and miR-26b and miR-148b (r=0.5, $p=0.04$). No significant linear relationships were observed for expression of miR-26b and miR-29c, miR-148c and miR-203, or miR-29c and miR-203. Combined, these observations suggest that several miRs that function in the regulation of *DNMT3b* are co-regulated.

Changes in miR Expression Levels in Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines after Pre-miR and Antagomir Transfection

To determine the mechanistic role of specific miRs in the dysregulation of *DNMT3b* among breast cancer cell lines, the complementary approach of modulating miR levels by transfection of pre-miR precursors (to enforce miR expression in cells lacking a given miR) or transfection of antagomirs (to knock down miR expression in cells that express normal levels of a given miR) was employed. Transfection of hypermethylator cell lines Hs578T, HCC1937, and SUM185 with pre-miR precursors for miR-148b, miR-26b, and miR-29c resulted in restoration of expression of these miRs **(Figure 3.14a-c)**. Following pre-miR transfection, Hs578T cells displayed 210-fold, 160-fold, and 240-fold increased levels of miR-148b, miR-26b, and miR-29c **(Figure 3.14a)**. Likewise, pre-miR transfection produced 430-fold, 2,100-fold, and 580-fold increases in miR-148b, miR-26b, and miR-29c levels in HCC1937 cells **(Figure 3.14b)**, and 54,000-fold, 4,700-fold, and 2,200-fold increases in miR-148b, miR-26b, and miR-29c levels in SUM185 cells **(Figure 3.14c)**. Non-target control pre-miR precursors did not produce any significant increase in miR-148b, miR-26b, and miR-29c levels in any of these cell lines **(Figure 3.14a-c)**.

Transfection of non-hypermethylator cell lines BT20, MDA-MB-415, and MDA-MB-468 with antagomirs directed against miR-148b, miR-26b, and miR-29c resulted in a significant knockdown of miR-148b, miR-26b, and miR-29c levels **(Figure 3.15a-c)**. Antagomir transfection of BT20 cells resulted in reduction of miR-148b, miR-26b, and miR-29c levels by 76%, 69%, and 73%, respectively **(Figure 3.15a)**. Likewise, antagomir transfection of MDA-MB-415 cells produced 76%, 49%, and 48% reductions in miR-148b, miR-26b, and miR-29c levels **(Figure 3.15b)**, and antagomir transfection of MDA-MB-468 cells resulted in 72%, 69%, and 35% reduction in miR-148b, miR-26b, and miR-29c levels

(Figure 3.15c). Non-target control antagomirs did not produce significant alterations in the level of miR-148b, miR-26b, and miR-29c in any of these cell lines **(Figure 3.15a-c)**.

Perturbation of Regulatory miR Expression Alters *DNMT3b* Levels in Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines

Enforced expression of miR-148b, miR-26b, and miR-29c in hypermethylator cell lines Hs578T, HCC1937, and SUM185 resulted in statistically significant reduction in *DNMT3b* expression levels **(Figure 3.16)**. In Hs578T cells, miR-29c expression reduced *DNMT3b* levels by 73%, and expression of miR-148b and miR-26b produced 62% reduction in *DNMT3b* levels **(Figure 3.16)**. Similar results were obtained in HCC1937 cells with 58%-64% reductions of DNMT3b levels in response to enforced expression of miR-148b, miR-26b, and miR-29c **(Figure 3.16)**. The most dramatic effect of enforced pre-miR expression on *DNMT3b* levels was observed in SUM185 cells. Expression of miR-29c in SUM185 cells resulted in an 88% decrease in *DNMT3b* mRNA **(Figure 3.16)**. Likewise, expression of miR-148b and miR-26b in SUM185 cells produced 80% and 82% reduction in *DNMT3b* levels **(Figure 3.16)**. Transfection of non-target control pre-miR precursors did not produce any significant change in *DNMT3b* levels in Hs578T, HCC1937, and SUM185 cells **(Figure 3.16)**. Western analysis of cell lysates from Hs578T, HCC1937, and SUM185 cells following pre-miR transfection failed to detect significant alterations in DNMT3b protein levels, probably due to the transient nature of this assay system. Likewise, assessment of methylation-sensitive gene expression (for *CEACAM6, CST6,* and *SCNN1A*) in Hs578T cells after enforced expression of miR-148b, miR-26b, and miR-29c did not reveal changes in

levels of expression compared to control cells, consistent with the lack of change in DNMT3b protein levels.

Antagomir-mediated knockdown of miR-148b, miR-26b, and miR-29c in non-hypermethylator cell lines MDA-MB-468, MDA-MB-415, and BT20 resulted in statistically significant increases in *DNMT3b* expression levels **(Figure 3.17)**. The most dramatic effects were observed in MDA-MB-468 cells, where miR-148b knockdown produced a 3.2-fold increase in *DNMT3b* mRNA, whereas knockdown of miR-26b and miR-29c resulted in 2-fold and 2.6-fold increases in *DNMT3b* levels, respectively **(Figure 3.17)**. Comparable increases in DNMT3b expression levels (1.8-fold to 2-fold) were observed in BT20 cells following knockdown of miR-148b, miR-26b, and miR-29c **(Figure 3.17)**. More modest increases of *DNMT3b* levels (1.2-fold to 1.4-fold) were observed in MDA-MB-415 cells after knockdown of miR-148b, miR-26b, and miR-29c; though modest, these alterations were statistically significant. Transfection of non-target control antagomirs did not produce any significant change in *DNMT3b* levels in these cell lines **(Figure 3.17)**. Similar to the results obtained with pre-miR-transfected hypermethylator cell lines, western analysis of cell lysates from MDA-MB-468, MDA-MB-415, and BT20 cells following antagomir transfection failed to detect significant alterations in DNMT3b protein levels. Further, assessment of methylation-sensitive gene expression (for *CEACAM6, CST6,* and *SCNN1A*) in MDA-MB-468 cells after antagomir-mediated knockdown of miR-148b, miR-26b, and miR-29c did not reveal changes in levels of expression compared to control cells, consistent with the lack of change in DNMT3b protein levels in this short-term assay system.

Results Related to MicroRNA Analysis in Primary Breast Cancers

Hypermethylator Breast Cancer Cells Express Diminished Levels of Regulatory miRs

We utilized a cohort of 70 primary human breast cancers of known molecular subtype (36 luminal A, 13 luminal B, 5 Her2+, 16 basal-like) and 18 normal mammoplasty tissues to analyze expression of microRNAs that contribute to regulation of *DNMT3b* (miR-26a, miR-26b, miR-29a, miR-29b, miR-29c, miR-148a, miR-148b, miR-203, and miR-222). Average miR expression in each of the intrinsic subtypes of breast cancer is shown in the **Table 3.3**. Significantly reduced average expression of miR-29c distinguished basal-like breast cancers from other molecular subtypes **(Table 3.3)**. The average expression of miR-26a was also reduced in basal-like breast cancers compared to its expression in other subtypes of primary breast cancers, but the difference was not statistically significant **(Table 3.3)**.

The methylation status of a subset of 33 cancers (6 luminal A, 6 luminal B, 5 HER2+, and 16 basal-like cancers) was established through examination of methylation-sensitive biomarker gene expression. Individual cancers were classified as hypermethylators when their expression signature reflected diminished levels of ≥ 7 biomarker genes. Among this cohort of 33 cancers, 12 (36%) were classified as hypermethylators **(Figure 3.18)**. 9/12 (75%) hypermethylator cancers corresponded to basal-like molecular subtype, and this hypermethylator group contains 56% (9/16) of all basal-like cancers examined **(Figure 3.19)**. The remaining hypermethylator cancers corresponded to the luminal A (n=1), luminal B (n=1), and HER2+ (n=1) subtypes. This finding is consistent with the observation of a large degree of correspondence and overlap between basal-like cancers and hypermethylator breast cancers. The miR expression status within this subset of 33 cancers is shown in **Figure 3.20**.

We compared the expression patterns of the miRs between the two subsets of basal-like breast cancers; hypermethylators (n=9, 56%) and non-hypermethylators (n=7, 44%).

While there was variability in expression among the miRs examined, in general the hypermethylator cancers expressed diminished levels of regulatory miRs compared to the non-hypermethylator cancers **(Figure 3.19)**. miR-29c did not display the pattern of expression observed with the majority of miRs. Since loss of miR-29c differentiated the basal-like cancers from other subtypes of breast cancers, the absence of differential expression among the basal-like cancers suggests that loss of miR-29c to be a feature of this molecular subtype, irrespective of the methylator status.

The average expression of miR-29a and miR-26a among hypermethylator basal-like cancers was significantly diminished compared to the average expression of these miRs among non-hypermethylator basal-like cancers ($p<0.05$) **(Figure 3.21)**. Differences in average expression of miR-29b and miR-26b in hypermethylator cancers versus non-hypermethylator cancers were not statistically significant **(Figure 3.21)**, although there was a distinct trend towards lower expression in the hypermethylator tumors ($p=0.11$ and $p=0.08$). 9/9 (100%) hypermethylator cancers expressed low levels of miR-29b, miR-26a, and miR-26b, and 8/9 (89%) of these expressed low levels of miR-29a **(Figure 3.22a-d)**. However, among non-hypermethylator cancers, miR-29a and miR-26a were normally expressed in 4/7 (57%) cancers, and miR-29b and miR-26b were expressed at normal levels in 3/7 (43%) non-hypermethylator cancers **(Figure 3.22a-d)**. Interestingly, the three non-hypermethylator cancers with low levels of expression of miR-29a exhibited low levels of expression of miR-26a, miR-29b, and miR-26b. In addition, these three cancers express low levels of miR-148b and miR-203. miR-29c is expressed at normal levels in 3/7 (43%) non-hypermethylator cancers. However, the 4 non-hypermethylator cancers with diminished miR-29c do not include all three cancers that lost the expression of multiple miRs **(Figure 3.22e)**. Among

hypermethylator cancers, 6/9 (67%) expressed low levels of miR-29c **(Figure 3.22e)**. 7/9 (78%) and 8/9 (89%) cancers had diminished levels of expression of miR-148a and miR-148b respectively **(Figure 3.22f-g)**. These miRs were expressed at normal levels in 5/7 (71%, miR-148a) and 4/7 (57%, miR-148b) non-hypermethylator cancers **(Figure 3.22f-g)**. 7/9 (78%) hypermethylator cancers expressed miR-203 at low levels, while 3/7 (43%) non-hypermethylator cancers expressed miR-203 at easily detectable levels **(Figure 3.22h)**. miR-222 was expressed at low levels in 5/9 (56%) hypermethylator cancers. 6/7 (87%) non-hypermethylator cancers expressed miR-222 at normal levels **(Figure 3.22i)**.

Diminished Expression of miR-29a, miR-29b, miR-26a, miR-26b, miR-148a, and miR-148b Predict Hypermethylator Status Among Breast Cancers

We observed differential expression of miR-29a, miR-29b, miR-26a, miR-26b, miR-148a, and miR-148b among basal-like breast cancers with strong trends towards diminished expression in hypermethylators compared to non-hypermethylator cancers. To assess the value of individual miR expression levels in the prediction of the methylation status of a certain tumor, a Bayesian analysis was performed. Correct assignments (CA) were used as a guiding principle to determine the threshold values for each of the differentially expressed miRs indicated in **Figure 3.22**. The expression level of miR-26a (CA=81%) emerged as the best individual predictor of methylator status among basal-like breast cancers, followed by miR-29a (CA=75%), miR-29b (CA=75%), miR-26b (CA=75%), miR-148a (CA=75%), and miR-148b (CA=75%). These miRs individually displayed excellent sensitivity (range: 78-100%) and negative predictive value (NPV range: 71-100%), as well as good specificity (range: 43-71%) and positive predictive value (PPV range: 69-78%). The remaining miRs

displayed poor predictive value for determination of methylation status among breast cancers (CA=63%-69) **(Table 3.4)**.

miR Scores Correlate With Methylation-sensitive Gene Expression Scores Among Primary Breast Cancers

miR scores were generated for each breast cancer, reflecting the number of miRs with diminished expression. Hypermethylator basal-like breast cancers frequently express diminished levels of this panel of miRs. 8/9 (89%) hypermethylator basal-like cancers express ≥6 regulatory miRs at diminished levels **(Figure 3.23)**, resulting in higher miR scores. Three hypermethylator cancers express diminished levels of all nine miRs examined **(Figure 3.23)**. In contrast to the hypermethylator cancers, non-hypermethylator basal-like breast cancers typically express the majority of these regulatory miRs at higher levels. 4/7 (57%) non-hypermethylator cancers express ≥7 miRs at higher levels **(Figure 3.23)**, resulting in lower miR scores. Hypermethylator basal-like cancers exhibit an average miR score of 7.6 ± 0.5, whereas, non-hypermethylator basal-like cancers exhibit an average miR score of 4 ± 1.3 (p=0.039).

A linear correlation analysis was performed to determine if miR scores significantly correlate with the expression score among basal-like breast cancers. The expression score reflects the combined relative gene expression status for methylation-sensitive biomarker genes associated with the hypermethylation defect (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, TFF3,* and *SCNNIA*) [150]. A strong inverse correlation (r=-0.67, p=0.003) was observed between miR score and gene expression score **(Figure 3.24)**. The cancers that exhibit diminished expression of multiple regulatory miRs (high miR score) tend

to express low levels of methylation-sensitive genes (gene expression score) and cancers that express higher levels of regulatory miRs (low miR score) tend to express methylation-sensitive genes at higher levels **(Figure 3.24**). Previous studies demonstrated significant relationships between overexpression of DNMT3b and gene expression scores for methylation-sensitive genes [150]. miR expression patterns revealed two groups among basal-like breast cancers corresponding to low expression (n=11) and high expression (n=5) **(Figure 3.25)**. The subset of basal-like breast cancers with reduced expression overlaps considerably with the hypermethylator subset of basal-like breast cancers. 8/9 hypermethylators correspond to the low expression group. These results strongly support the suggestion that loss of miR expression may account for the expression of hypermethylation defect that is characterized by DNMT3b-overexpression.

Table 3.1. *Changes in IC_{50} for Doxorubicin, Paclitaxel, and 5-Fluorouracil in Hypermethylator Breast Cancer Cells after Pharmacologic Inhibition of DNA Methyltransferase Activity and/or Targeted Inhibition of DNMT3b.*

	MDA-MB-453 Cells			BT549 Cells			Hs578T Cells		
Pretreatment	DOX (%)[4]	PAX (%)[4]	5-FU (%)[4]	DOX (%)[4]	PAX (%)[4]	5-FU (%)[4]	DOX (%)[4]	PAX (%)[4]	5-FU (%)[4]
No Pretreatment	0.086 ± 0.006	0.497 ± 0.025	0.817 ± 0.133	0.099 ± 0.003	1.974 ± 0.081	1.183 ± 0.145	0.230 ± 0.001	2.605± 0.012	1.211 ± 0.073
250 nM 5aza[1]	0.080 ± 0.012 (7%)	0.499 ± 0.016 (+4%)	0.865 ± 0.249 (+6%)	0.065 ± 0.007* (32%)	1.486 ± 0.171 (24%)	0.634 ± 0.052 (46%)	0.229 ± 0.004 (0%)	2.596 ± 0.049 (0%)	1.229 ± 0.055 (+2%)
500 nM 5aza[1]	0.034 ± 0.002* (60%)	0.311 ± 0.026¥ (37%)	0.065 ± 0.010* (92%)	0.052 ± 0.006¥ (47%)	1.015 ± 0.067† (48%)	0.472 ± 0.054* (60%)	0.110 ± 0.004† (52%)	1.466 ± 0.035‡ (43%)	0.371 ± 0.059† (69%)
RNAi Scram[2]	0.080 ± 0.001 (7%)	0.497 ± 0.006 (0%)	0.799 ± 0.140 (2%)	0.094 ± 0.001 (4%)	1.967 ± 0.047 (0%)	1.176 ± 0.013 (1%)	0.221 ± 0.001 (4%)	2.544± 0.015 (2%)	1.191 ± 0.057 (2%)
DNMT3b KD[2]	0.048 ± 0.002* (44%)	0.376 ± 0.016* (24%)	0.145 ± 0.036* (82%)	0.086 ± 0.002* (13%)	1.660 ± 0.121	0.791 ± 0.076 (33%)	0.155 ± 0.005† (32%)	1.839 ± 0.028‡ (29%)	0.562 ± 0.037† (53%)
KD + 250 nM 5aza[3]	0.040 ± 0.001* (43%)	0.386 ± 0.005* (22%)	0.152 ± 0.099* (81%)	0.065 ± 0.002† (32%)	1.527 ±0.202 (23%)	0.632 ± 0.034 (46%)	0.161 ± 0.006¥ (30%)	1.837 ± 0.012‡ (29%)	0.573 ± 0.041† (53%)
KD + 500 nM 5aza[3]	0.035 ± 0.002* (59%)	0.313 ± 0.022¥ (37%)	0.067 ± 0.014* (92%)	0.054 ± 0.001¥ (45%)	1.082 ± 0.098† (45%)	0.470 ± 0.049* (60%)	0.111 ± 0.002‡ (51%)	0.573 ± 0.044† (53%)	0.359 ± 0.030† (70%)

[1]Cell lines were pretreated with 5-aza (250 or 500 nM) for 7 days.
[2]RNAi Scram refers to cell lines transfected with pLVHTM^{SCRAM} and DNMT3b KD refers to cells that were transfected with pLVHTM^{DNMT3b}.
[3]DNMT3b KD cell lines were also pretreated with 5-aza (250 or 500 nM) for 7 days.
[4]Percent reduction in IC_{50} compared to parental breast cancer cell lines with no pretreatment.
Values for IC_{50} are expressed as averages ± standard error of the mean (S.E.M.).
Statistical significance versus parental breast cancer cell lines with no pretreatment: *$p<0.05$, ¥$p<0.01$, †$p<0.005$, ‡$p<0.0005$.
Doses of chemotherapeutic drugs expressed as μM (DOX), nM (PAX), and mM (5FU).

Table 3.2. *Bayesian Analyses Show That Loss of miR Expression is Associated with Expression of Hypermethylation Defect in Breast Cancer Cell Lines.* Threshold values for Bayesian analysis are as follows: miR-29a, 1.0; miR-29b, 0.15; miR-29c, 0.62; miR-148a, 2.8, miR-148b, 6.0; miR-26a, 4.0; miR-26b, 3.63; miR-203, 0.2; and miR-222, 2.0.

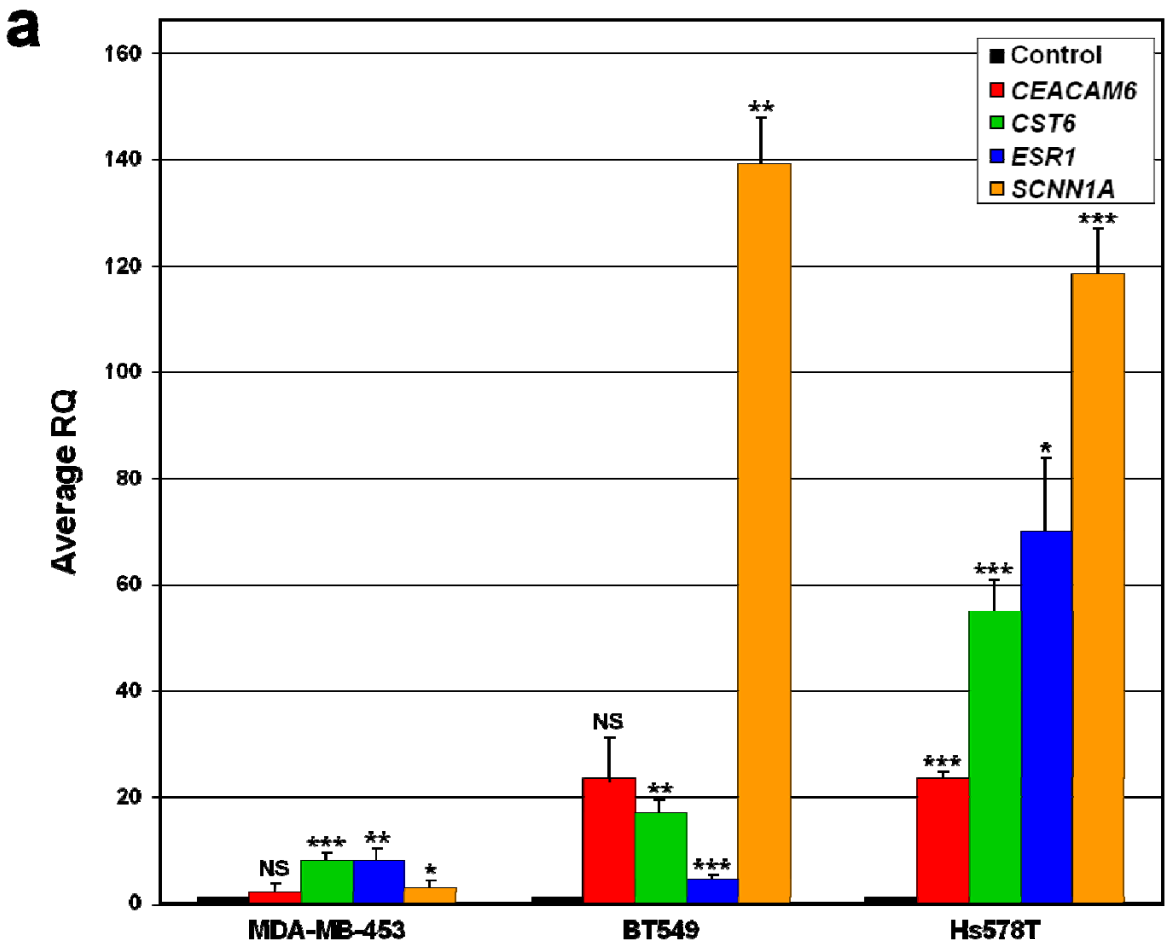
	Sensitivity	**Specificity**	**Positive Predictive Value**	**Negative Predictive Value**	**Correct Assignment**
miR-29a	60%	66%	75%	50%	63%
miR-29b	60%	66%	75%	50%	63%
miR-29c	60%	83%	86%	56%	69%
miR-148a	90%	83%	90%	83%	88%
miR-148b	100%	83%	91%	100%	94%
miR-26a	80%	100%	100%	75%	88%
miR-26b	90%	100%	100%	86%	94%
miR-203	80%	83%	89%	71%	81%
miR-222	30%	17%	38%	13%	25%

Table 3.3. *Average miR Expression Among the Molecular Subtypes of Breast Cancer.* The table shows the average expression (relative to the expression in normal breast tissue ± SEM) of the regulatory miRs in each of the intrinsic subtypes of breast cancer.

	Luminal A (n=36)	**Luminal B (n=13)**	**HER2+(n=5)**	**Basal-like (n=16)**
miR-29a	2.37±0.33	2.44±0.58	2.02±0.46	2.22±0.64
miR-29b	7.39±1.24	7.95±1.62	4.85±1.46	8.93±2.72
miR-29c	5.74±1.07	8.48±3.37	3.22±0.67	1.89±0.38
miR-148a	3.56±0.50	3.41±0.86	2.77±0.60	3.66±0.81
miR-148b	4.13±0.58	4.29±0.63	3.01±0.73	3.74±1.09
miR-26a	1.77±0.19	1.69±0.33	1.35±0.48	1.13±0.38
miR-26b	2.63±0.34	3.02±0.53	1.61±0.49	2.11±0.72
miR-203	3.67±0.78	11.75±3.34	18.65±9.56	17.70±7.29
miR-222	1.36±0.43	1.34±0.32	2.35±1.01	1.70±0.54

Table 3.4. *Bayesian Analyses Show That Loss of miR Expression is Associated with Expression of Hypermethylation Defect in Primary Cancers.* Threshold values for Bayesian analysis are as follows: miR-29a, 2.40; miR-29b, 14.00; miR-29c, 2.60; miR-148a, 2.70; miR-148b, 3.50; miR-26a, 1.30; miR-26b, 4.00; miR-203, 10.00; and miR-222, 0.76.

	Sensitivity	**Specificity**	**Positive Predictive Value**	**Negative Predictive Value**	**Correct Assignment**
miR-29a	89%	58%	73%	80%	75%
miR-29b	100%	43%	69%	100%	75%
miR-29c	67%	43%	60%	50%	63%
miR-148a	78%	71%	78%	71%	75%
miR-148b	89%	57%	73%	80%	75%
miR-26a	100%	57%	75%	100%	81%
miR-26b	100%	43%	69%	100%	75%
miR-203	78%	43%	64%	60%	63%
miR-222	56%	86%	83%	60%	69%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Average RQ
160
140
120
100
80
60
40
20
0
Control
CEACAM6
CST6
ESR1
SCNN1A
NS
***
**
*
NS
**
***
**
***
*
MDA-MB-453
BT549
Hs578T

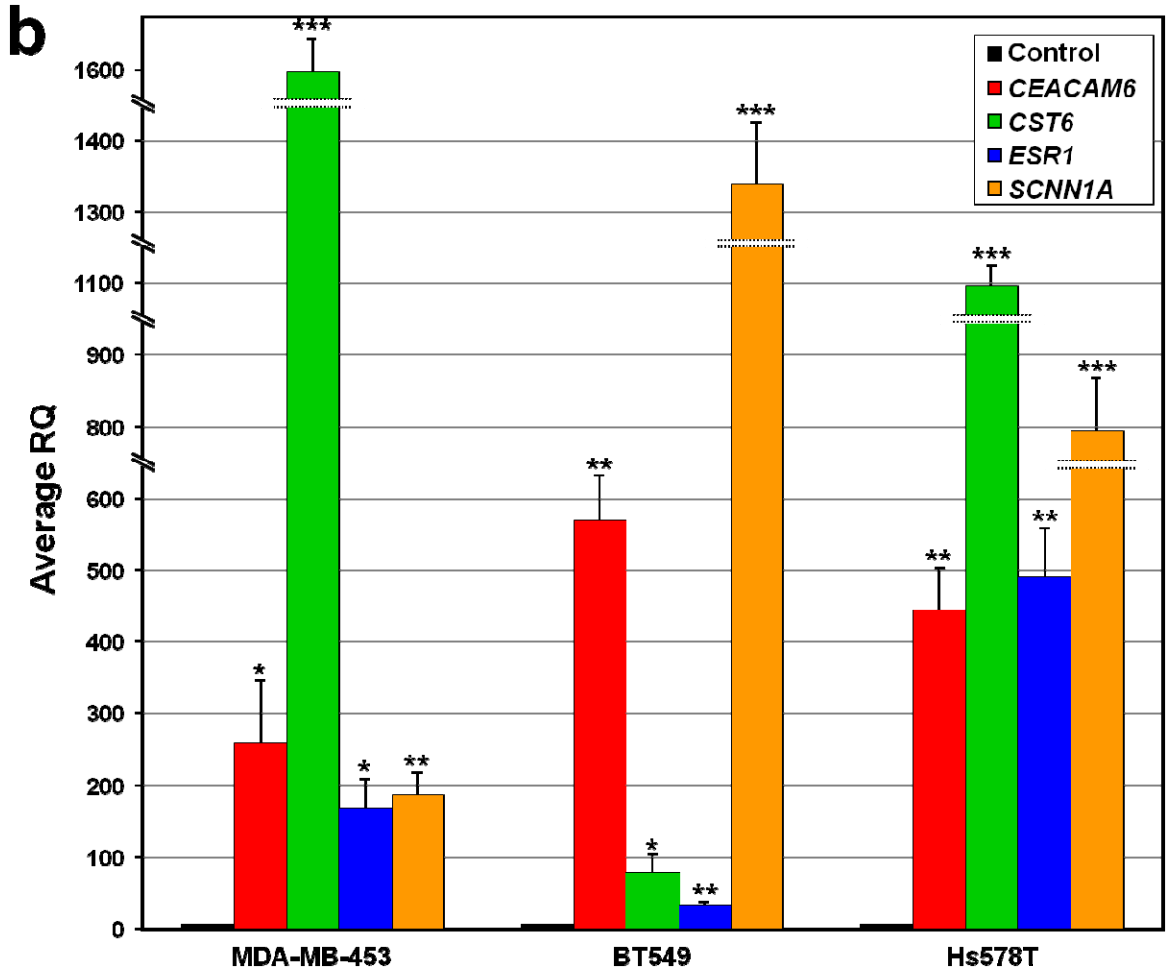
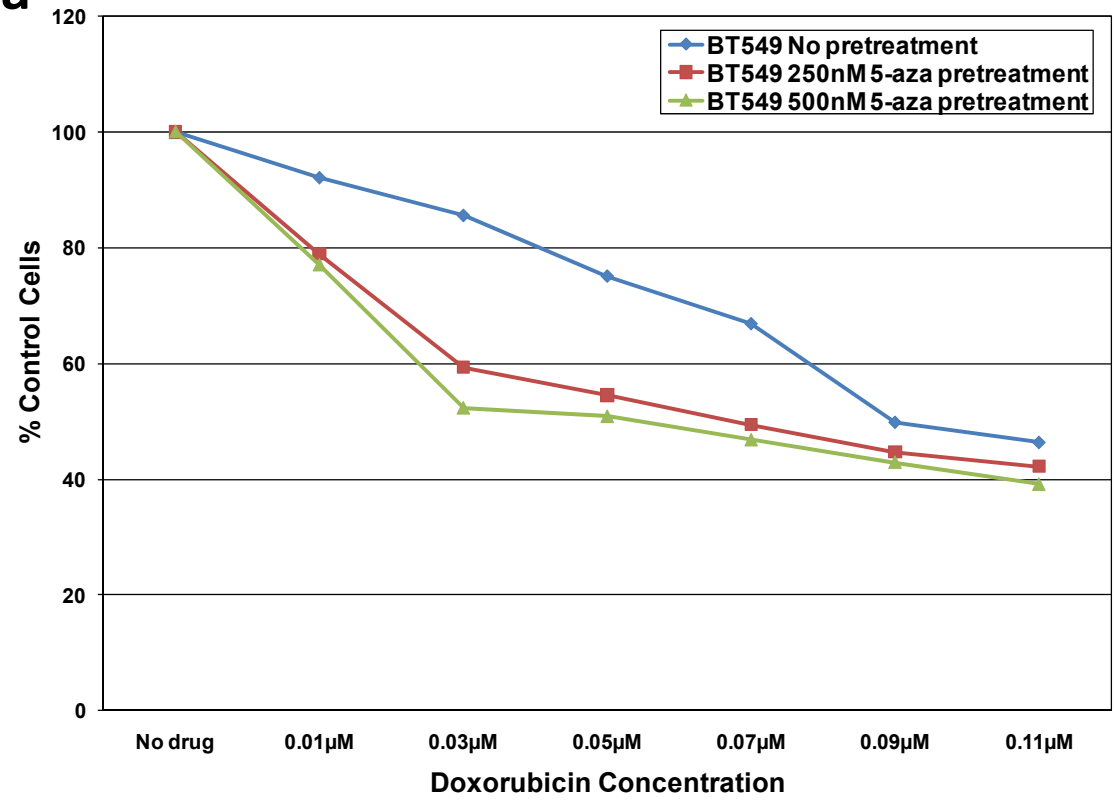
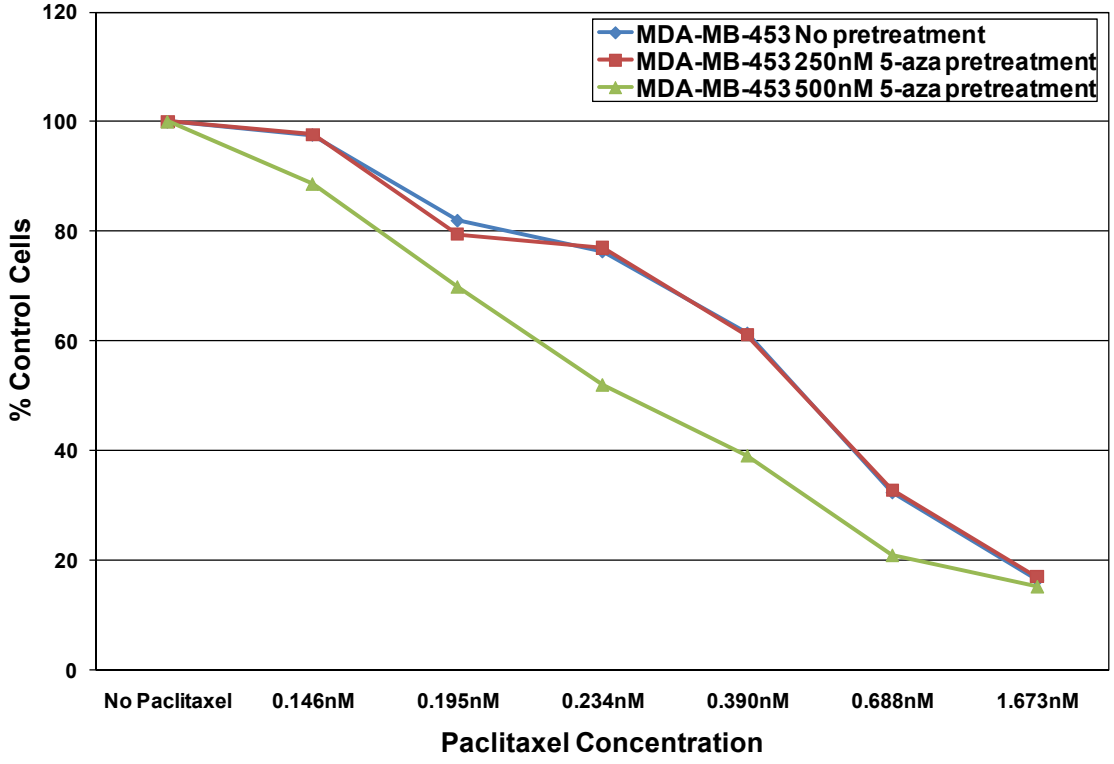

b
Average RQ
0
100
200
300
400
500
600
800
900
1100
1300
1400
1600
Control
CEACAM6
CST6
ESR1
SCNN1A
MDA-MB-453
BT549
Hs578T

Figure 3.1. *Re-expression of Methylation-Sensitive Genes in Hypermethylator Breast Cancer Cell Lines Following Demethylating Treatment*. **(a**) MDA-MB-453, BT549, and Hs578T cells were treated with 250 nM 5-aza for 7 days and real-time PCR was performed for *CEACAM6, CST6, ESR1,* and *SCNN1A*. **(b)** MDA-MB-453, BT549, and Hs578T cells were treated with 500 nM 5-aza for 7 days and real-time PCR was performed for *CEACAM6, CST6, ESR1,* and *SCNN1A*. The bars represent the fold-change in levels of expression for each gene and cell line relative to the untreated control cells (where RQ = 1). NS = not statistically significant, $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$.

a


BT549 No pretreatment
BT549 250nM 5-aza pretreatment
BT549 500nM 5-aza pretreatment
% Control Cells
120
100
80
60
40
20
0
No drug
0.01μM
0.03μM
0.05μM
0.07μM
0.09μM
0.11μM
Doxorubicin Concentration

b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MDA-MB-453 250nM 5-aza pretreatment
MDA-MB-453 500nM 5-aza pretreatment
% Control Cells
120
100
80
60
40
20
0
No Paclitaxel
0.146nM
0.195nM
0.234nM
0.390nM
0.688nM
1.673nM
Paclitaxel Concentration

C

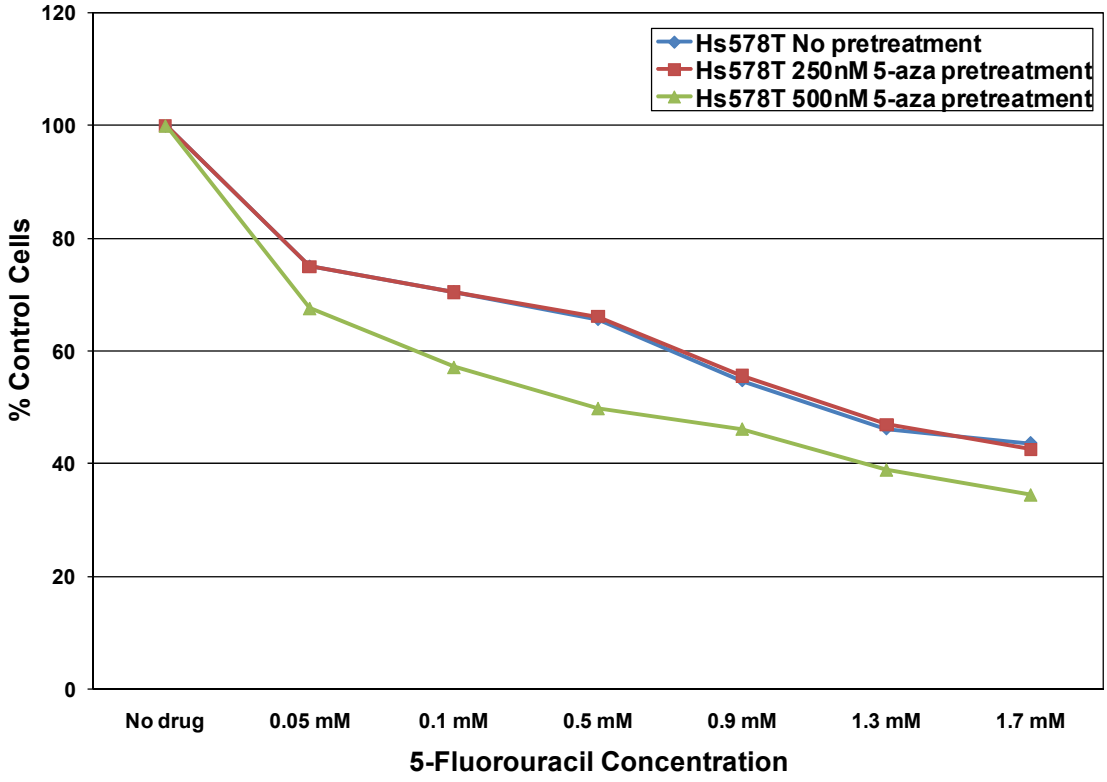
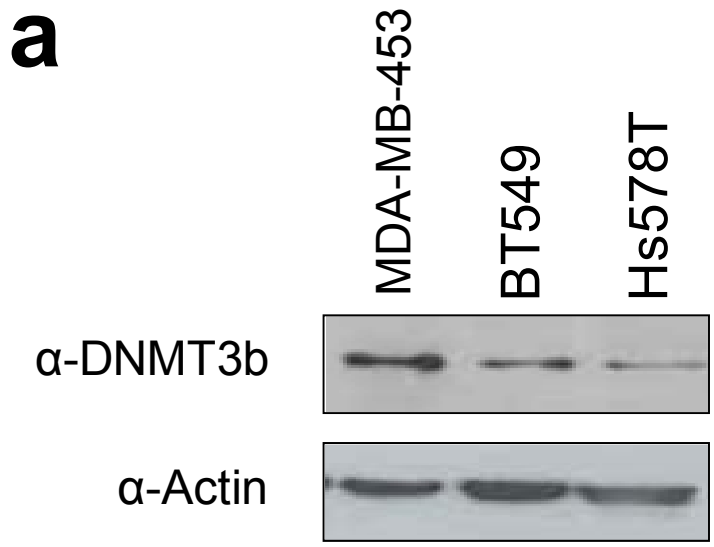
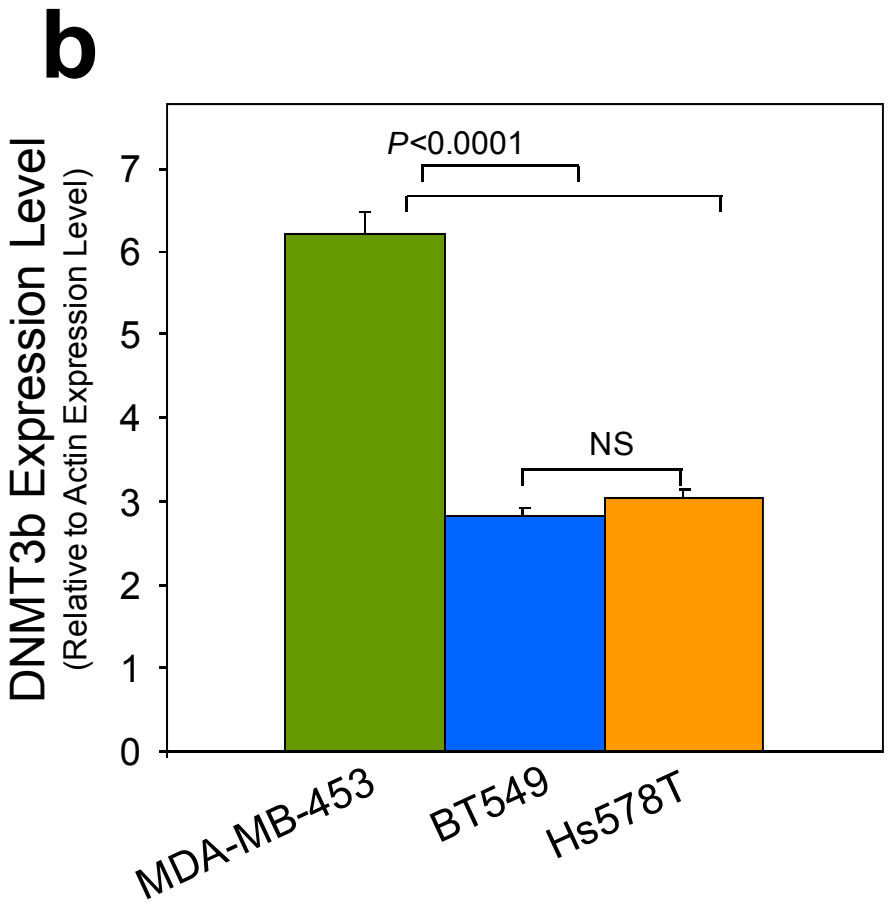

120
100
80
60
40
20
0
% Control Cells
Hs578T No pretreatment
Hs578T 250nM 5-aza pretreatment
Hs578T 500nM 5-aza pretreatment
No drug
0.05 mM
0.1 mM
0.5 mM
0.9 mM
1.3 mM
1.7 mM
5-Fluorouracil Concentration

Figure 3.2. *Demethylating Treatment with 5-Aza Sensitizes Hypermethylator Breast Cancer Cells to Chemotherapy.* The blue lines represent breast cancer cells with no pretreatment, red lines represent cells pretreated with 250 nM 5-aza for 7 days, and green lines represent cells pretreated with 500 nM 5-aza for 7 days. Changes in chemotherapeutic sensitivity are shown as percentage of viable cells remaining (relative to untreated control cells) after 72 hrs of drug exposure for the indicated doses. (**a**) BT549 breast cancer cells exhibit increased DOX efficacy after 250 nM and 500 nM 5-aza pretreatment. (**b**) MDA-MB-453 breast cancer cells exhibit increased PAX efficacy after 500 nM 5-aza pretreatment. (**c**) Hs578T breast cancer cells exhibit increased 5-FU efficacy after 500 nM 5-aza pretreatment.


a
MDA-MB-453
BT549
Hs578T
α-DNMT3b
α-Actin


b
DNMT3b Expression Level
(Relative to Actin Expression Level)
P<0.0001
NS
0
1
2
3
4
5
6
7
MDA-MB-453
BT549
Hs578T

c

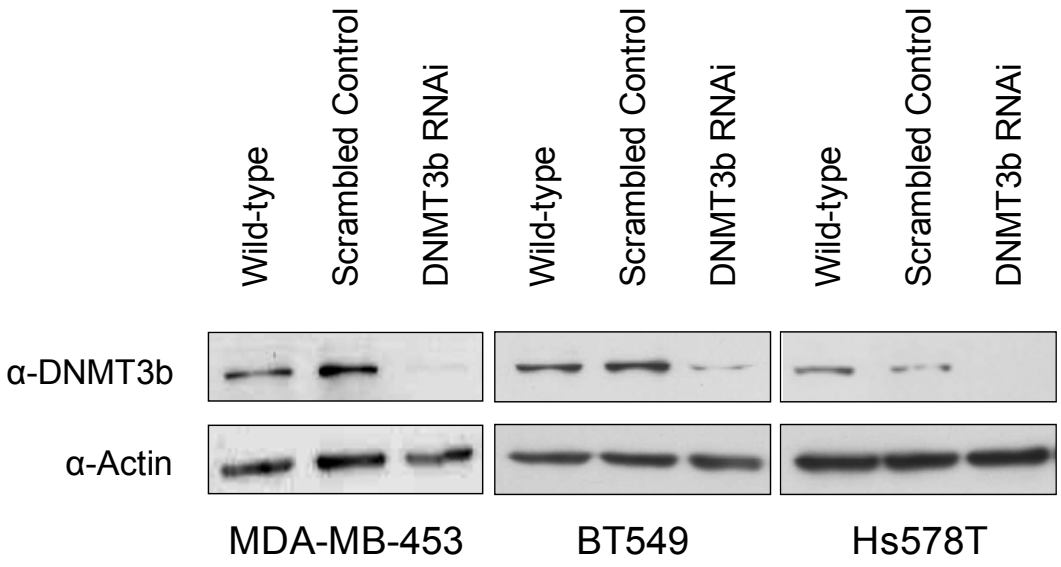
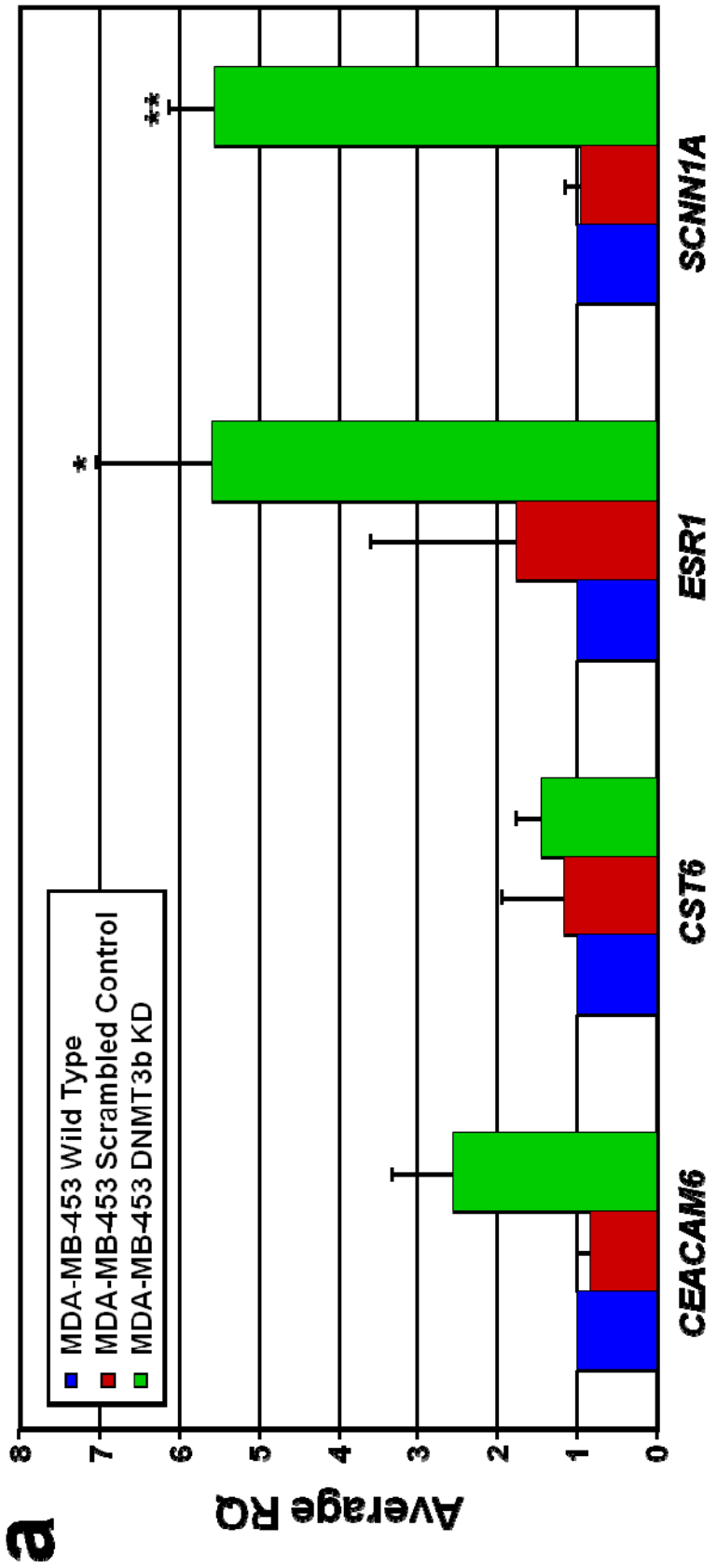
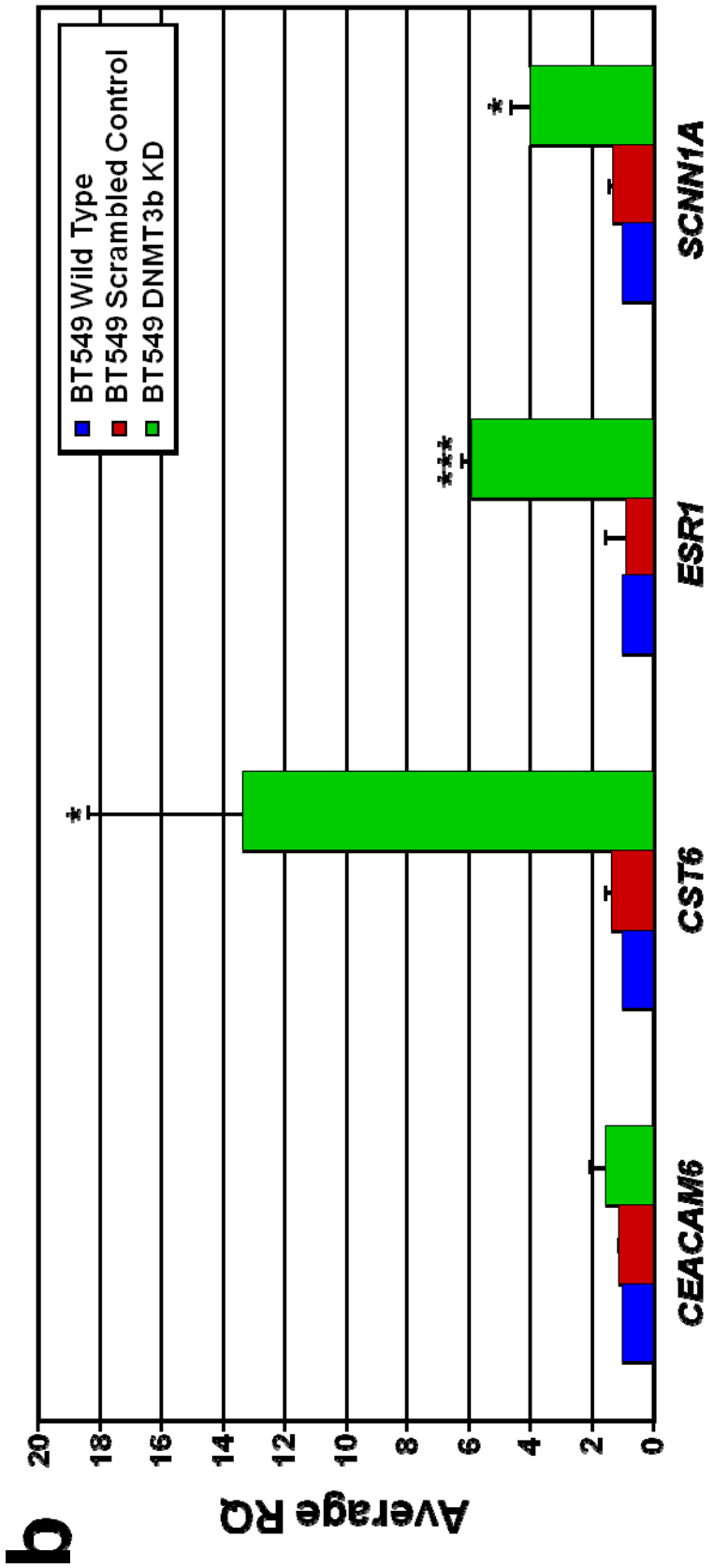

Wild-type
Scrambled Control
DNMT3b RNAi
Wild-type
Scrambled Control
DNMT3b RNAi
Wild-type
Scrambled Control
DNMT3b RNAi
α-DNMT3b
α-Actin
MDA-MB-453
BT549
Hs578T

Figure 3.3. *RNAi-mediated Knockdown of DNMT3b Results in Reduction in DNMT3b Protein Levels in Hypermethylator Breast Cancer Cell Lines.* **(a)** Western blot analysis of DNMT3b protein levels in MDA-MB-453, BT549, and Hs578T cells. Actin levels are shown as a loading control. **(b)** Quantification of DNMT3b protein levels relative to actin. **(c)** Western blot analysis of DNMT3b protein levels in MDA-MB-453, BT549, and Hs578T cells after RNAi-mediated knockdown of DNMT3b. Western blot analysis was performed by Dr. Ashley G. Rivenbark (Department of Biochemistry and Biophysics, UNC). Actin levels are shown as a loading control. NS = not statistically significant.


a
Average RQ
0
1
2
3
4
5
6
7
8
MDA-MB-453 Wild Type
MDA-MB-453 Scrambled Control
MDA-MB-453 DNMT3b KD
*
**
CEACAM6
CST6
ESR1
SCNN1A


b
Average RQ
0
2
4
6
8
10
12
14
16
18
20
BT549 Wild Type
BT549 Scrambled Control
BT549 DNMT3b KD
*
***
*
CEACAM6
CST6
ESR1
SCNN1A

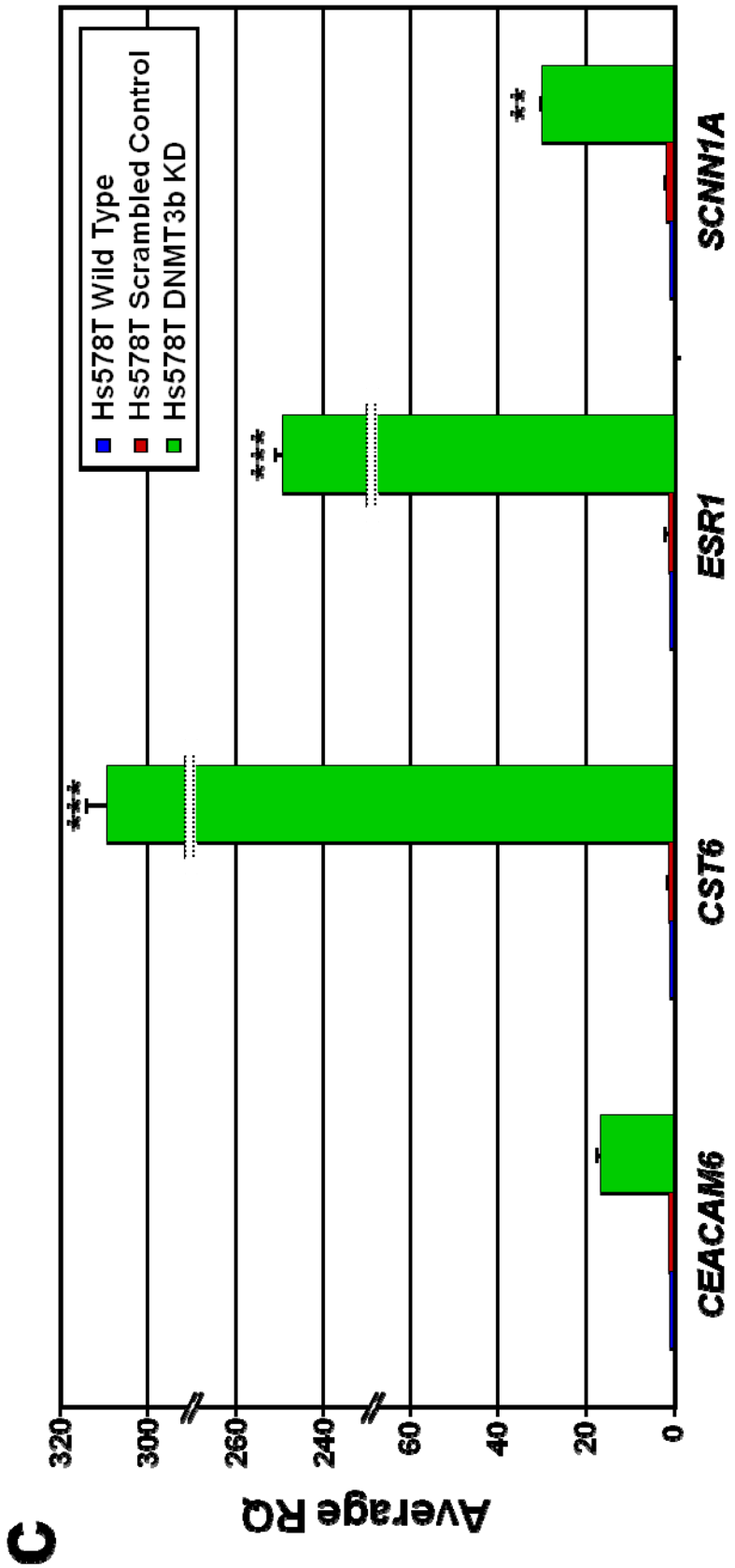

C
Average RQ
320
300
260
240
60
40
20
0
Hs578T Wild Type
Hs578T Scrambled Control
Hs578T DNMT3b KD
***
***
**
CEACAM6
CST6
ESR1
SCNN1A

Figure 3.4. *RNAi-mediated Knockdown of DNMT3b Results in Re-expression of Methylation-Sensitive Genes in Hypermethylator Breast Cancer Cell Lines.* Hypermethylator breast cancer cells re-express methylation-sensitive DNMT3b target genes after DNMT3b KD. The blue bars represent real-time PCR results for wild-type cells, the red bars represent the results in scram-transfected cells and green bars represent the results in DNMT3b KD cells compared to wild-type control cells (where RQ = 1). (**a**) MDA-MB-453 breast cancer cells re-express *CEACAM6, CST6, ESR1,* and *SCNN1A* after DNMT3b KD. (**b**) BT549 breast cancer cells re-express *CEACAM6, CST6, ESR1,* and *SCNN1A* after DNMT3b KD. (**c**) Hs578T breast cancer cells re-express *CEACAM6, CST6, ESR1,* and *SCNN1A* after DNMT3b KD. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$.

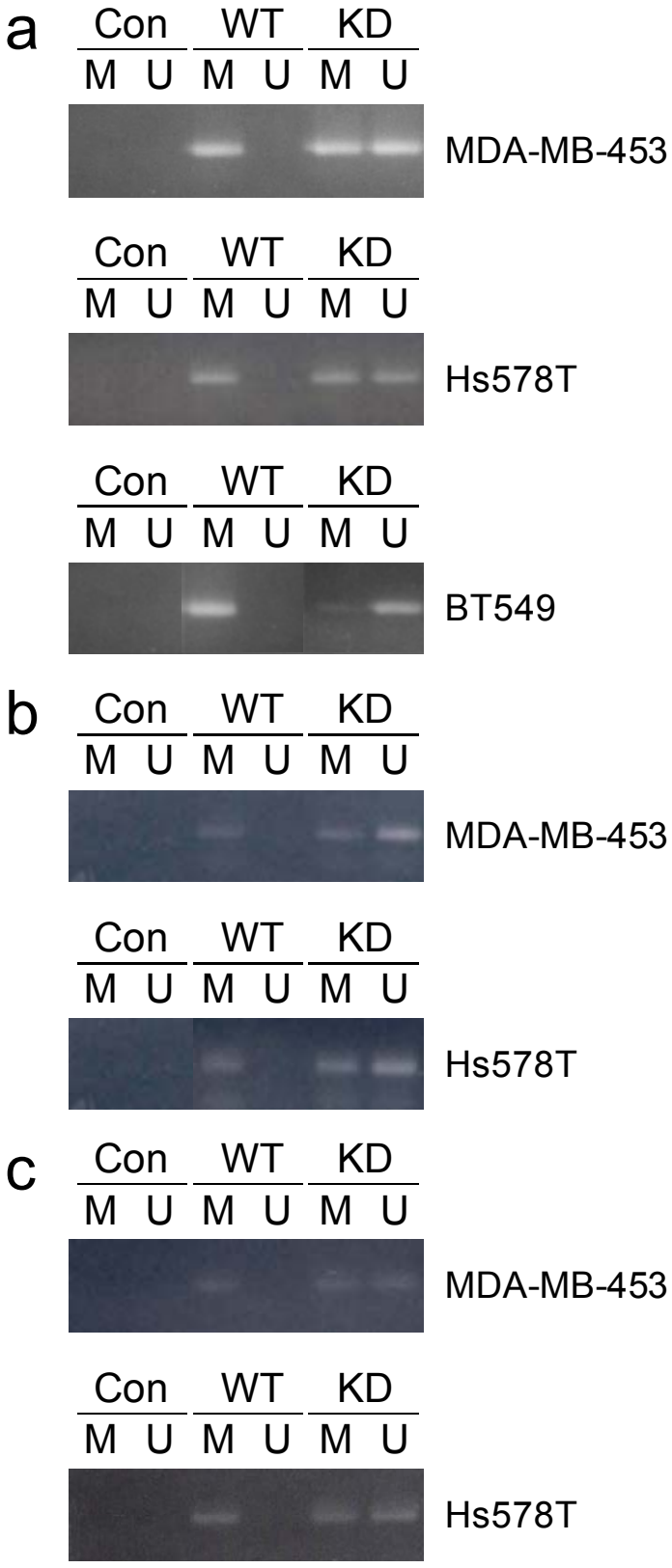
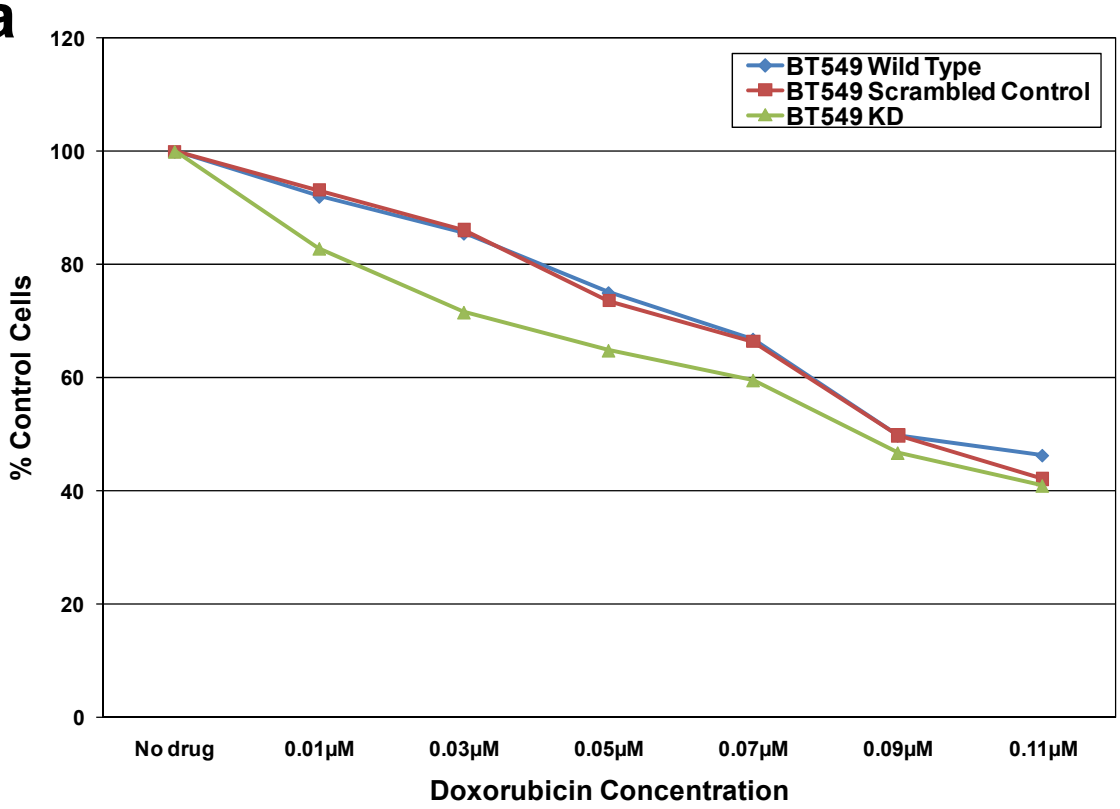
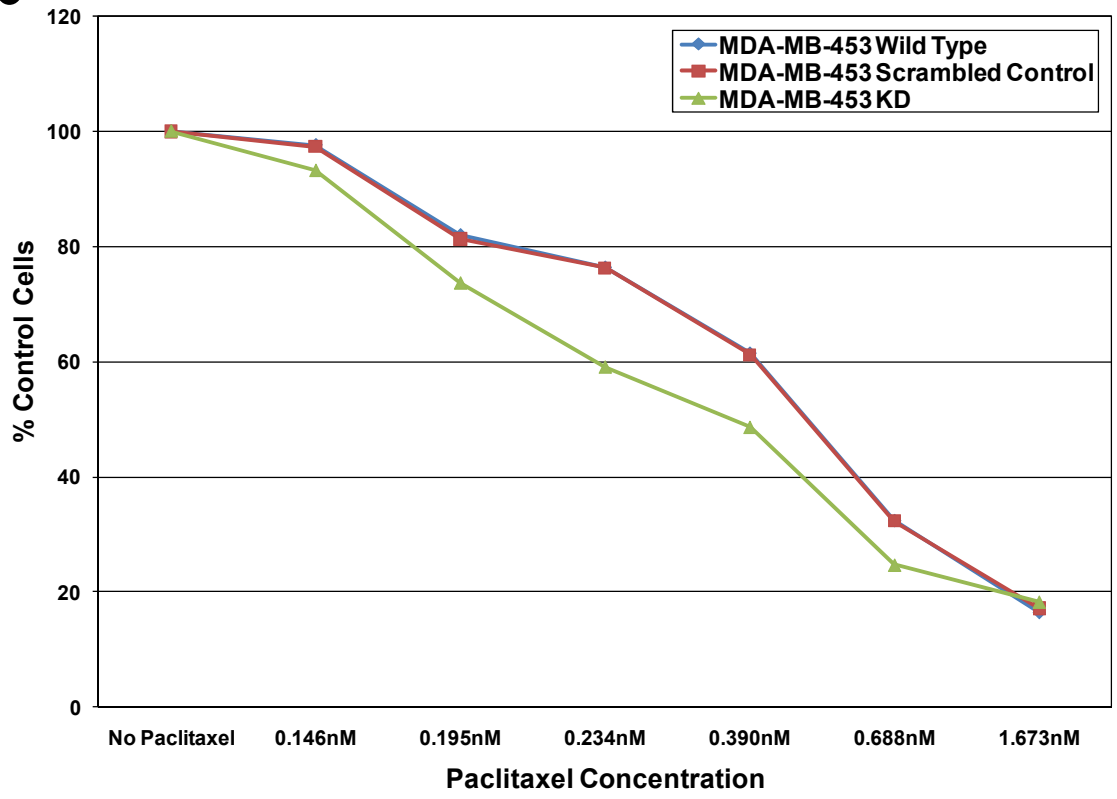

a
Con WT KD
M U M U M U
MDA-MB-453
Con WT KD
M U M U M U
Hs578T
Con WT KD
M U M U M U
BT549
b
Con WT KD
M U M U M U
MDA-MB-453
Con WT KD
M U M U M U
Hs578T
c
Con WT KD
M U M U M U
MDA-MB-453
Con WT KD
M U M U M U
Hs578T

Figure 3.5. *RNAi-mediated Knockdown of DNMT3b Results in Demethylation of Methylation-Sensitive Genes in Hypermethylator Breast Cancer Cell Lines.* Representative agarose gels of methylation-specific PCR (MSP) products corresponding to *CST6, ESR1,* and *SCNN1A* are shown. M = methylated MSP product, U = unmethylated MSP product. The abbreviations are as follows: Con = no DNA control, WT = wild-type, KD = DNMT3b knockdown. **(a)** Changes in methylation status of *CST6* in MDA-MB-453, Hs578T, and BT549 cells after DNMT3b knockdown. **(b)** Changes in methylation status of *ESR1* in MDA-MB-453 and Hs578T cells after DNMT3b knockdown. **(c)** Changes in methylation status of *SCNN1A* in MDA-MB-453 and Hs578T cells after DNMT3b knockdown.

a


BT549 Wild Type
BT549 Scrambled Control
BT549 KD
% Control Cells
120
100
80
60
40
20
0
No drug
0.01μM
0.03μM
0.05μM
0.07μM
0.09μM
0.11μM
Doxorubicin Concentration

b


MDA-MB-453 Wild Type
MDA-MB-453 Scrambled Control
MDA-MB-453 KD
% Control Cells
120
100
80
60
40
20
0
No Paclitaxel
0.146nM
0.195nM
0.234nM
0.390nM
0.688nM
1.673nM
Paclitaxel Concentration

C

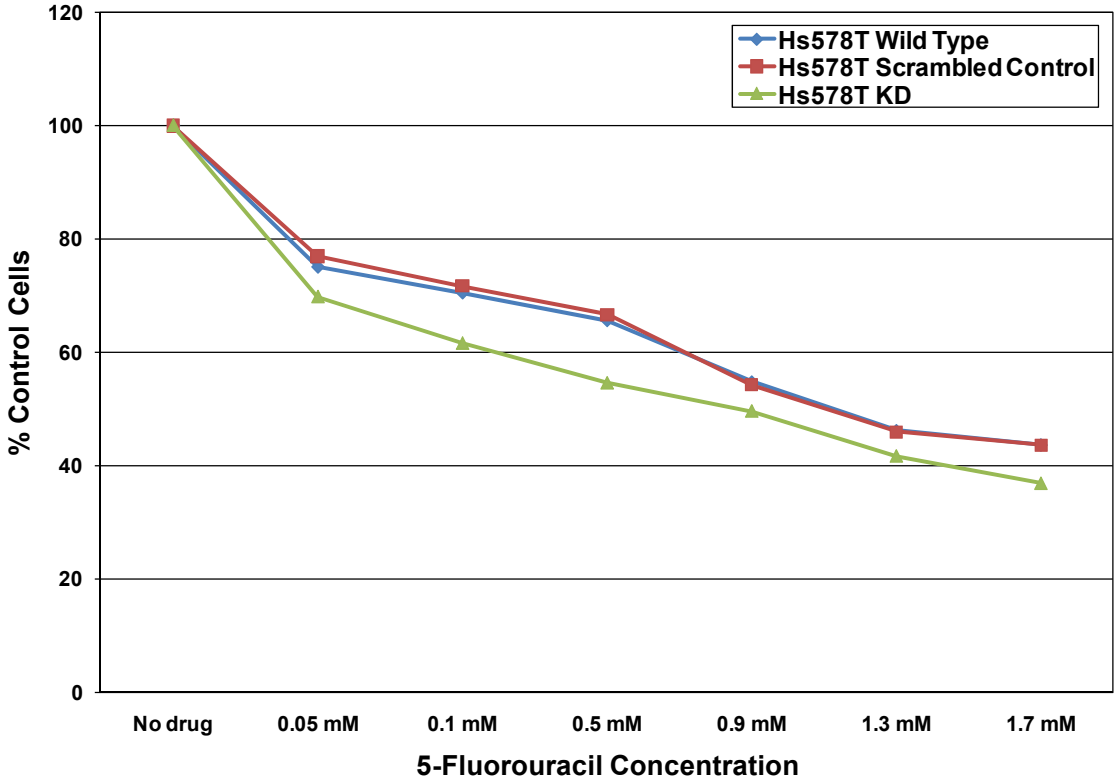
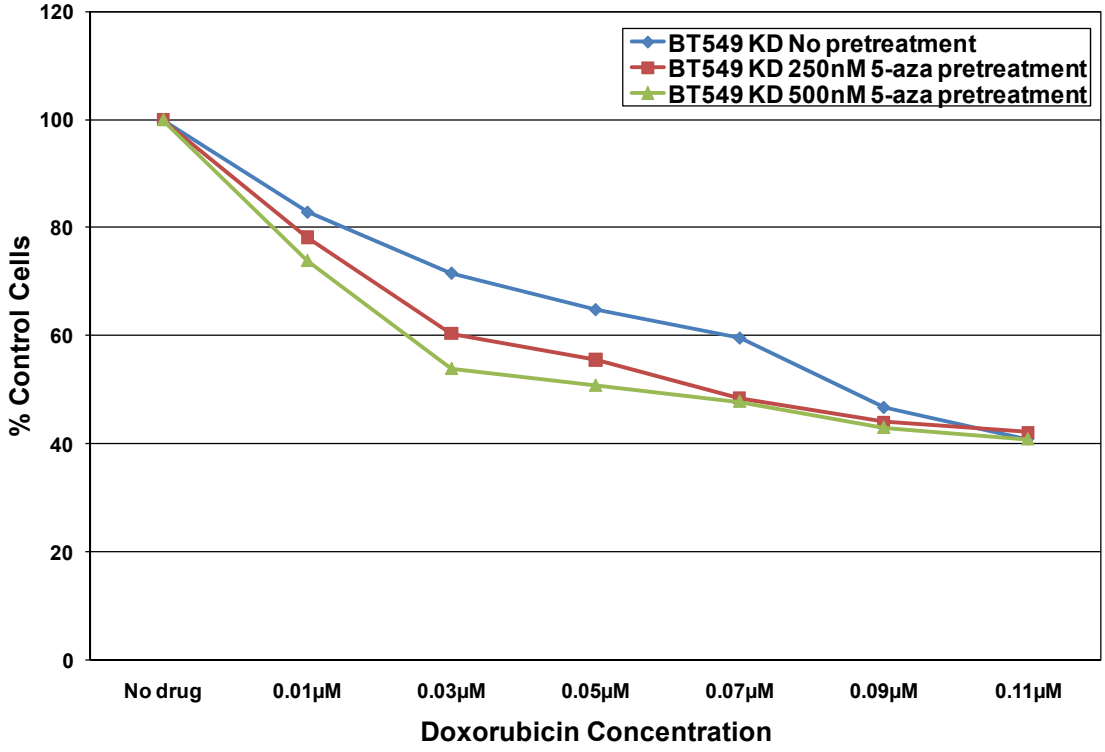
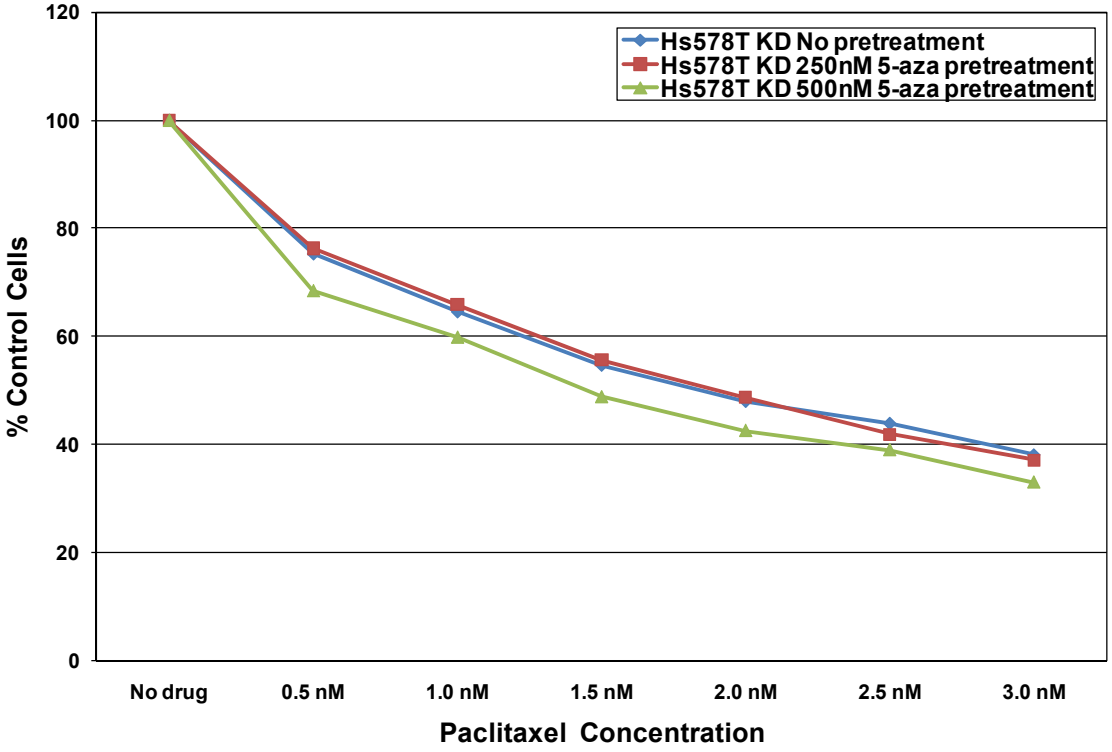

Hs578T Wild Type
Hs578T Scrambled Control
Hs578T KD
% Control Cells
120
100
80
60
40
20
0
No drug
0.05 mM
0.1 mM
0.5 mM
0.9 mM
1.3 mM
1.7 mM
5-Fluorouracil Concentration

Figure 3.6. *Hypermethylator Breast Cancer Cell Lines Exhibit Increased Sensitivity to Chemotherapeutic Drugs after DNMT3b Knockdown.* The blue lines represent wild-type breast cancer cells, red lines represent cells transfected with scrambled control vector, and green lines represent cells after DNMT3b knockdown. Changes in chemotherapeutic sensitivity are shown as percentage of viable cells remaining (relative to wild-type control cells) after 72 hrs of drug exposure for the indicated doses. (**a**) BT549 breast cancer cells show increased DOX efficacy after DNMT3b knockdown. (**b**) MDA-MB-453 breast cancer cells show increased PAX efficacy after DNMT3b knockdown. (**c**) Hs578T breast cancer cells show increased 5-FU efficacy after DNMT3b knockdown.

a


BT549 KD No pretreatment
BT549 KD 250nM 5-aza pretreatment
BT549 KD 500nM 5-aza pretreatment
% Control Cells
120
100
80
60
40
20
0
No drug
0.01μM
0.03μM
0.05μM
0.07μM
0.09μM
0.11μM
Doxorubicin Concentration

b


120
100
80
60
40
20
0
% Control Cells
Hs578T KD No pretreatment
Hs578T KD 250nM 5-aza pretreatment
Hs578T KD 500nM 5-aza pretreatment
No drug
0.5 nM
1.0 nM
1.5 nM
2.0 nM
2.5 nM
3.0 nM
Paclitaxel Concentration

C

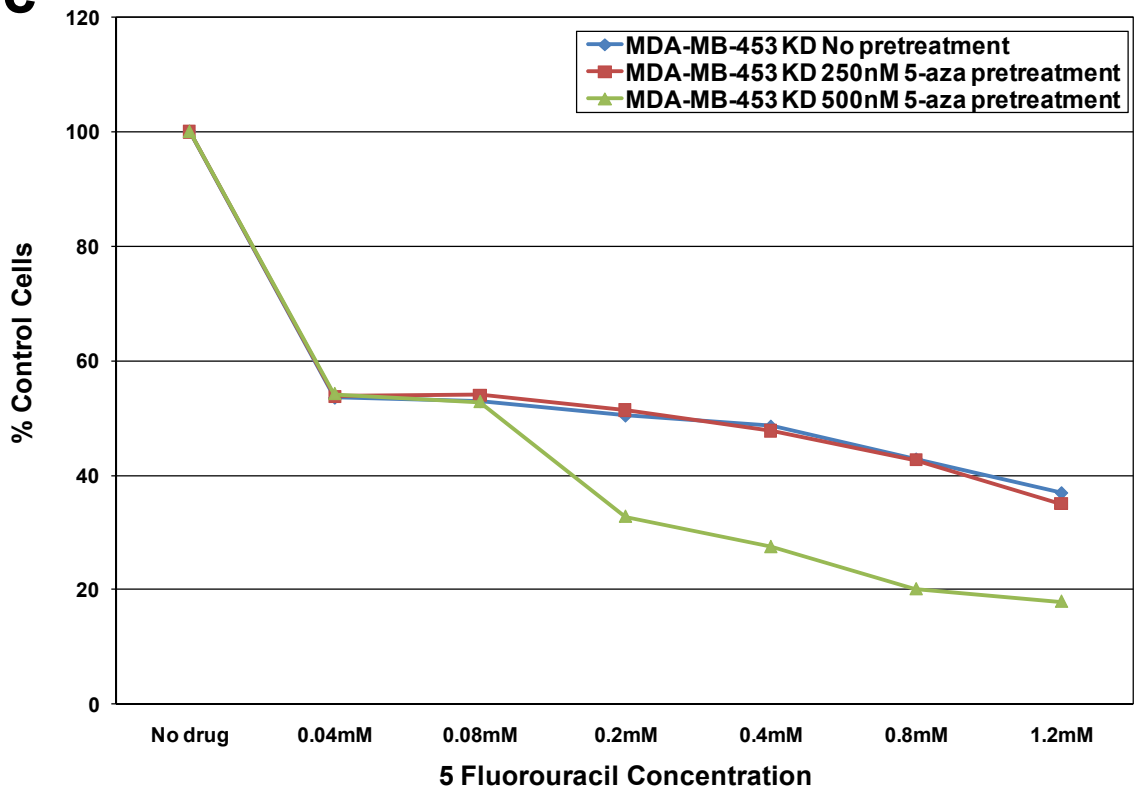
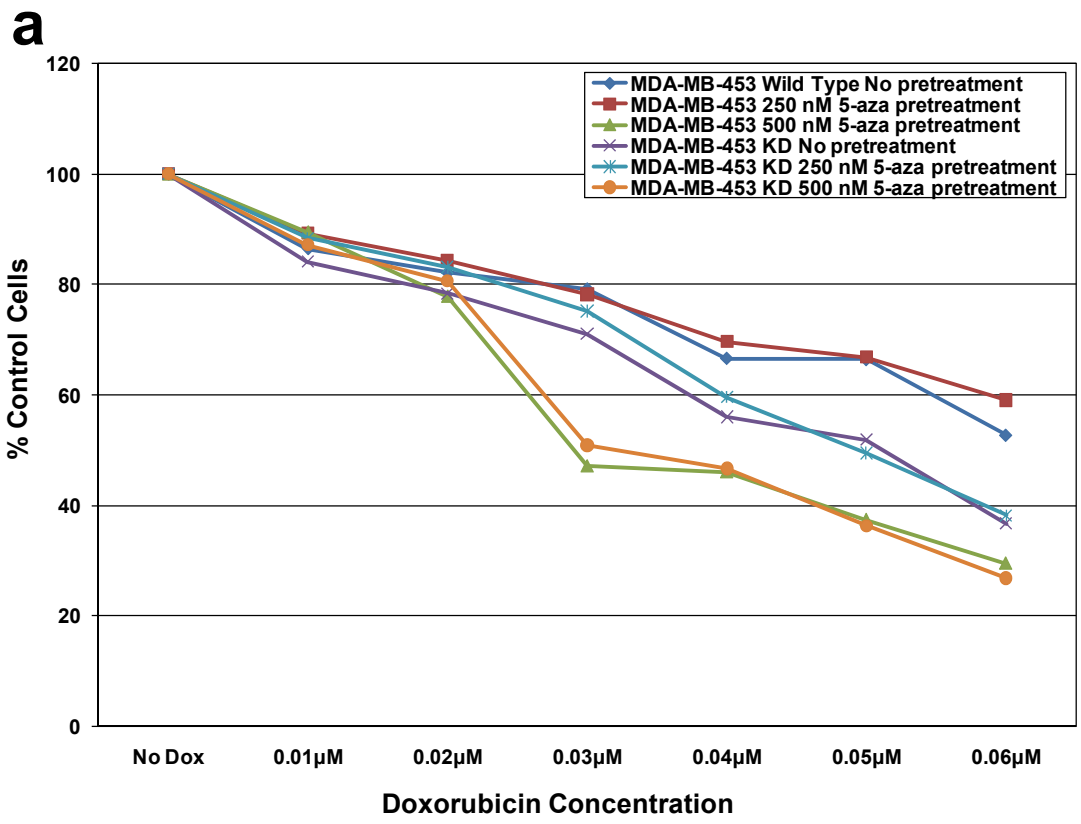
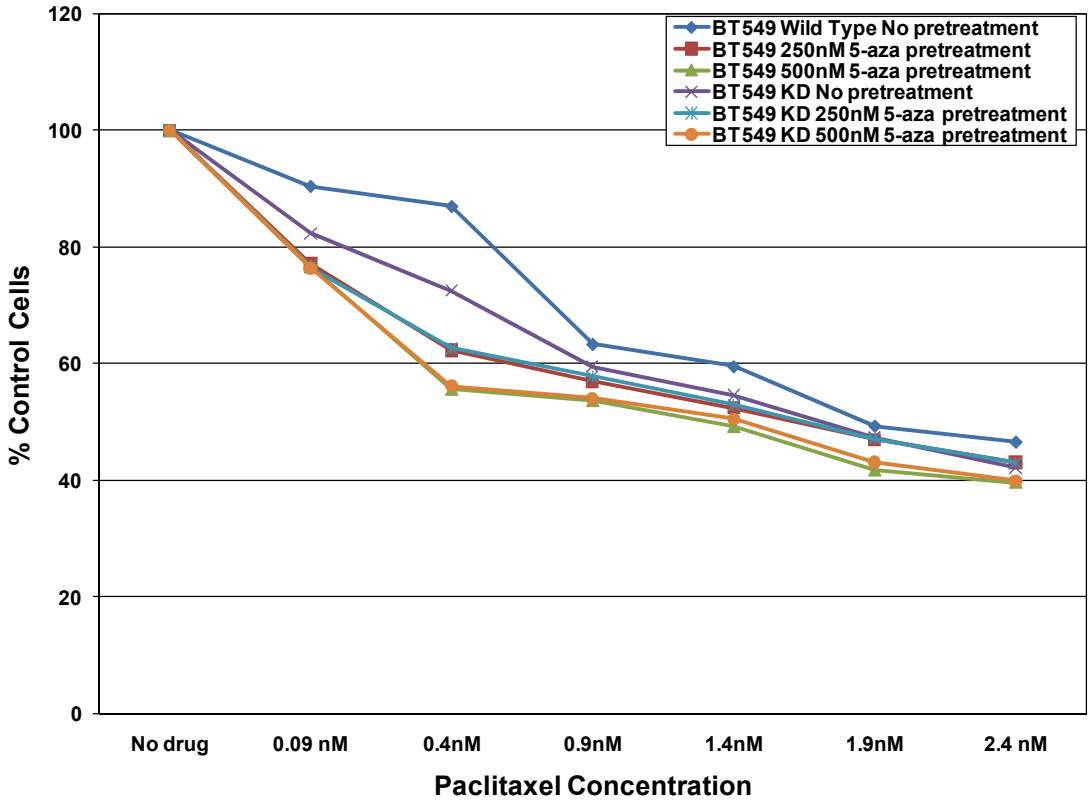

MDA-MB-453 KD No pretreatment
MDA-MB-453 KD 250nM 5-aza pretreatment
MDA-MB-453 KD 500nM 5-aza pretreatment
% Control Cells
120
100
80
60
40
20
0
No drug
0.04mM
0.08mM
0.2mM
0.4mM
0.8mM
1.2mM
5 Fluorouracil Concentration

Figure 3.7. *Demethylating Treatment With 5-Aza Sensitizes Hypermethylator Breast Cancer Cells to Chemotherapy after DNMT3b Knockdown.* The blue lines represent DNMT3b KD breast cancer cells with no pretreatment, red lines represent DNMT3b KD cells pretreated with 250 nM 5-aza for 7 days, and green lines represent DNMT3b KD cells pretreated with 500 nM 5-aza for 7 days. Changes in chemotherapeutic sensitivity are shown as percentage of viable cells remaining (relative to untreated DNMT3b KD control cells) after 72 hrs of drug exposure for the indicated doses. (**a**) BT549 DNMT3b KD breast cancer cells show increased DOX efficacy after 250 nM and 500 nM 5-aza pretreatment. (**b**) Hs578T DNMT3b KD breast cancer cells show increased PAX efficacy after 500 nM 5-aza pretreatment. (**c**) MDA-MB-453 DNMT3b KD breast cancer cells show increased 5-FU efficacy after 500 nM 5-aza pretreatment.


a
120
100
80
60
40
20
0
% Control Cells
MDA-MB-453 Wild Type No pretreatment
MDA-MB-453 250 nM 5-aza pretreatment
MDA-MB-453 500 nM 5-aza pretreatment
MDA-MB-453 KD No pretreatment
MDA-MB-453 KD 250 nM 5-aza pretreatment
MDA-MB-453 KD 500 nM 5-aza pretreatment
No Dox
0.01μM
0.02μM
0.03μM
0.04μM
0.05μM
0.06μM
Doxorubicin Concentration

b


BT549 Wild Type No pretreatment
BT549 250nM 5-aza pretreatment
BT549 500nM 5-aza pretreatment
BT549 KD No pretreatment
BT549 KD 250nM 5-aza pretreatment
BT549 KD 500nM 5-aza pretreatment
120
100
80
60
40
20
0
% Control Cells
No drug
0.09 nM
0.4nM
0.9nM
1.4nM
1.9nM
2.4 nM
Paclitaxel Concentration

C

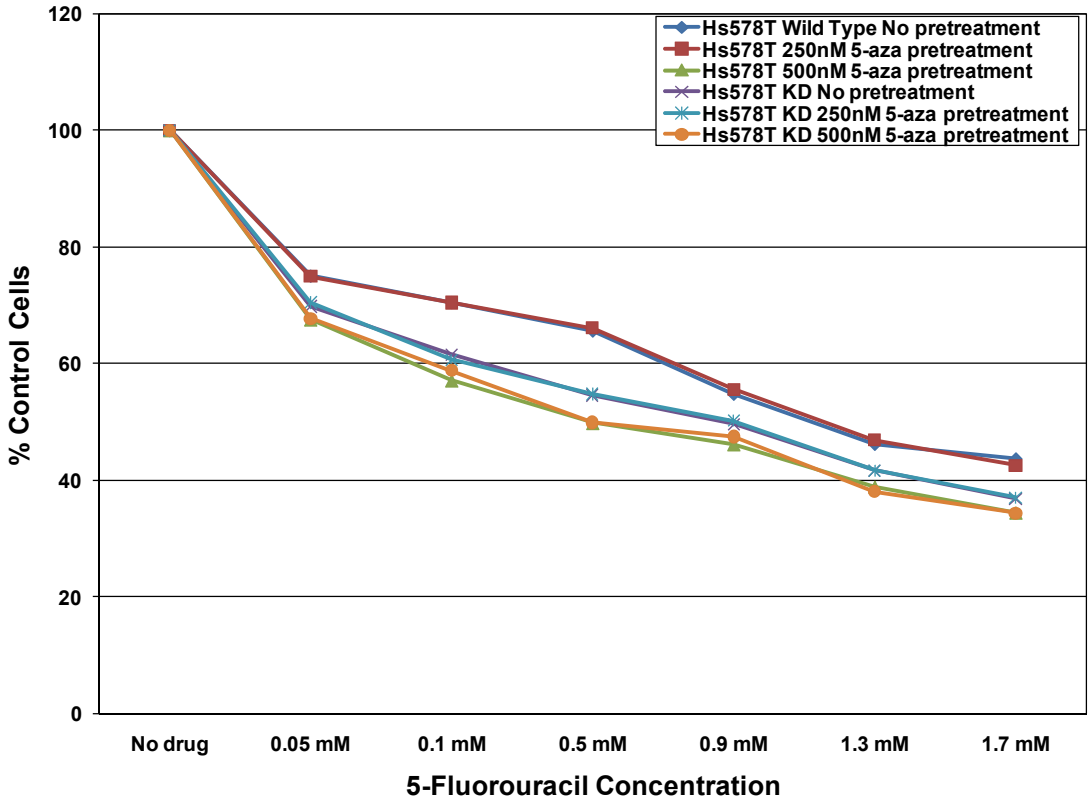

120
100
80
60
40
20
0
% Control Cells
Hs578T Wild Type No pretreatment
Hs578T 250nM 5-aza pretreatment
Hs578T 500nM 5-aza pretreatment
Hs578T KD No pretreatment
Hs578T KD 250nM 5-aza pretreatment
Hs578T KD 500nM 5-aza pretreatment
No drug
0.05 mM
0.1 mM
0.5 mM
0.9 mM
1.3 mM
1.7 mM
5-Fluorouracil Concentration

Figure 3.8. *Comparison of Sensitizing Effects of Demethylating Treatment, DNMT3b Knockdown, and Combined Demethylating Treatment and DNMT3b Knockdown in Hypermethylator Breast Cancer Cells to Chemotherapy.* The dark blue lines represent breast cancer cells with no pretreatment, red lines represent wild-type cells pretreated with 250 nM 5-aza for 7 days, green lines represent wild-type cells pretreated with 500 nM 5-aza for 7 days, purple lines represent DNMT3b KD breast cancer cells with no pretreatment, light blue lines represent DNMT3b KD cells pretreated with 250 nM 5-aza for 7 days, and orange lines represent DNMT3b KD cells pretreated with 500 nM 5-aza for 7 days. Changes in chemotherapeutic sensitivity are shown as percentage of viable cells remaining (relative to untreated control cells) after 72 hrs of drug exposure for the indicated doses. (**a**) MDA-MB-453 breast cancer cells show increased DOX efficacy. (**b**) BT549 breast cancer cells show increased PAX efficacy. (**c**) Hs578T breast cancer cells show increased 5-FU efficacy.

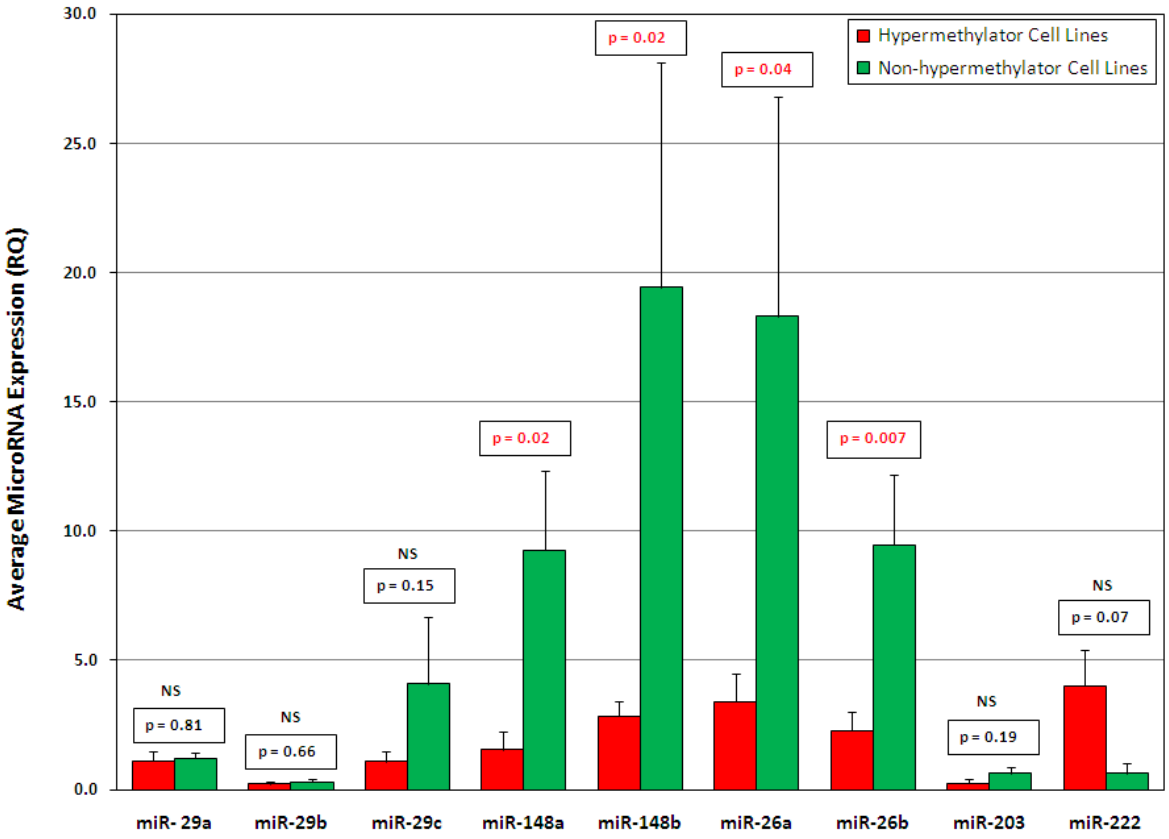
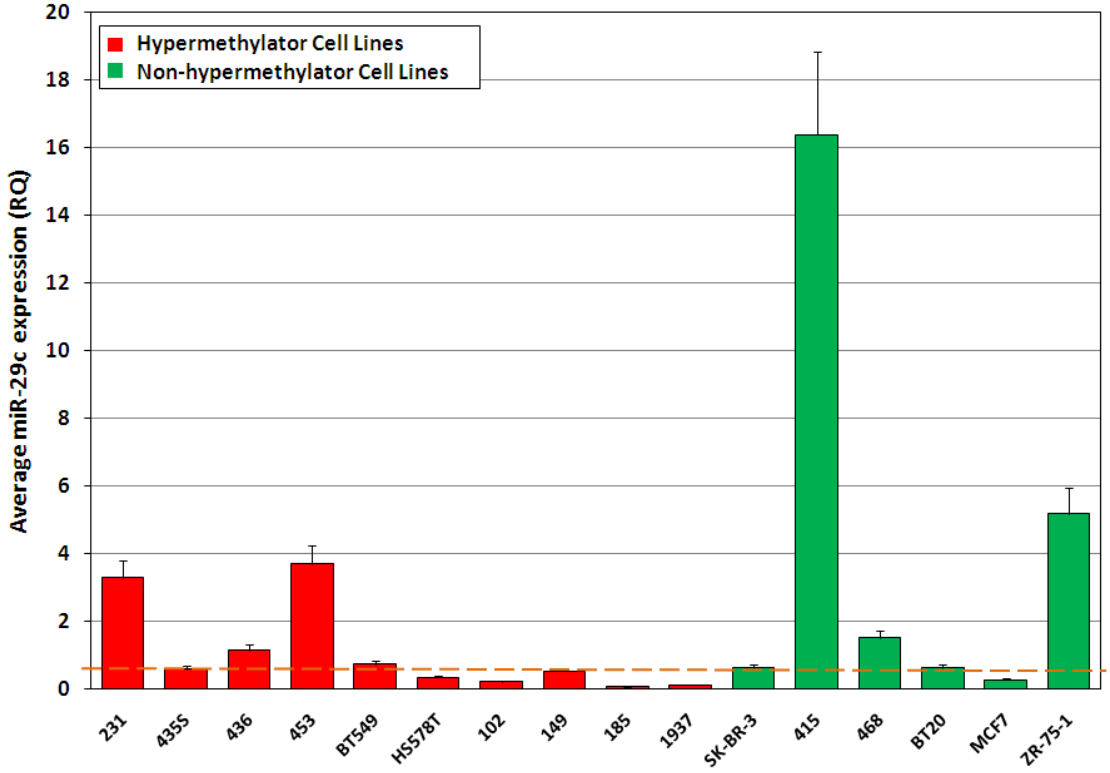
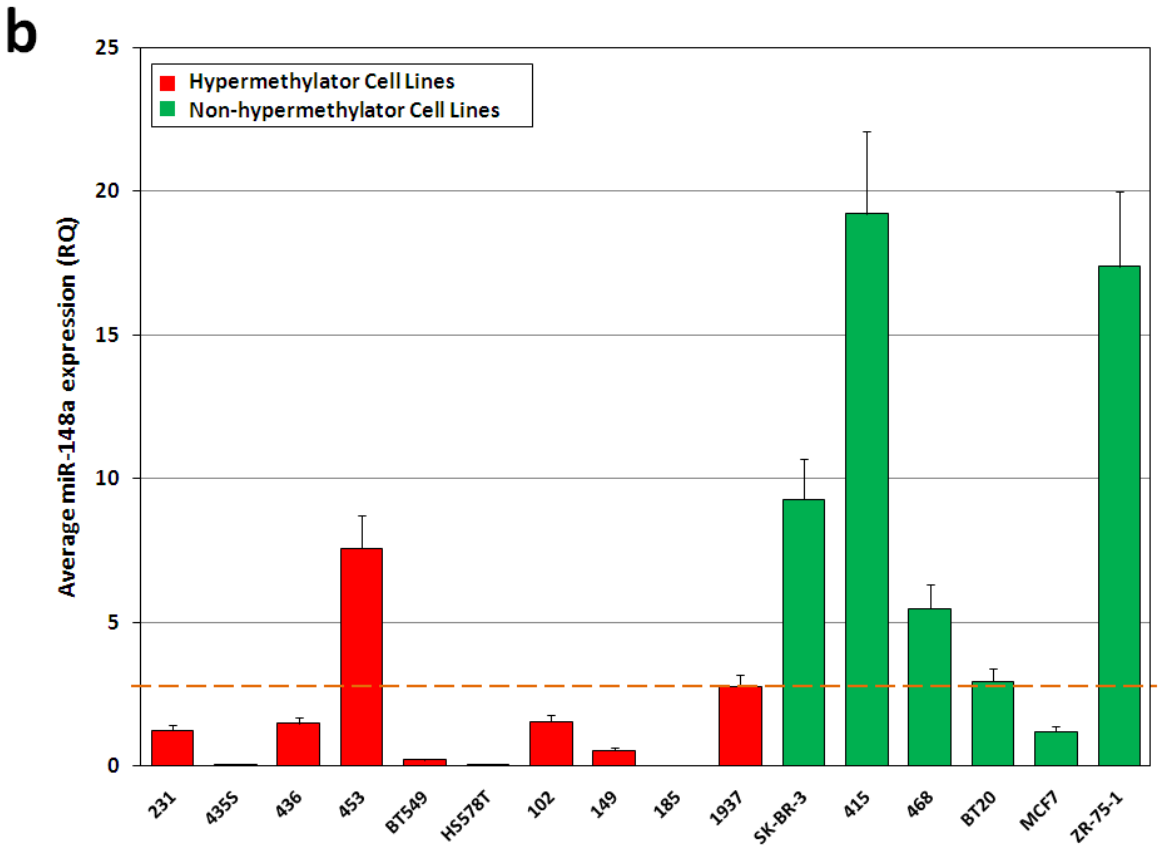
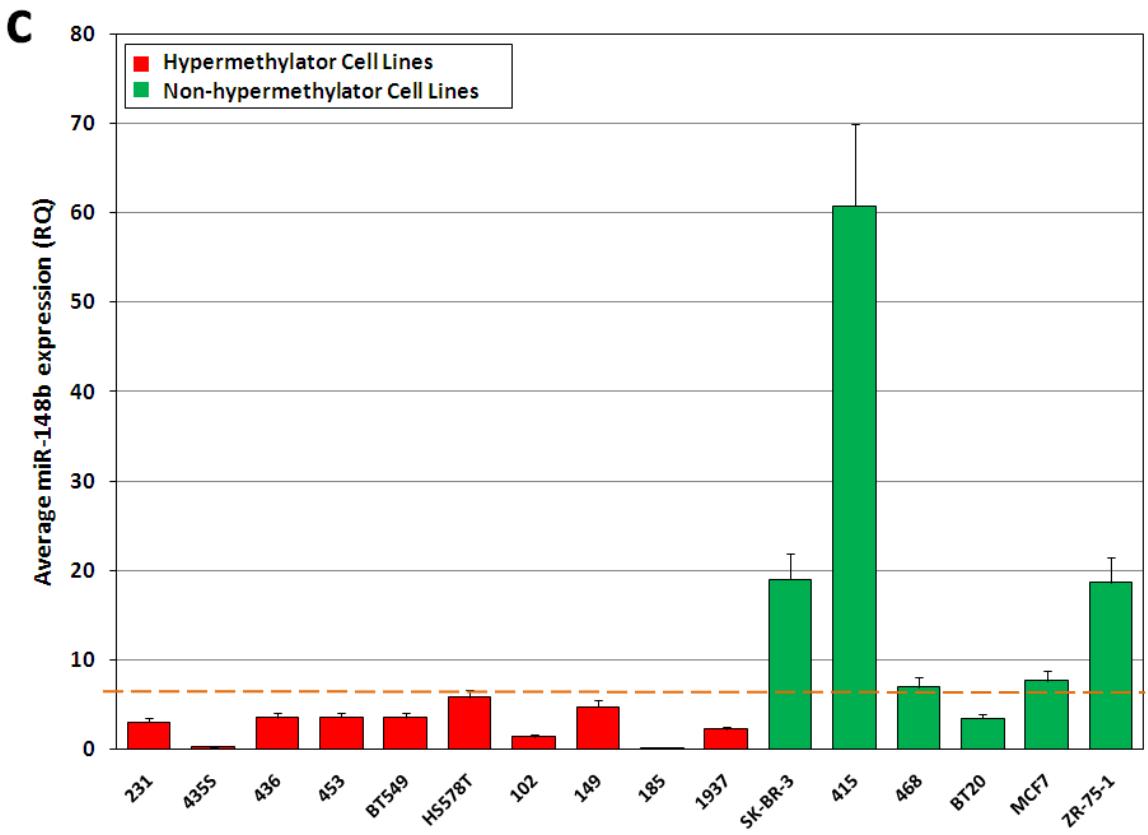

Average MicroRNA Expression (RQ)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
NS
p = 0.81
NS
p = 0.66
NS
p = 0.15
p = 0.02
p = 0.02
p = 0.04
p = 0.007
NS
p = 0.19
NS
p = 0.07
miR- 29a
miR-29b
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
miR-222

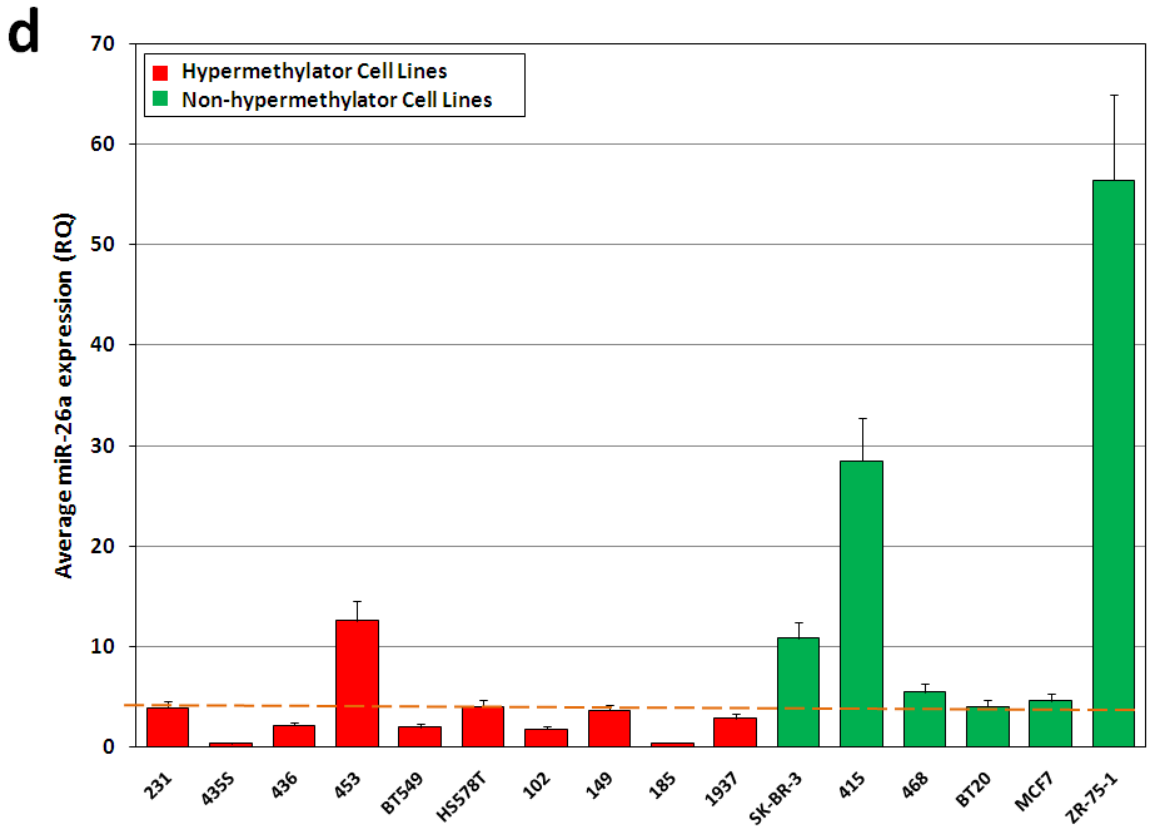
Figure 3.9. *Differential miR Expression Among Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines.* Red bars represent average miR expression among hypermethylator cell lines (n=10), and green bars represent average miR expression among non-hypermethylator cell lines (n=6). Comparison of the observed expression levels between hypermethylator cell lines and non-hypermethylator cell lines was accomplished using an unpaired t-test (two-tailed) and corresponding *p* values are given (NS – not significant).

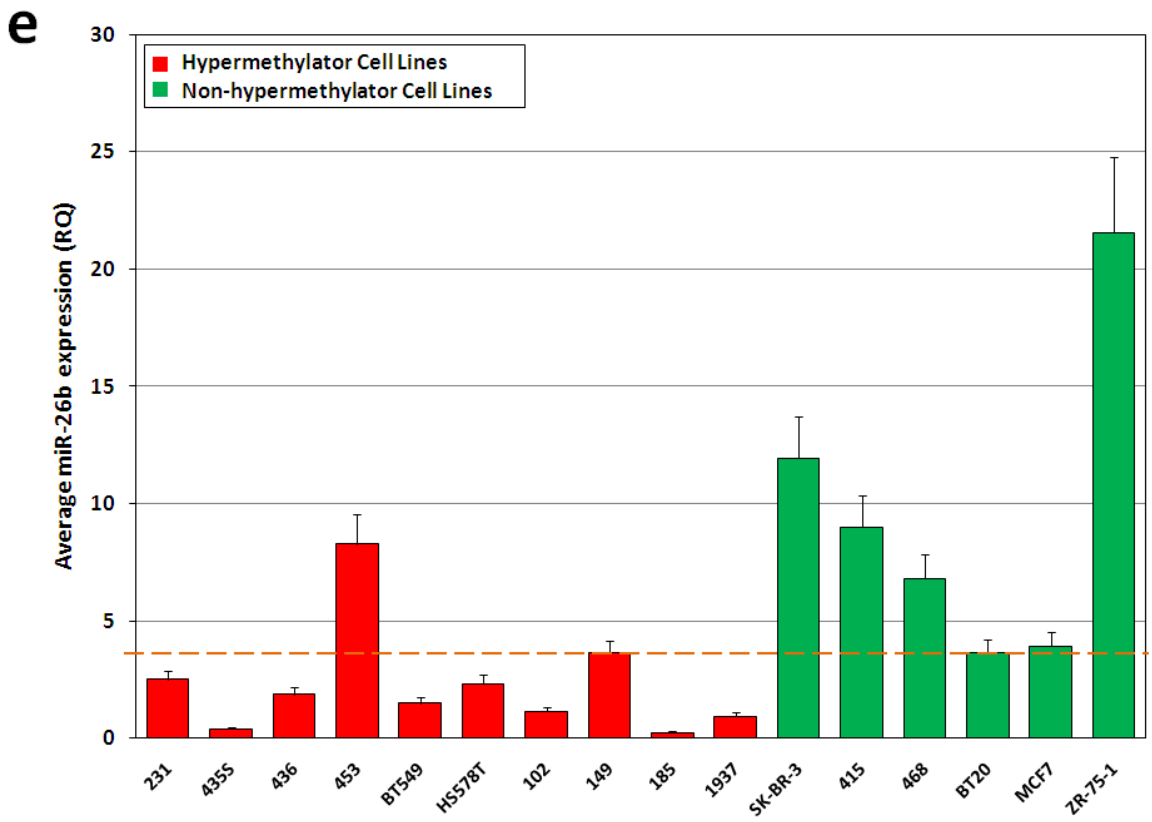
a


Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-29c expression (RQ)
20
18
16
14
12
10
8
6
4
2
0
231
435S
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1


b
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-148a expression (RQ)
25
20
15
10
5
0
231
435S
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1


C
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-148b expression (RQ)
80
70
60
50
40
30
20
10
0
231
4355
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1


d
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-26a expression (RQ)
70
60
50
40
30
20
10
0
231
435S
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1


e
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-26b expression (RQ)
30
25
20
15
10
5
0
231
435S
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1

f

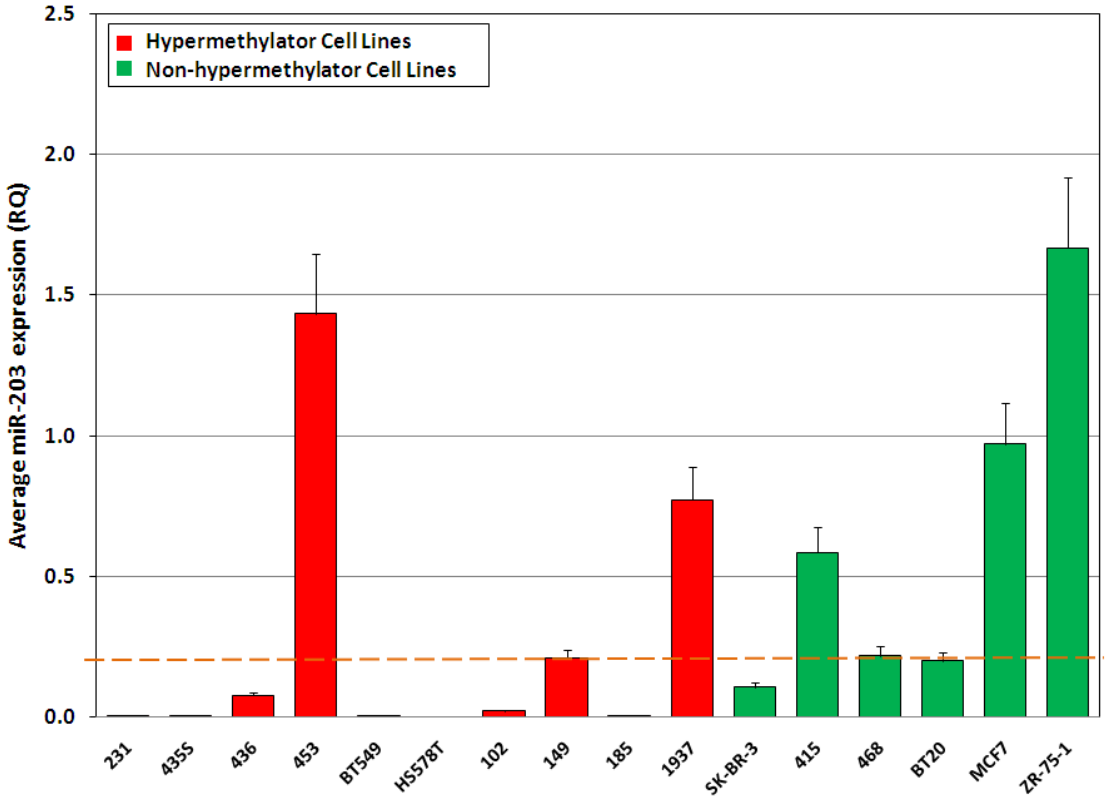
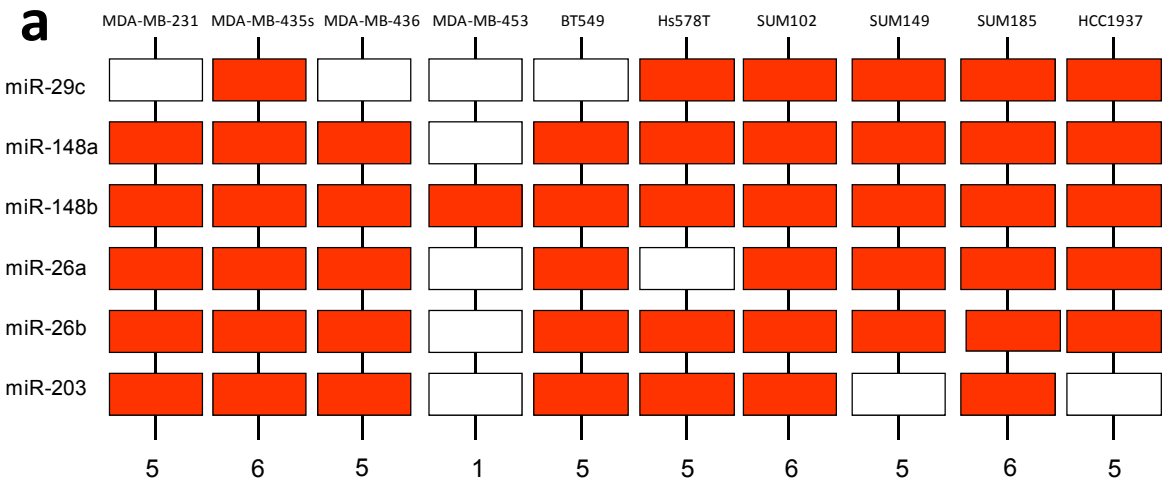

Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
Average miR-203 expression (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
231
435S
436
453
BT549
HS578T
102
149
185
1937
SK-BR-3
415
468
BT20
MCF7
ZR-75-1

Figure 3.10. *miR Expression Among Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines.* (**a-f**) Analysis of miR expression among hypermethylator and non-hypermethylator breast cancer cell lines. Hypermethylator cell lines are represented by red bars and non-hypermethylator cell lines are represented by green bars. The orange dashed line represents the optimal threshold value determined by Bayesian analysis for correct assignments related to methylation status of individual cell lines. Each real-time assay was performed in triplicate and error bars represent S.E.M. MDA-MB-231, MDA-MB-415, MDA-MB-435s, MDA-MB-436, and MDA-MB-453 cell line are designated 231, 415, 435s, 436, and 453, respectively; SUM102, SUM149, and SUM185 cell lines are represented as 102, 149, and 185, respectively; and HCC1937 is labeled 1937. (**a**) miR-29c expression, (**b**) miR-148a expression, (**c**) miR-148b expression, (**d**) miR-26a expression, (**e**) miR-26b expression, (**f**) miR-203 expression.


a
MDA-MB-231
MDA-MB-435s
MDA-MB-436
MDA-MB-453
BT549
Hs578T
SUM102
SUM149
SUM185
HCC1937
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
5
6
5
1
5
5
6
5
6
5

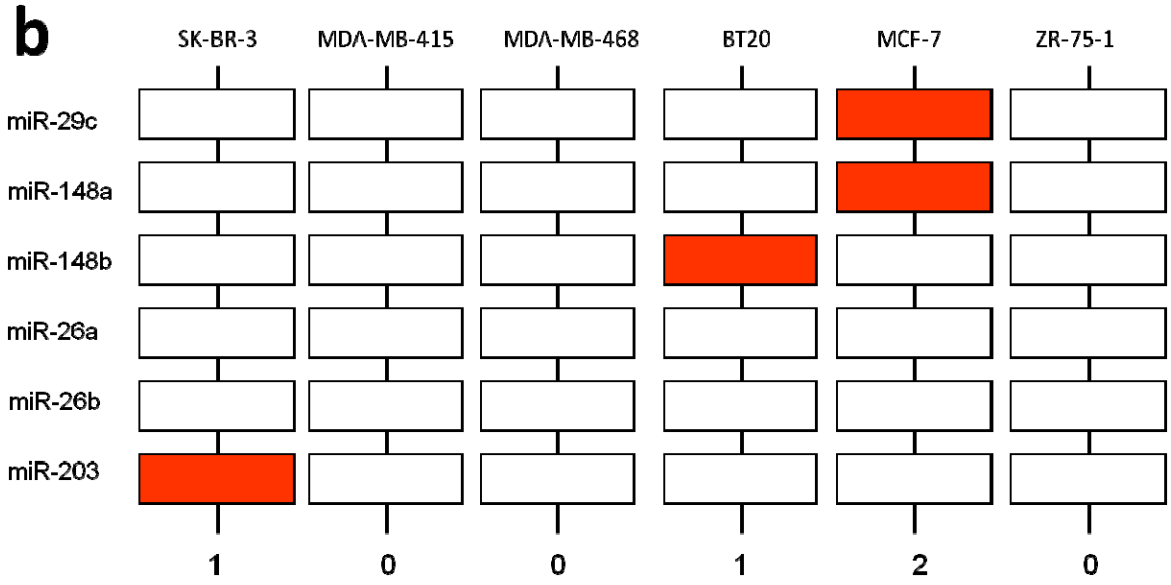
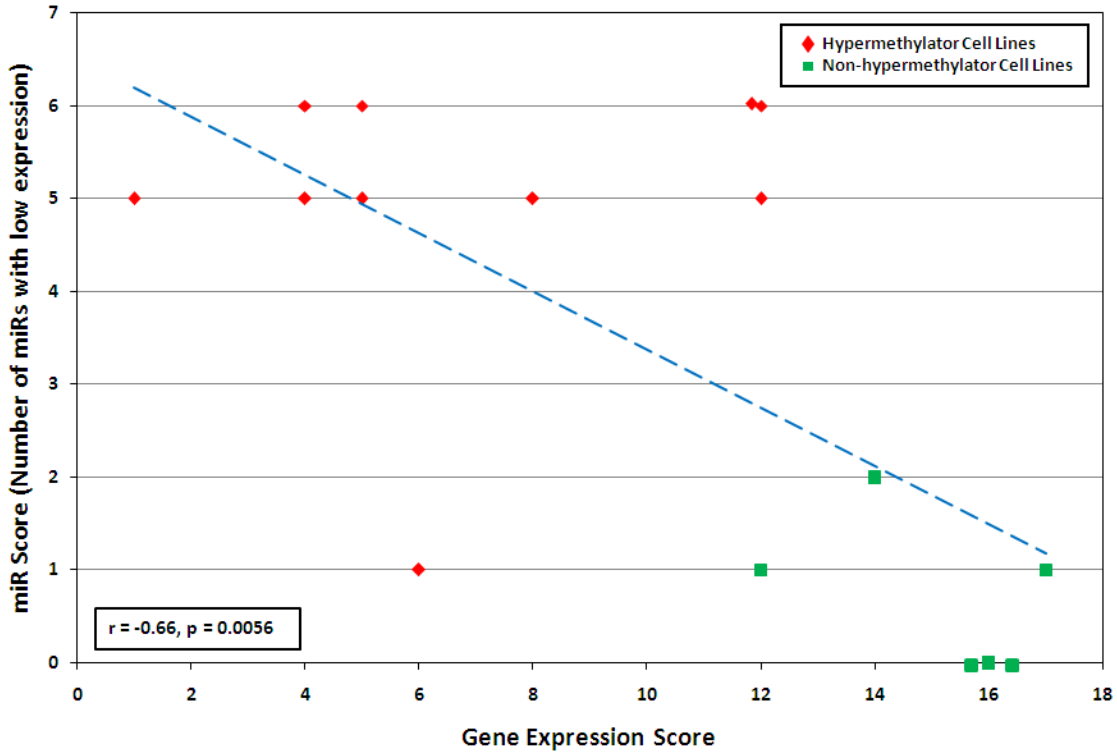

b
SK-BR-3
MDA-MB-415
MDA-MB-468
BT20
MCF-7
ZR-75-1
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
1
0
0
1
2
0

Figure 3.11. *miR Expression Patterns and miR Scores for Hypermethylator and Non-Hypermethylator Breast Cancer Cell Lines.* Red boxes indicate a measured level of expression for an individual miR that is below the threshold value established through Bayesian analysis, and white boxes indicate a measured level of expression of an individual miR that is above the threshold value established through Bayesian analysis. The numbers at the bottom of each column indicate the miR score which represents a measure of the number of miRs expressed at diminished levels in an individual cell line. (**a**) miR expression patterns and miR scores for hypermethylator breast cancer cell lines. (**b**) miR expression patterns and miR scores for non-hypermethylator breast cancer cell lines.

a


Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
miR Score (Number of miRs with low expression)
Gene Expression Score
r = -0.66, p = 0.0056
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18

b

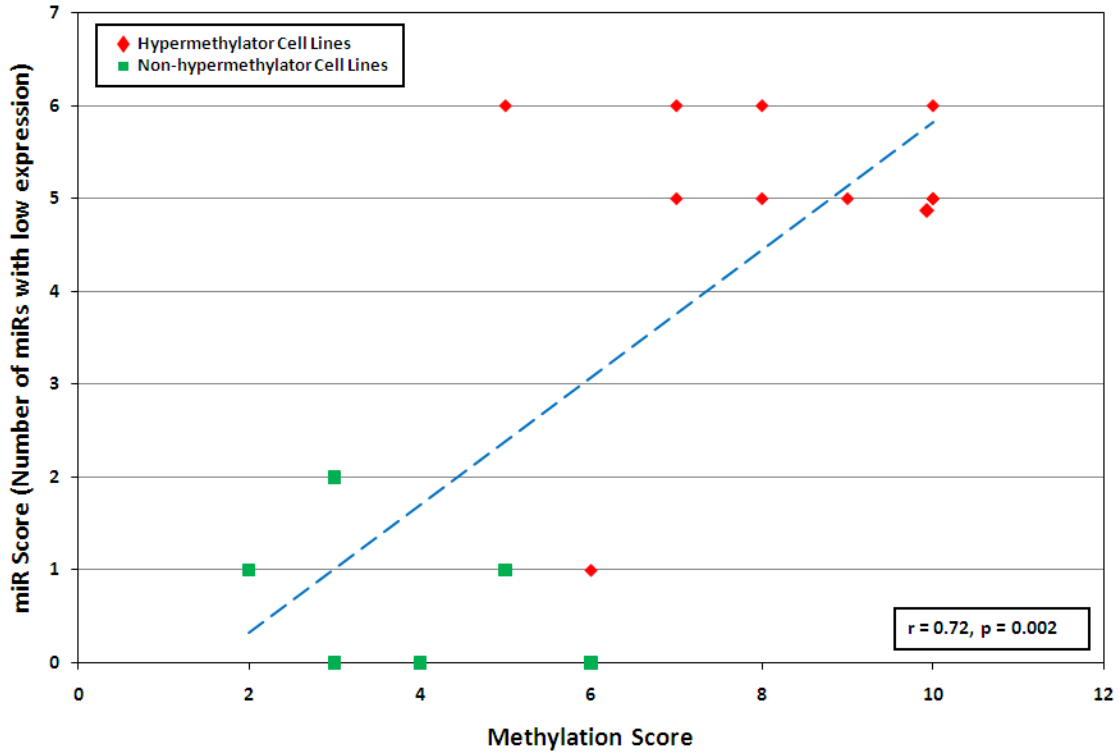
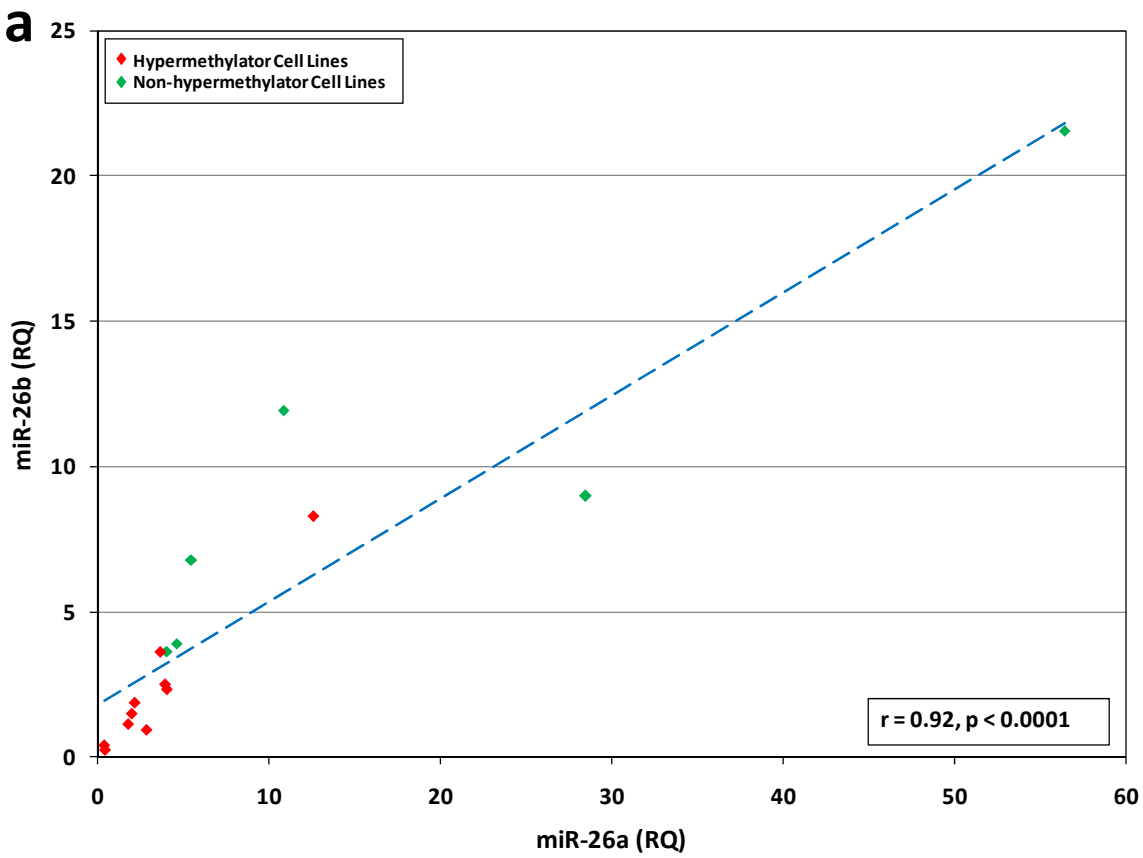
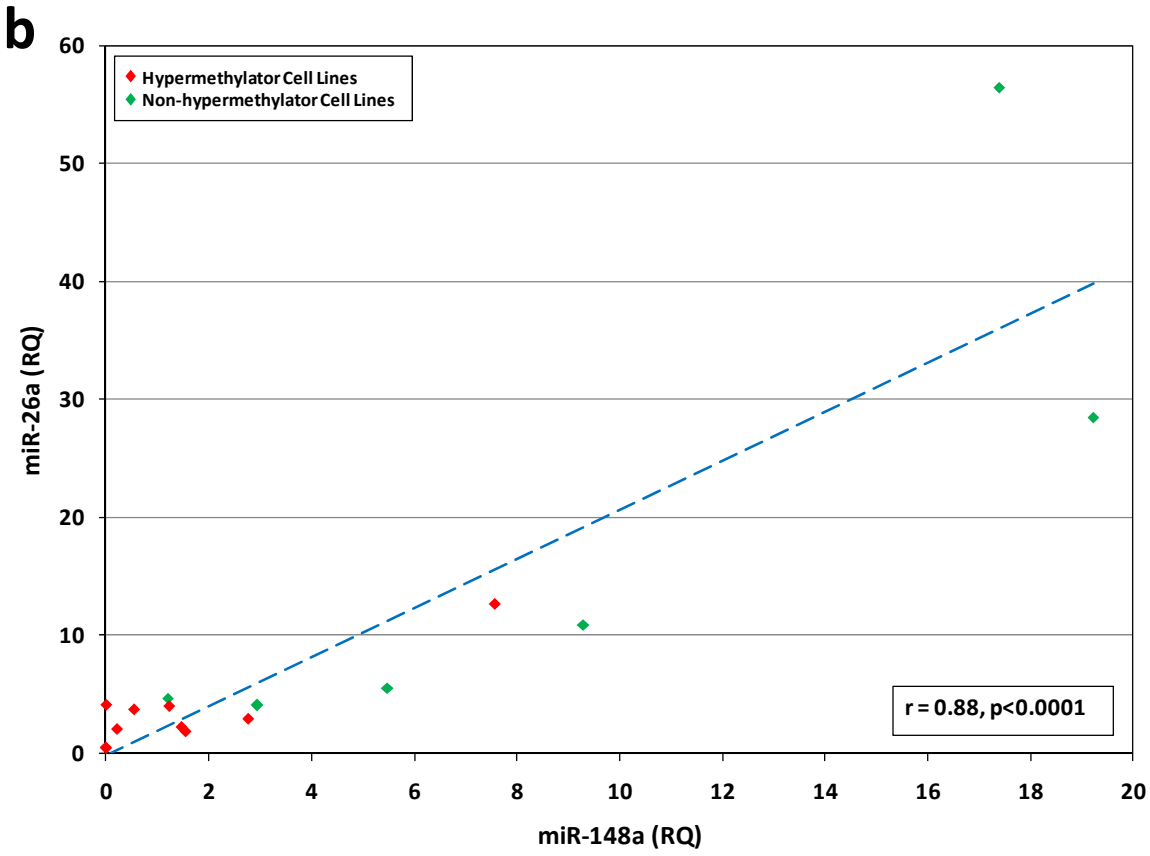
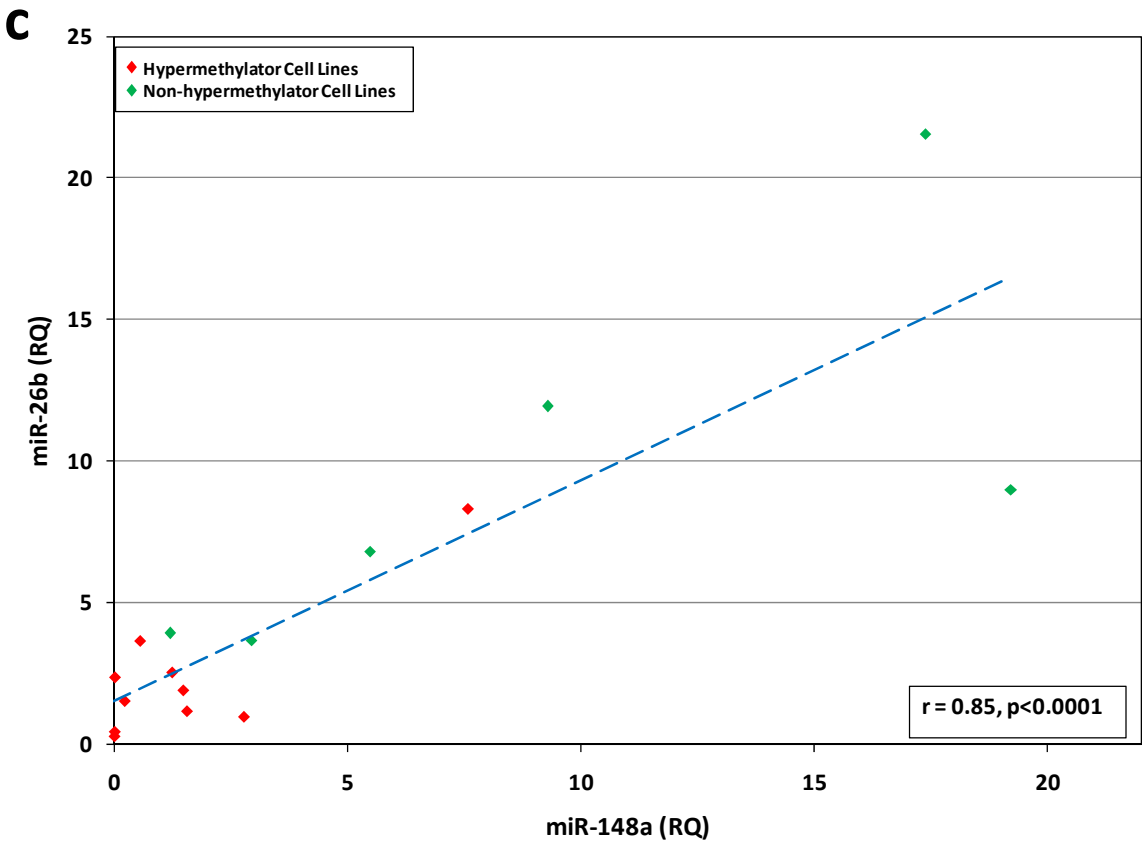
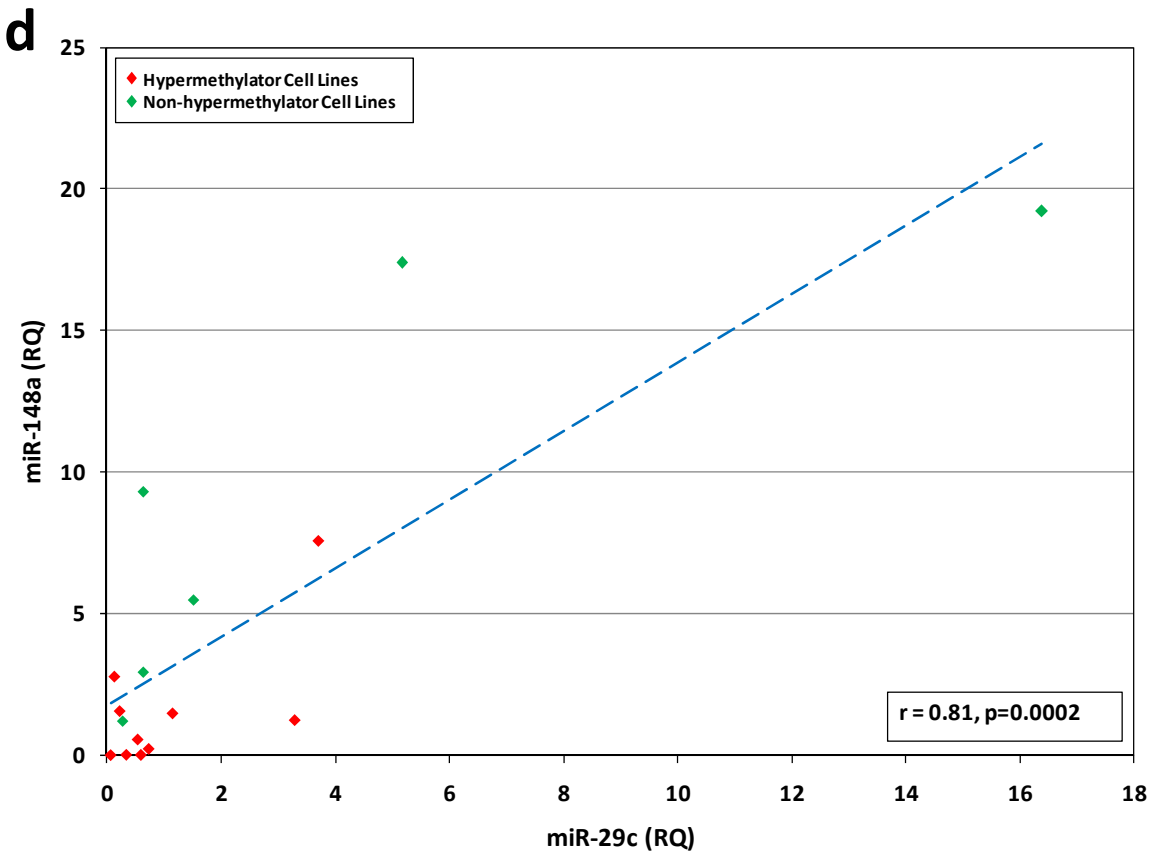

Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
miR Score (Number of miRs with low expression)
Methylation Score
r = 0.72, p = 0.002
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12

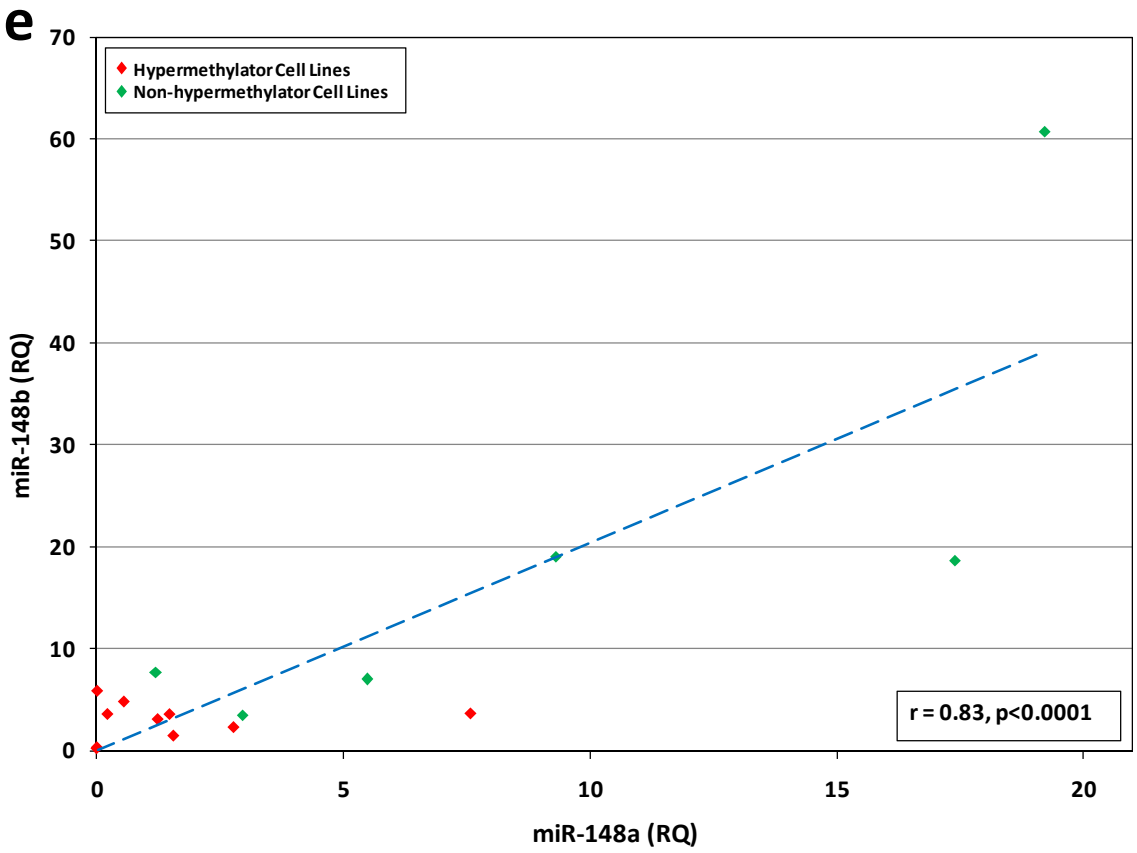
Figure 3.12. miR *Expression Patterns Correlate with Methylation-Sensitive Gene Expression Status and Promoter Methylation Status Among Breast Cancer Cell Lines*. Correlation of miR expression patterns (miR score) with gene expression levels (based on RT-PCR) and promoter methylation status (based on methylation-sensitive PCR) for methylation-sensitive genes among hypermethylator and non-hypermethylator breast cancer cell lines. Scores were calculated for differentially expressed miRs (miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203) and for well-characterized methylation sensitive genes (*CEACAM6, CDH1, CST6, ESR1, GNA11, MUC1, MYB, TFF3,* and *SCNNIA)*. Methylation-sensitive gene expression scores and promoter methylation scores were taken from previous studies [150]. (**a**) Relationship between miR score and gene expression score among hypermethylator cell lines (red diamonds) and non-hypermethylator cell lines (green squares). (**b**) Relationship between miR score and promoter methylation status among hypermethylator cell lines (red diamonds) and non-hypermethylator cell lines (green squares).


a
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
r = 0.92, p < 0.0001
miR-26b (RQ)
miR-26a (RQ)
0
5
10
15
20
25
0
10
20
30
40
50
60


b
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
r = 0.88, p<0.0001
miR-26a (RQ)
miR-148a (RQ)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20


c
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
miR-26b (RQ)
miR-148a (RQ)
r = 0.85, p<0.0001
0
5
10
15
20
25


d
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
r = 0.81, p=0.0002
miR-148a (RQ)
miR-29c (RQ)
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16
18


e
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
miR-148b (RQ)
miR-148a (RQ)
r = 0.83, p<0.0001
0
10
20
30
40
50
60
70
0
5
10
15
20

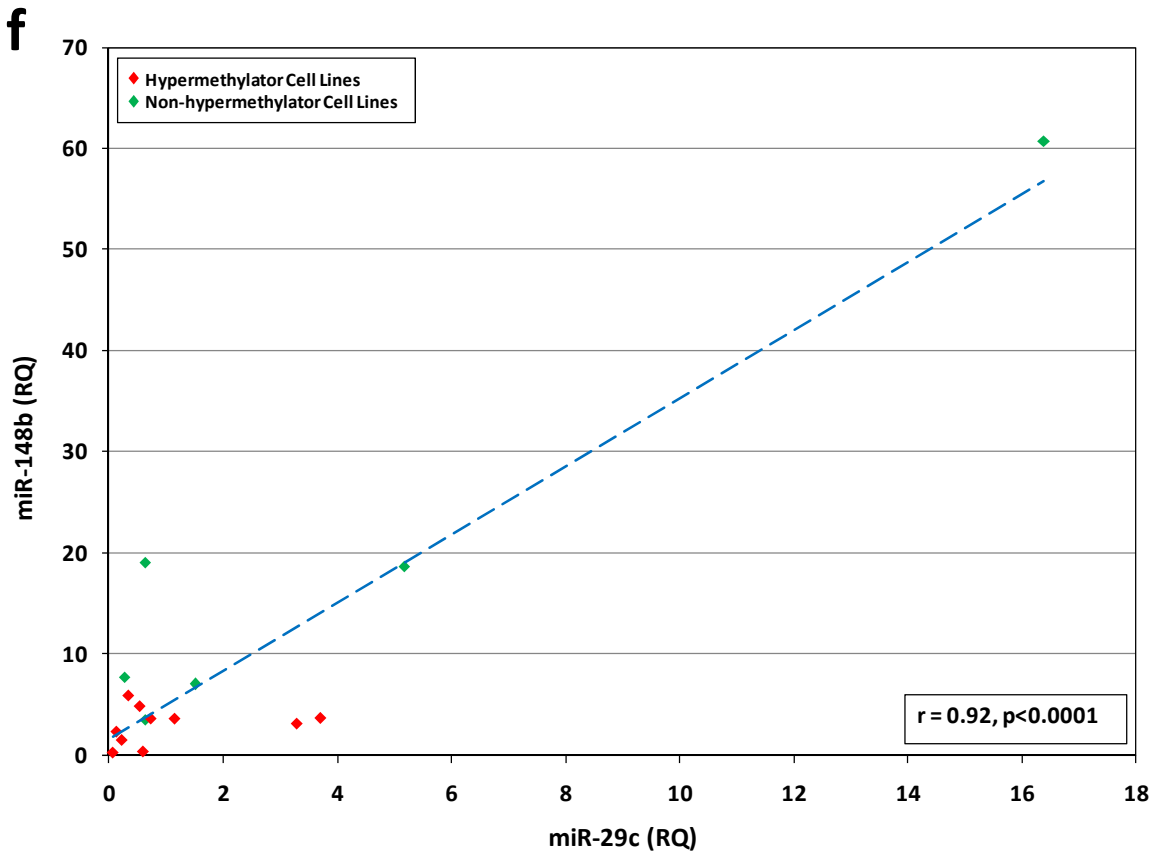
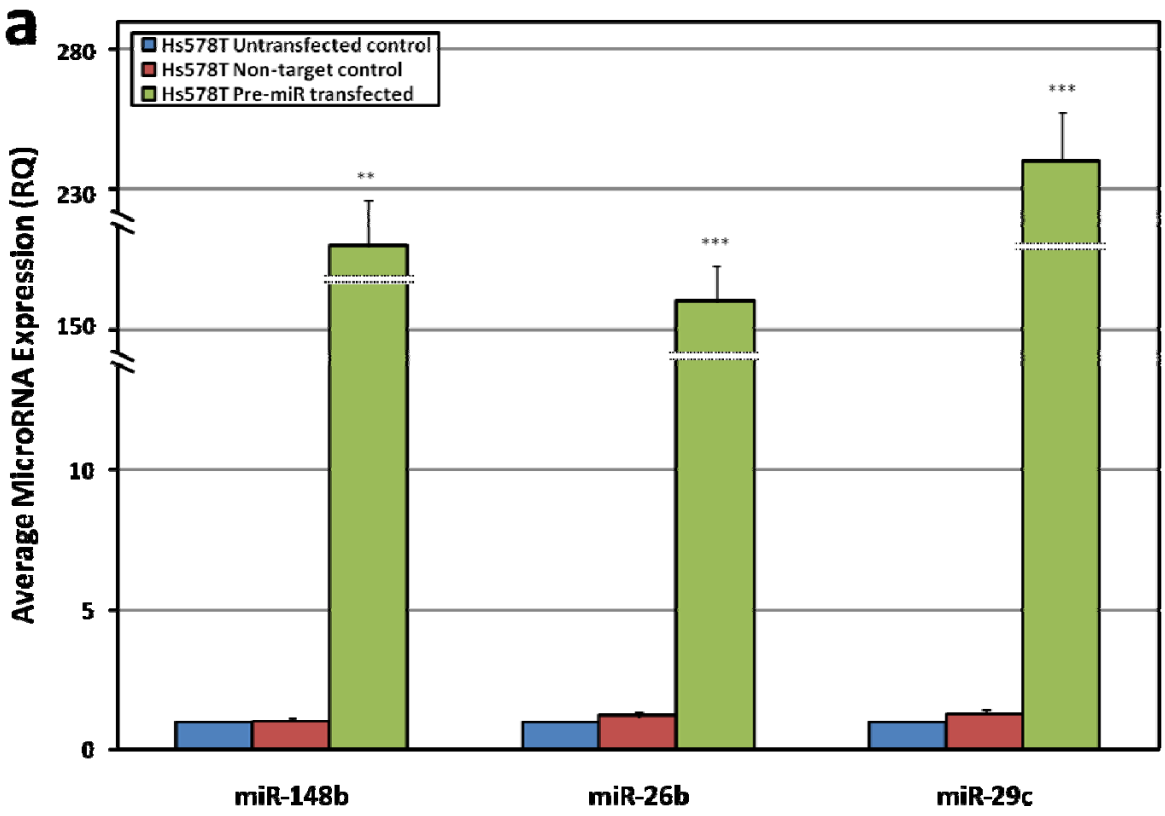
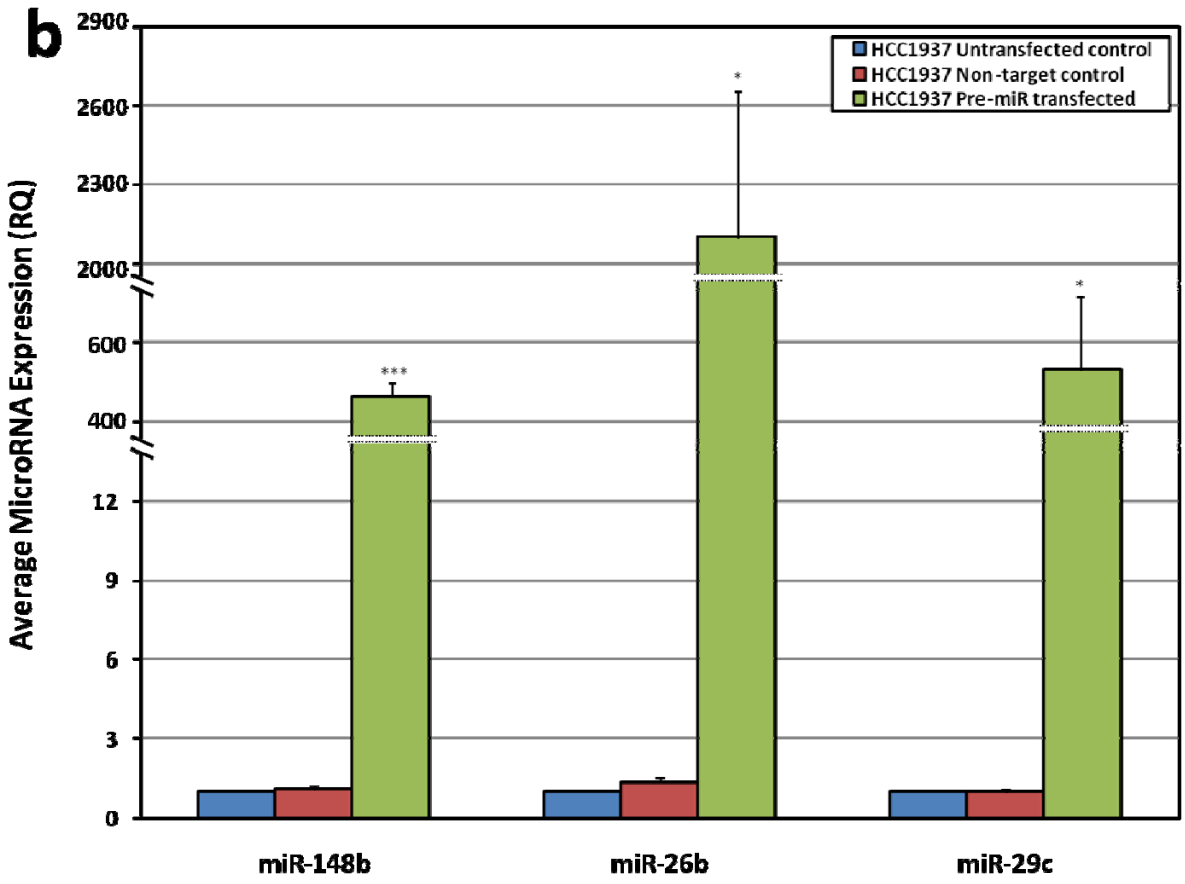

f
Hypermethylator Cell Lines
Non-hypermethylator Cell Lines
miR-148b (RQ)
miR-29c (RQ)
r = 0.92, p<0.0001
0
10
20
30
40
50
60
70
0
2
4
6
8
10
12
14
16
18

Figure 3.13. *Co-regulation of miR Expression.* Hypermethylator cell lines (red diamonds) and non-hypermethylator cell lines (green diamonds) demonstrate a statistically significant relationship between miR expression levels. The blue dashed line represents the linear regression trend line (*p* values are indicated). (**a**) Association of expression between miR-26a and miR-26b, (**b**) Association of expression between miR-148a and miR-26a, (**c**) Association of expression between miR-148a and miR-26b, (**d**) Association of expression between miR-29c and miR-148a, (**e**) Association of expression between miR-148a and miR-148b, (**f**) Association of expression between miR-29c and miR-148b.


a
Hs578T Untransfected control
Hs578T Non-target control
Hs578T Pre-miR transfected
Average MicroRNA Expression (RQ)
280
230
150
10
5
0
**
***
***
miR-148b
miR-26b
miR-29c


b
HCC1937 Untransfected control
HCC1937 Non-target control
HCC1937 Pre-miR transfected
Average MicroRNA Expression (RQ)
2900
2600
2300
2000
600
400
12
9
6
3
0
***
*
*
miR-148b
miR-26b
miR-29c

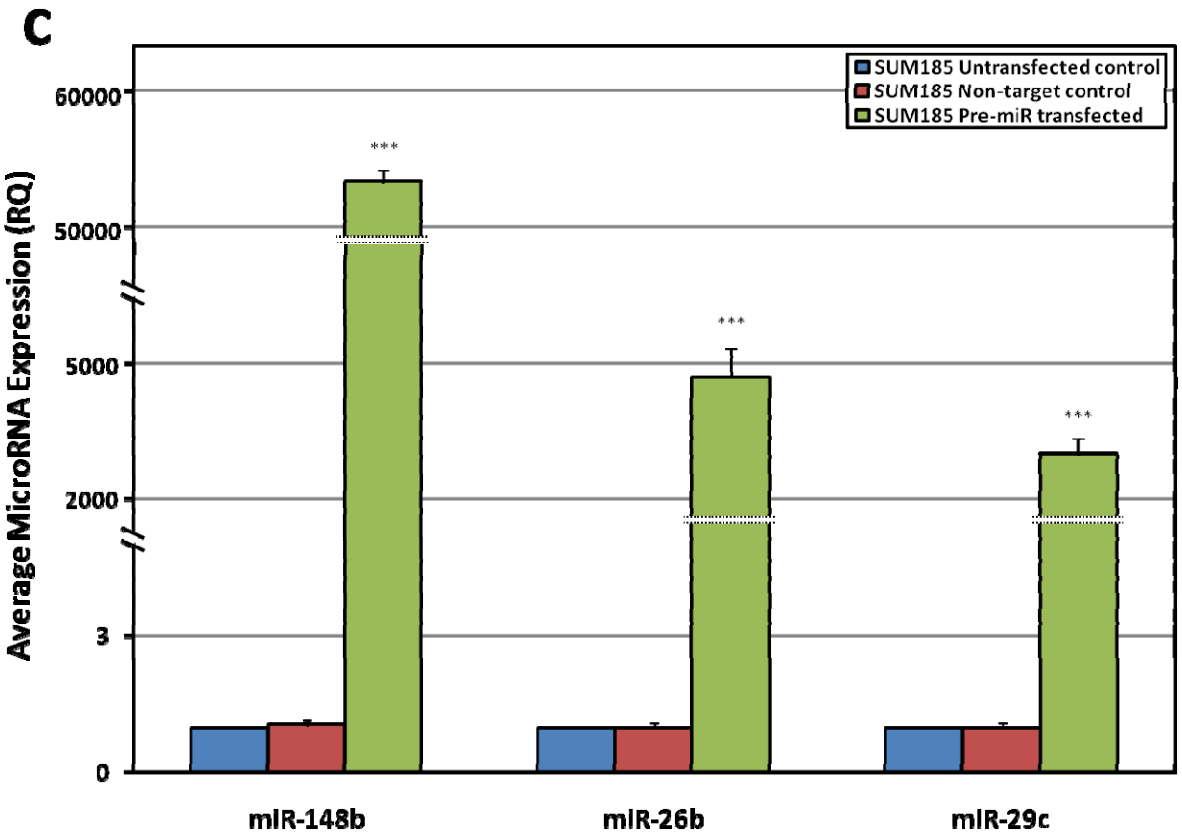
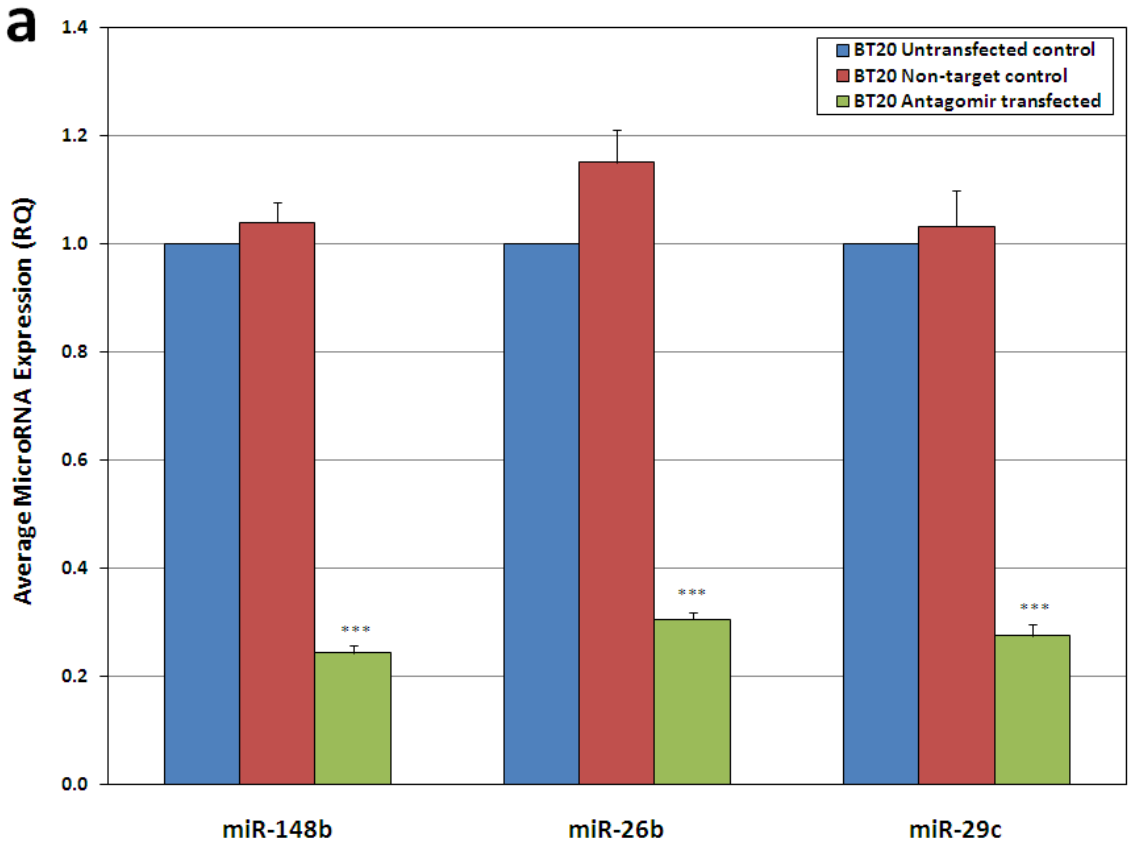
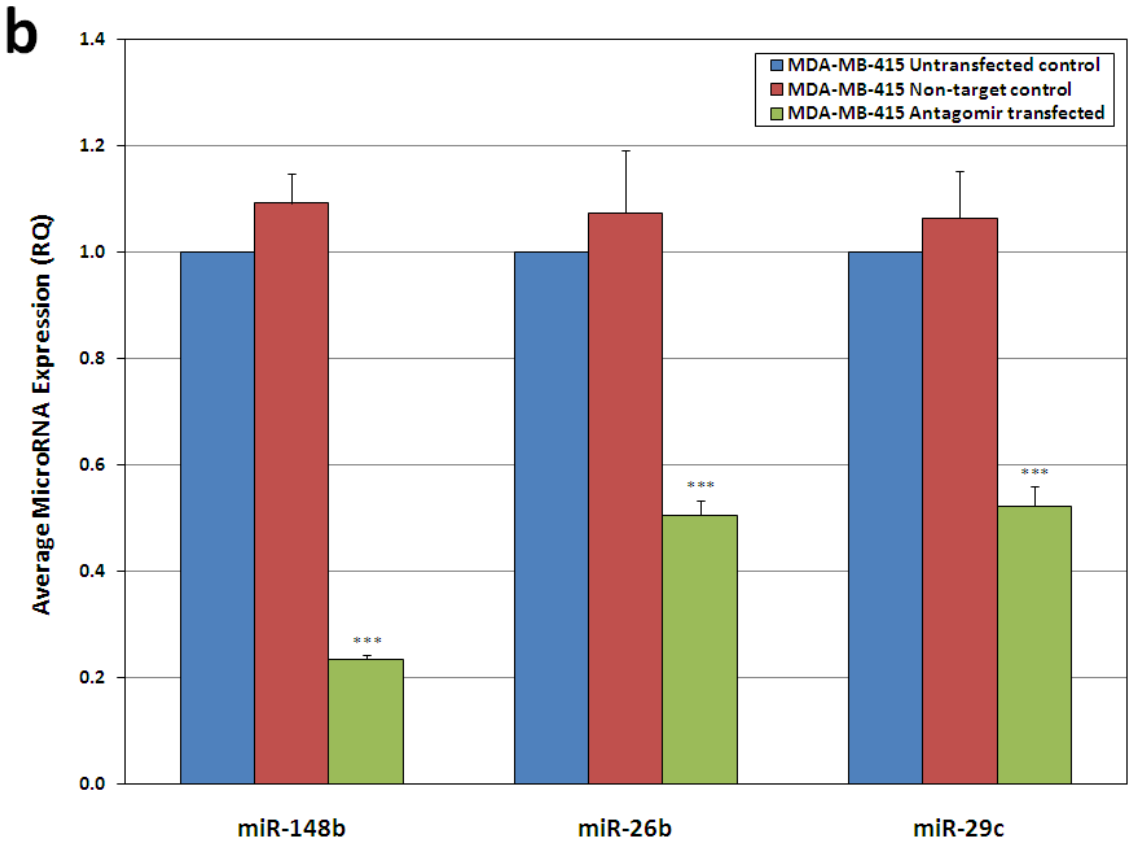

C
SUM185 Untransfected control
SUM185 Non-target control
SUM185 Pre-miR transfected
Average MicroRNA Expression (RQ)
60000
50000
5000
2000
3
0
***
***
***
miR-148b
miR-26b
miR-29c

Figure 3.14. *Changes in miR Expression Levels in Hypermethylator Breast Cancer Cell Lines After Pre-miR Transfection*. Blue bars represent miR expression levels in untransfected control cells, red bars represent miR expression levels in cells transfected with non-target control oligomers, and green bars represent miR expression levels in cells after indicated pre-miR transfections. (**a**) Hs578T breast cancer cells re-express miR-148b, miR-26b, and miR-29c after pre-miR transfection. (**b**) HCC1937 breast cancer cells re-express miR-148b, miR-26b, and miR-29c after pre-miR transfection. (**c**) SUM185 breast cancer cells re-express miR-148b, miR-26b, and miR-29c after pre-miR transfection. Each real-time assay was performed 3-6 times and error bars represent S.E.M. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, compared to untransfected control cells (unpaired t-test).


a
BT20 Untransfected control
BT20 Non-target control
BT20 Antagomir transfected
Average MicroRNA Expression (RQ)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
miR-148b
miR-26b
miR-29c


b
MDA-MB-415 Untransfected control
MDA-MB-415 Non-target control
MDA-MB-415 Antagomir transfected
Average MicroRNA Expression (RQ)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
miR-148b
miR-26b
miR-29c

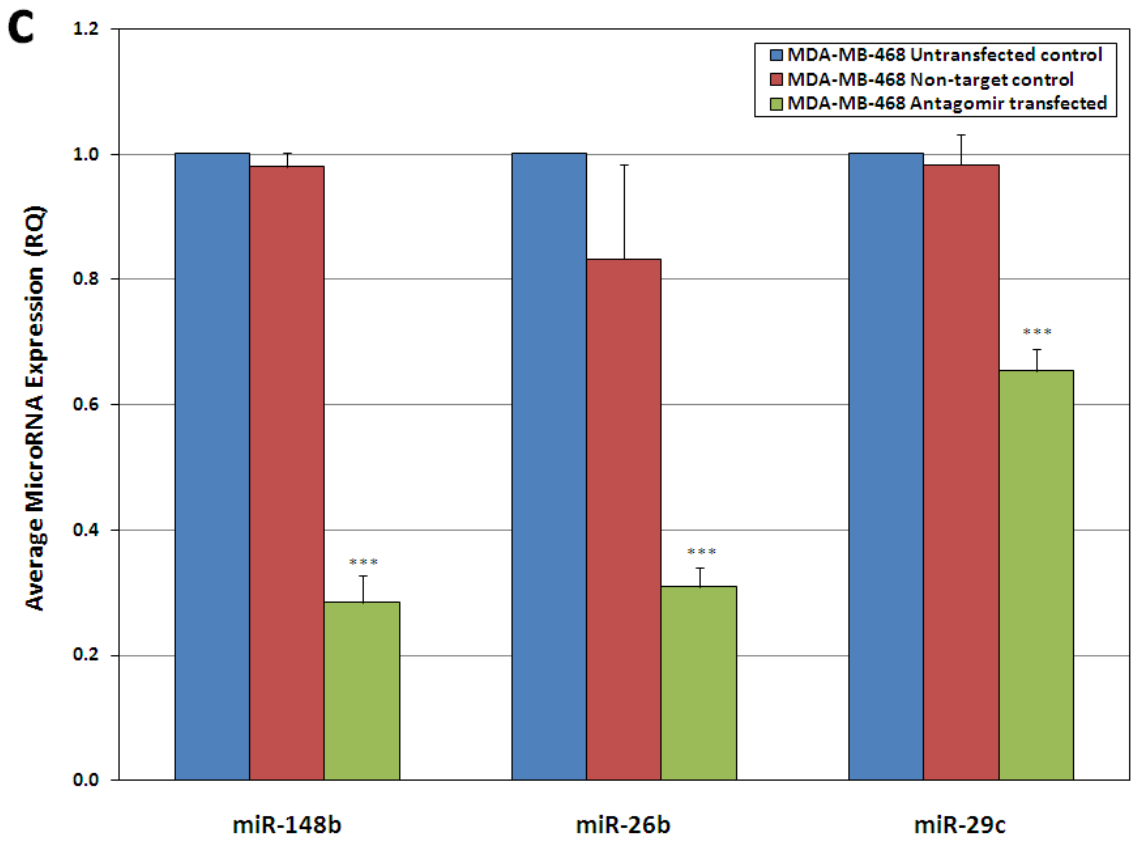

C
MDA-MB-468 Untransfected control
MDA-MB-468 Non-target control
MDA-MB-468 Antagomir transfected
Average MicroRNA Expression (RQ)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
miR-148b
miR-26b
miR-29c

Figure 3.15. *Changes in miR Expression Levels in Non-Hypermethylator Breast Cancer Cell Lines after Antagomir Transfection*. Blue bars represent miR expression levels in untransfected control cells, red bars represent miR expression levels in cells transfected with non-target control oligomers, and green bars represent miR expression levels in cells after indicated antagomir transfections. (**a**) BT20 breast cancer cells express diminished levels of miR-148b, miR-26b, and miR-29c after antagomir transfection. (**b**) MDA-MB-415 breast cancer cells express reduced levels of miR-148b, miR-26b, and miR-29c after antagomir transfection. (**c**) MDA-MB-468 breast cancer cells express reduced levels of miR-148b, miR-26b, and miR-29c after antagomir transfection. Each real-time assay was performed 3-6 times and error bars represent S.E.M. $^{*}p<0.05$, $^{**}p<0.005$, $^{***}p<0.0005$, compared to untransfected control cells (unpaired t-test).

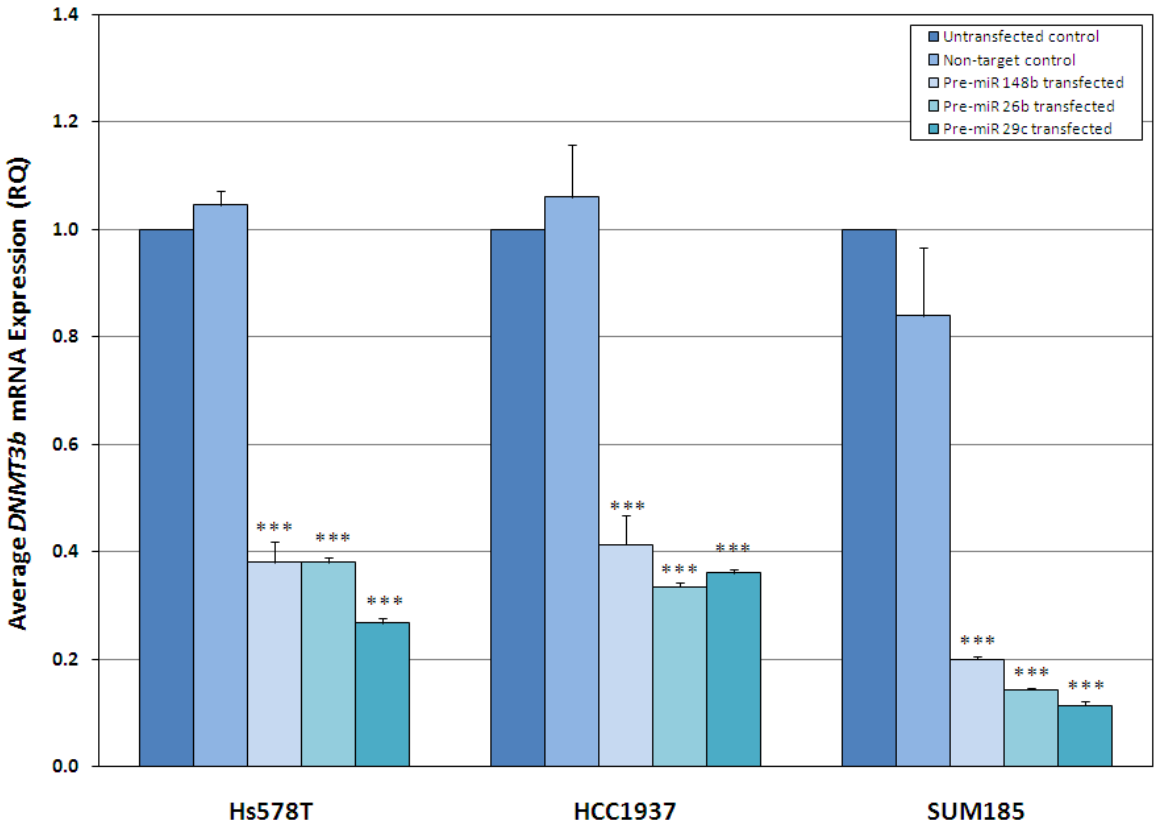

Average DNMT3b mRNA Expression (RQ)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Untransfected control
Non-target control
Pre-miR 148b transfected
Pre-miR 26b transfected
Pre-miR 29c transfected
***
Hs578T
HCC1937
SUM185

Figure 3.16. *Perturbation of Regulatory miR Expression affects DNMT3b Levels in Hypermethylator Cell Lines.* Hypermethylator breast cancer cells (Hs578T, HCC1937, and SUM185) exhibit significant reduction in *DNMT3b* mRNA levels following pre-miR transfection for miR-148b, miR-26b, and miR-29c. Each real-time assay was performed 3-6 times and error bars represent S.E.M. $^{**}p<0.005$, $^{***}p<0.0005$, compared to untransfected control cells (unpaired t-test).

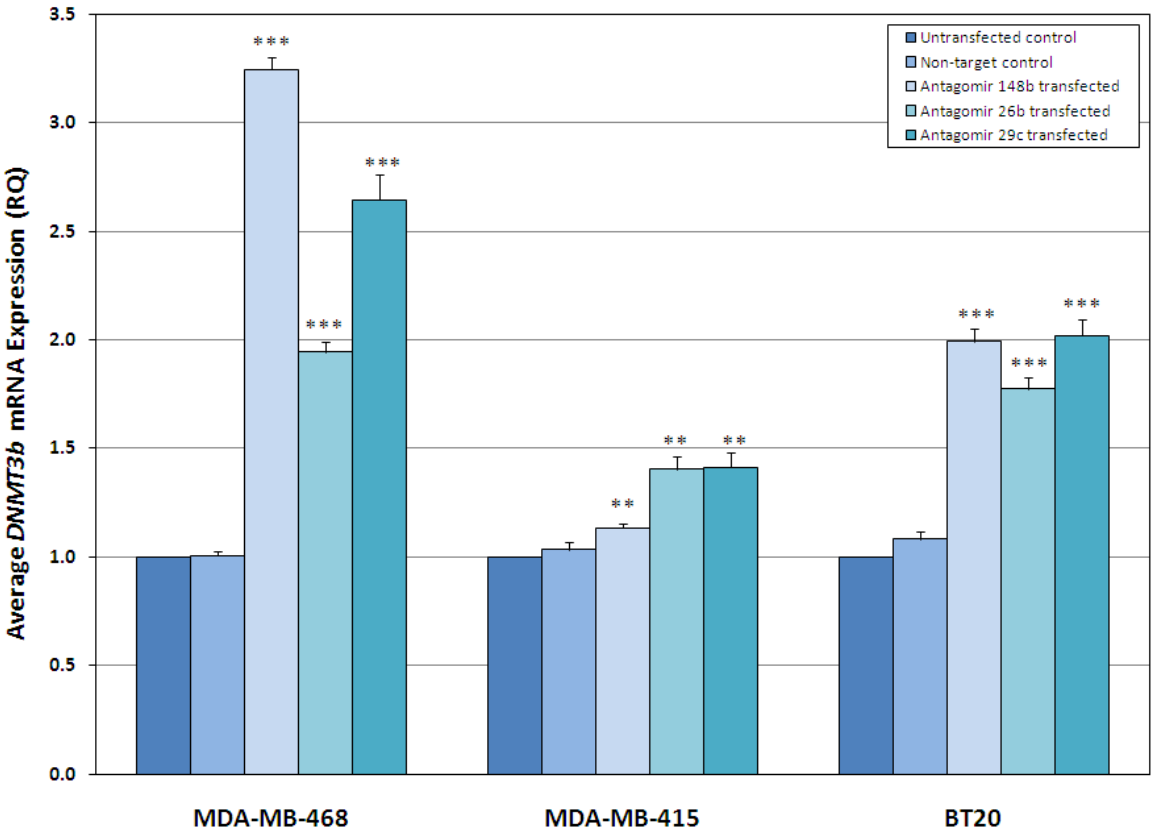

Average DNMT3b mRNA Expression (RQ)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Untransfected control
Non-target control
Antagomir 148b transfected
Antagomir 26b transfected
Antagomir 29c transfected
***
***
***
**
**
**
***
***
***
MDA-MB-468
MDA-MB-415
BT20

Figure 3.17. *Perturbation of Regulatory miR Expression affects DNMT3b Levels in Non-hypermethylator Cell Lines.* Non-hypermethylator breast cancer cells (MDA-MB-468, MDA-MB-415, and BT20) display significantly increased *DNMT3b* mRNA levels following transfection with antagomirs for miR-148b, miR-26b, and miR-29c. Each real-time assay was performed 3-6 times and error bars represent S.E.M. $^{**}p<0.005$, $^{***}p<0.0005$, compared to untransfected control cells (unpaired t-test).

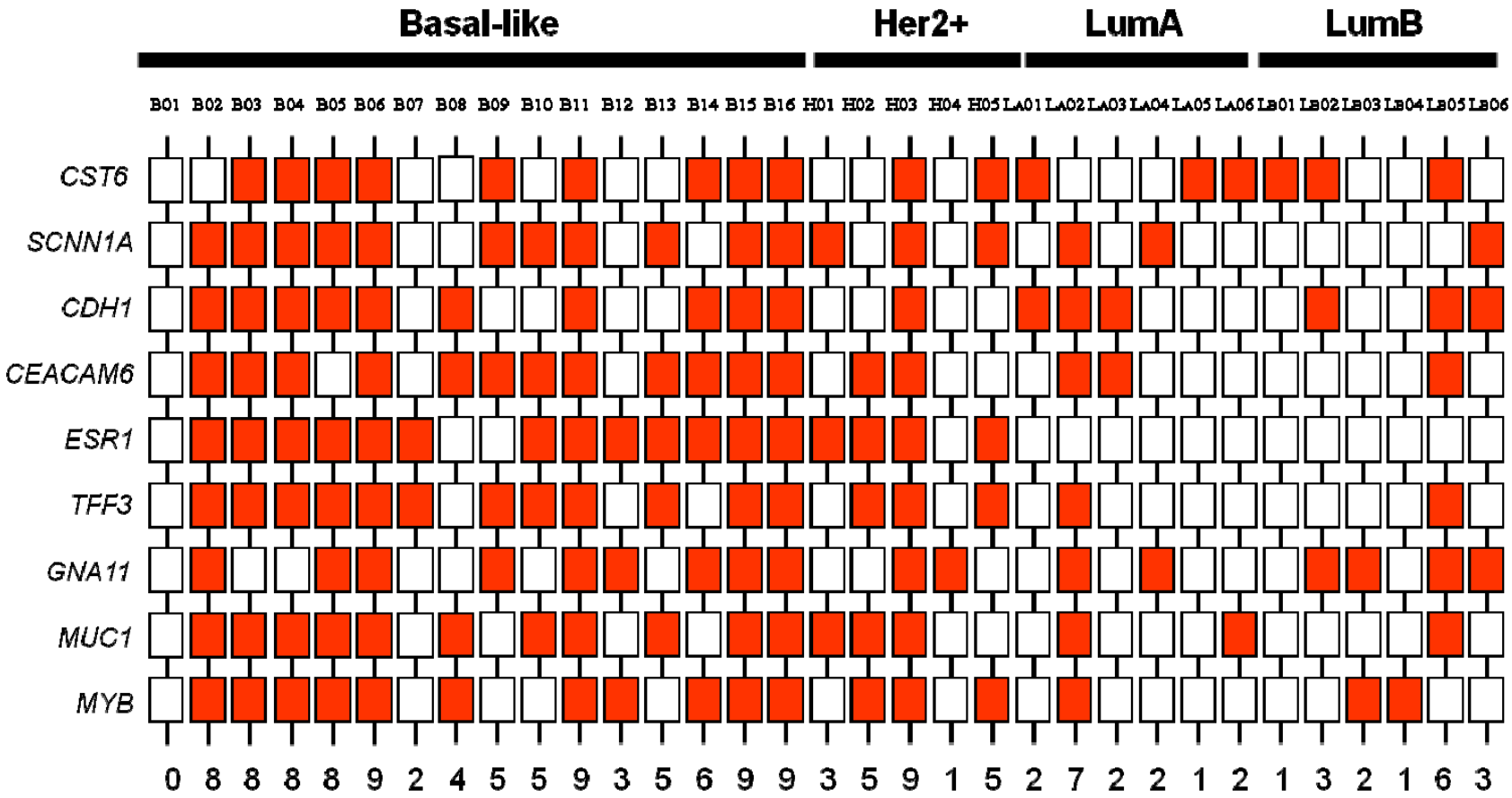

Basal-like
Her2+
LumA
LumB
B01 B02 B03 B04 B05 B06 B07 B08 B09 B10 B11 B12 B13 B14 B15 B16 H01 H02 H03 H04 H05 LA01 LA02 LA03 LA04 LA05 LA06 LB01 LB02 LB03 LB04 LB05 LB06
CST6
SCNN1A
CDH1
CEACAM6
ESR1
TFF3
GNA11
MUC1
MYB
0 8 8 8 8 9 2 4 5 5 9 3 5 6 9 9 3 5 9 1 5 2 7 2 2 1 2 1 3 2 1 6 3

Figure 3.18. *Gene Expression Patterns of Methylation-Sensitive Genes for Human Primary Breast Cancers*. Red boxes indicate a measured level of expression for an individual gene that is below the median level of expression for the dataset, and white boxes indicate a measured level of expression of an individual gene that is above the median level of expression for the dataset. The numbers at the bottom of each column indicate the number of methylation sensitive genes expressed at diminished levels in an individual cancer. 'B' represents basal-like cancers, 'H' represents HER2+ cancers, L_A represents luminal A cancers, and L_B represents luminal B cancers.

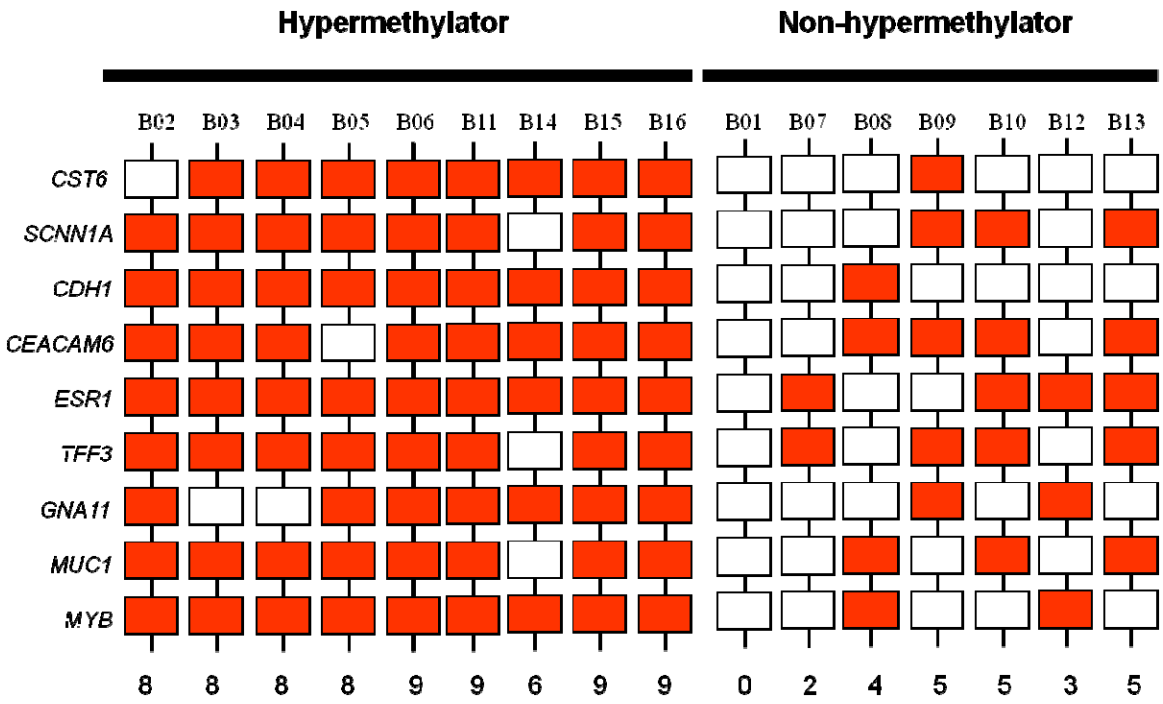

Hypermethylator
Non-hypermethylator
B02 B03 B04 B05 B06 B11 B14 B15 B16
B01 B07 B08 B09 B10 B12 B13
CST6
SCNN1A
CDH1
CEACAM6
ESR1
TFF3
GNA11
MUC1
MYB
8 8 8 8 9 9 6 9 9
0 2 4 5 5 3 5

Figure 3.19. *Classification of Basal-like Breast Cancers into Hypermethylators and Non-hypermethylators based on the Gene Expression Patterns of Methylation-Sensitive Genes*. Red boxes indicate a measured level of expression for an individual gene that is below the median level of expression for the dataset, and white boxes indicate a measured level of expression of an individual gene that is above the median level of expression for the dataset. The numbers at the bottom of each column indicate the number of methylation sensitive genes expressed at diminished levels in an individual cancer.

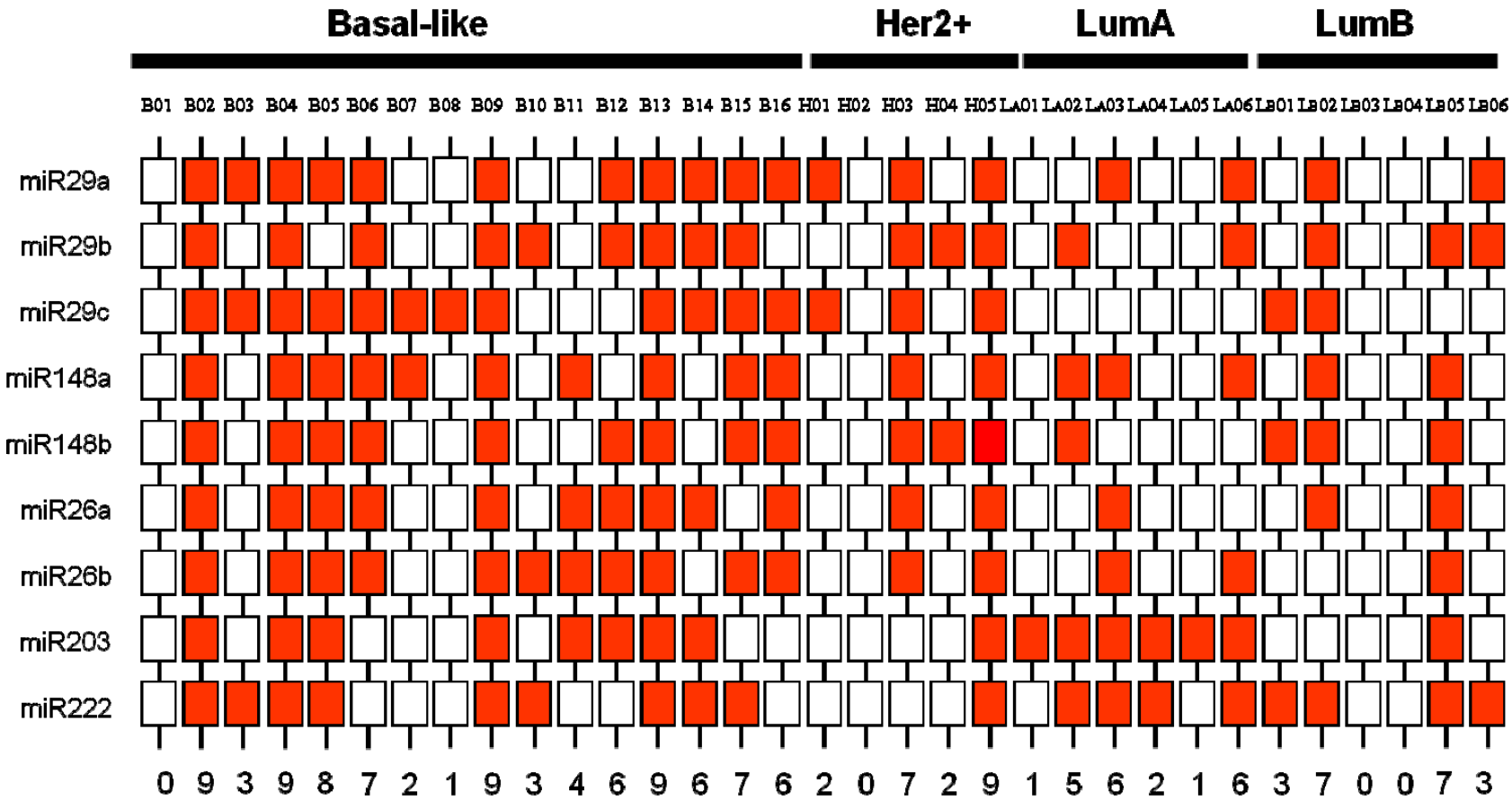

Basal-like
Her2+
LumA
LumB
B01 B02 B03 B04 B05 B06 B07 B08 B09 B10 B11 B12 B13 B14 B15 B16 H01 H02 H03 H04 H05 LA01 LA02 LA03 LA04 LA05 LA06 LB01 LB02 LB03 LB04 LB05 LB06
miR29a
miR29b
miR29c
miR148a
miR148b
miR26a
miR26b
miR203
miR222
0 9 3 9 8 7 2 1 9 3 4 6 9 6 7 6 2 0 7 2 9 1 5 6 2 1 6 3 7 0 0 7 3

Figure 3.20. *miR Expression Patterns and miR Scores for Human Primary Breast Cancers*. Red boxes indicate a level of expression for an individual miR below the median value, and white boxes indicate a level of expression of an individual miR that is above the median value for the dataset. The numbers at the bottom of each column indicate the miR score which represents a measure of the number of miRs expressed at diminished levels in an individual tumor. 'B' represents basal-like cancers, 'H' represents HER2+ cancers, L_A represents luminal A cancers, and L_B represents luminal B cancers.

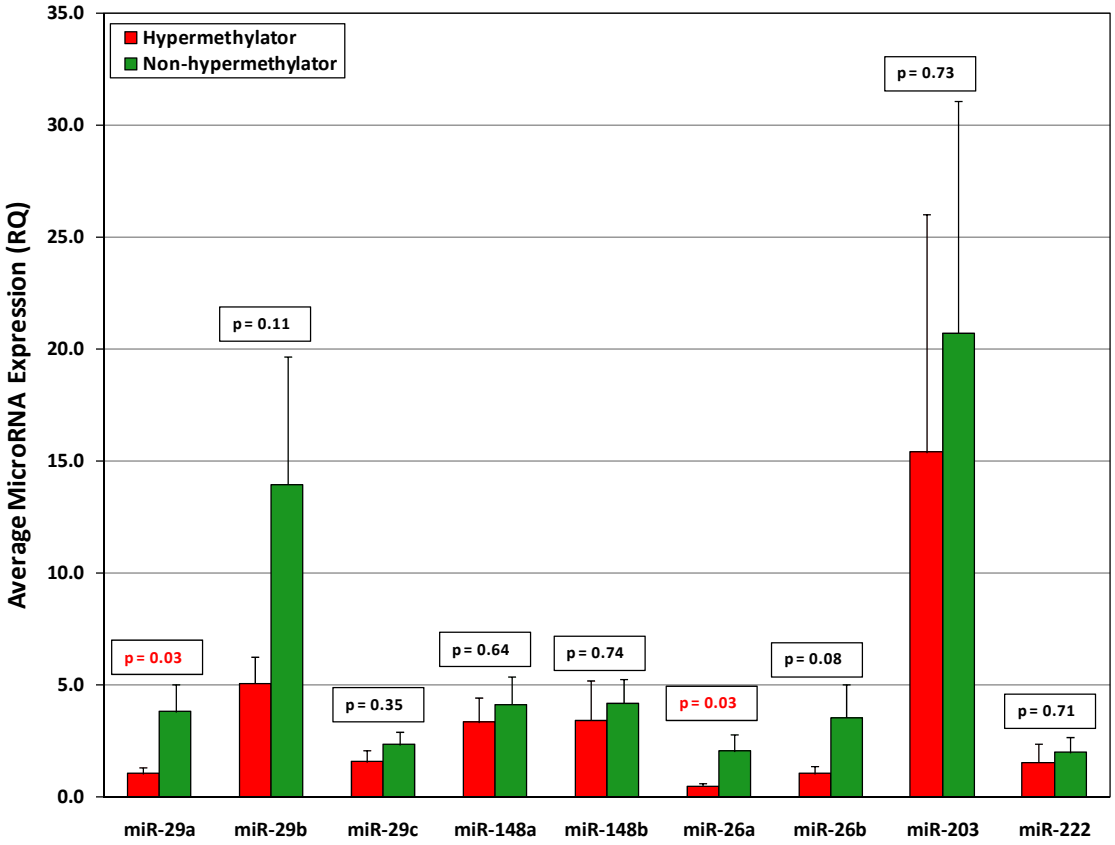
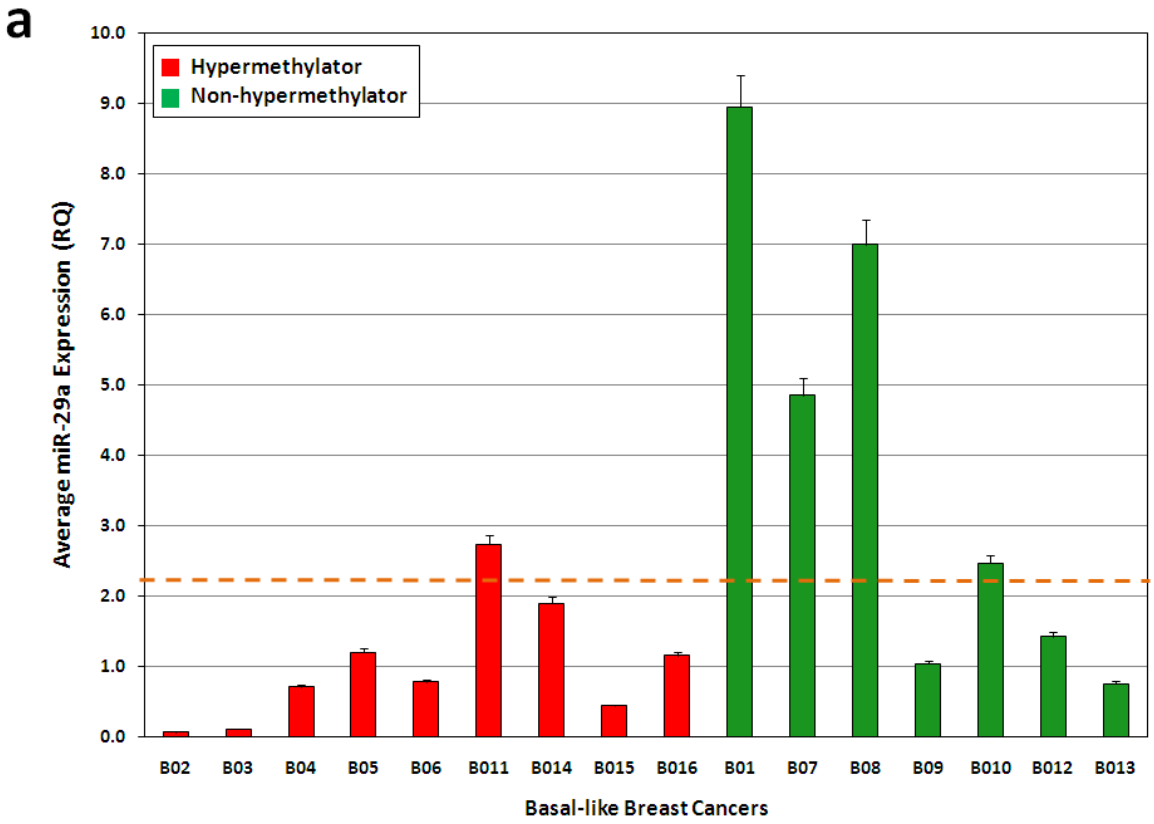
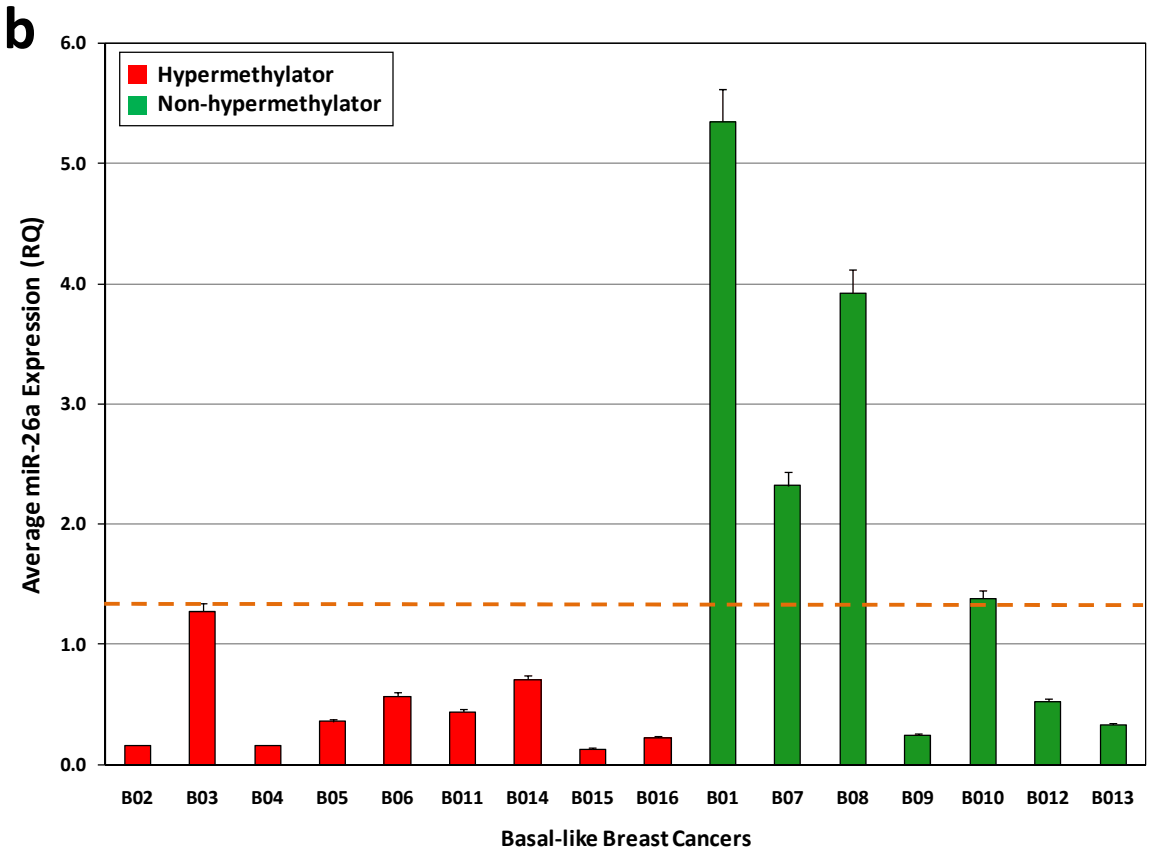
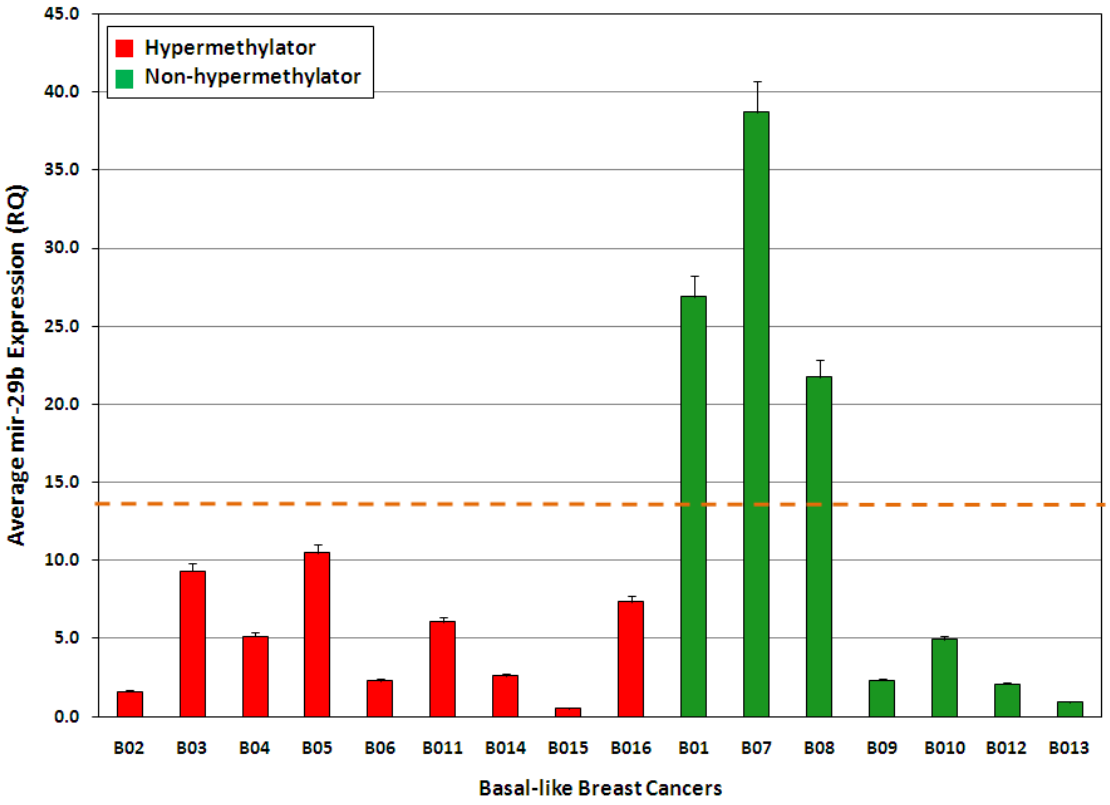

Hypermethylator
Non-hypermethylator
Average MicroRNA Expression (RQ)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
p = 0.03
p = 0.11
p = 0.35
p = 0.64
p = 0.74
p = 0.03
p = 0.08
p = 0.73
p = 0.71
miR-29a
miR-29b
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
miR-222

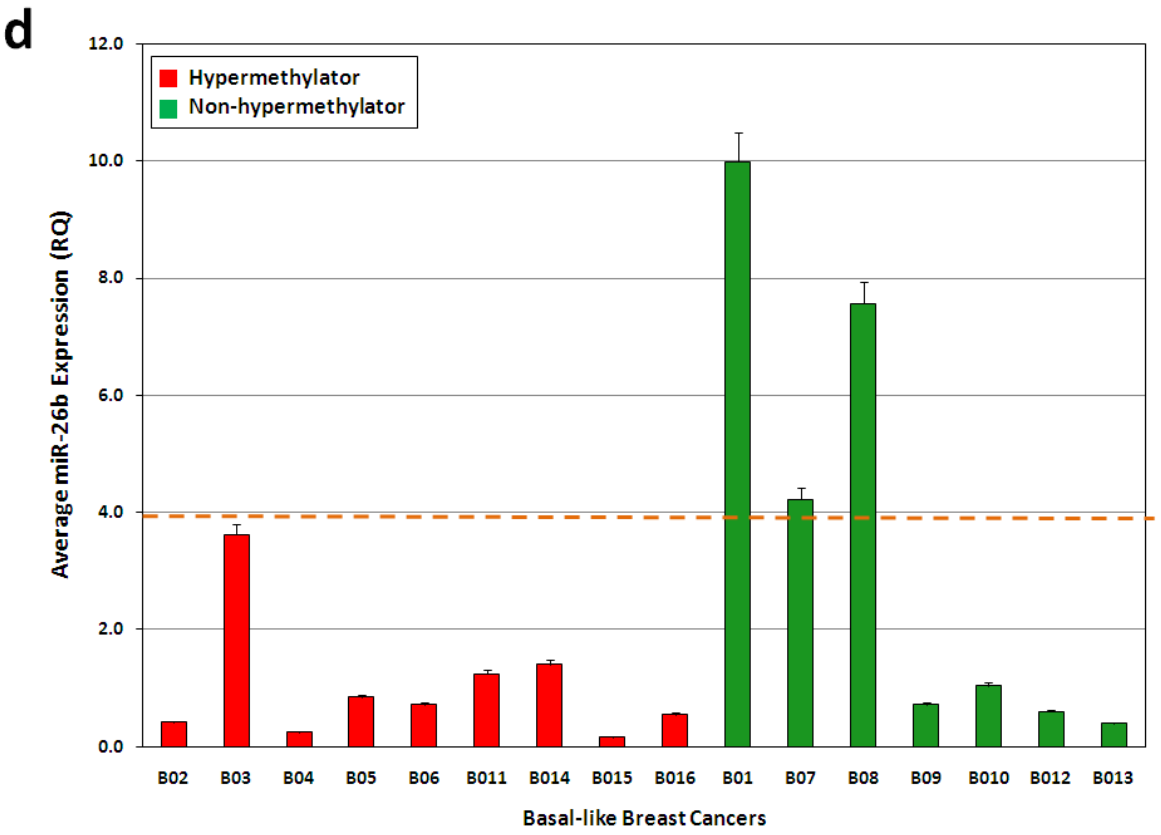
Figure 3.21. *Differential miR Expression in Hypermethylator and Non-Hypermethylator Basal-Like Breast Cancers.* (A) Red bars represent average miR expression among hypermethylator cancers (n=9), and green bars represent average miR expression among non-hypermethylator cancers (n=7). Comparison of the observed expression levels between hypermethylator cancers and non-hypermethylator cancers was accomplished using an unpaired t-test (two-tailed) and corresponding *p* values are given (NS - not significant).


a
Hypermethylator
Non-hypermethylator
Average miR-29a Expression (RQ)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

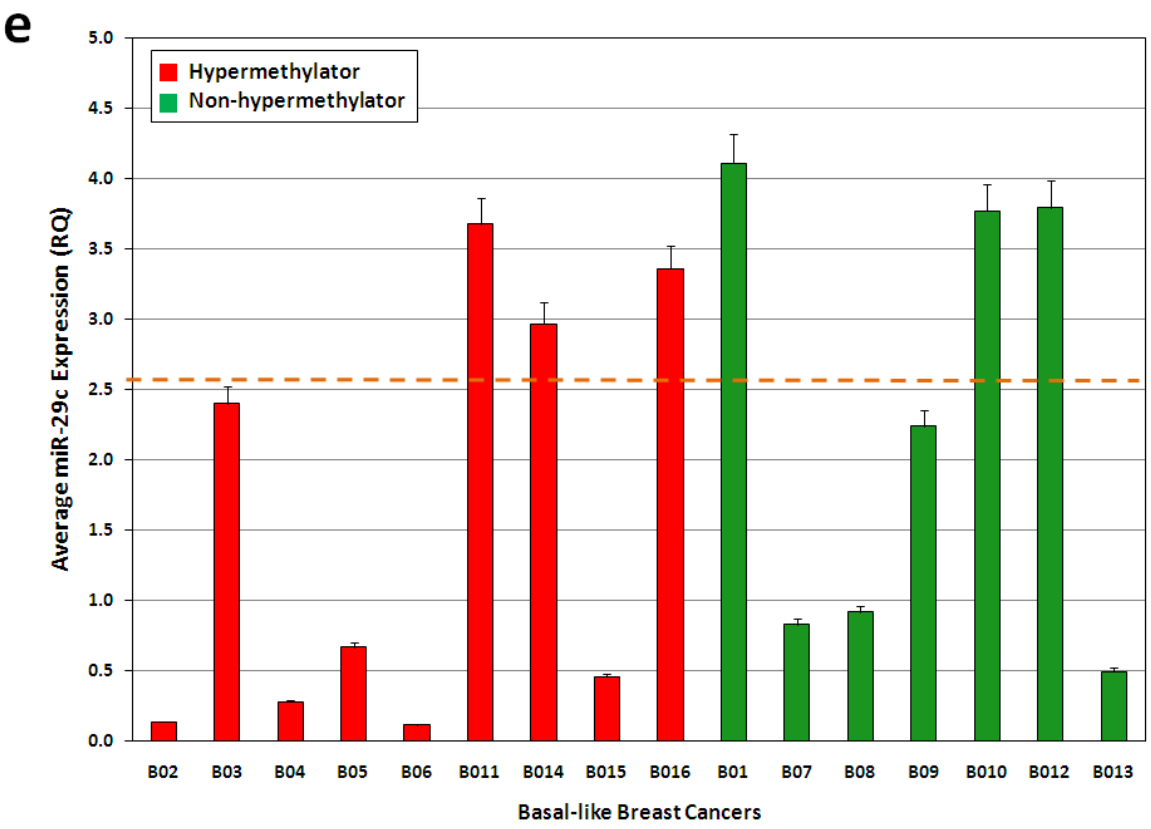

b
Hypermethylator
Non-hypermethylator
Average miR-26a Expression (RQ)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

C

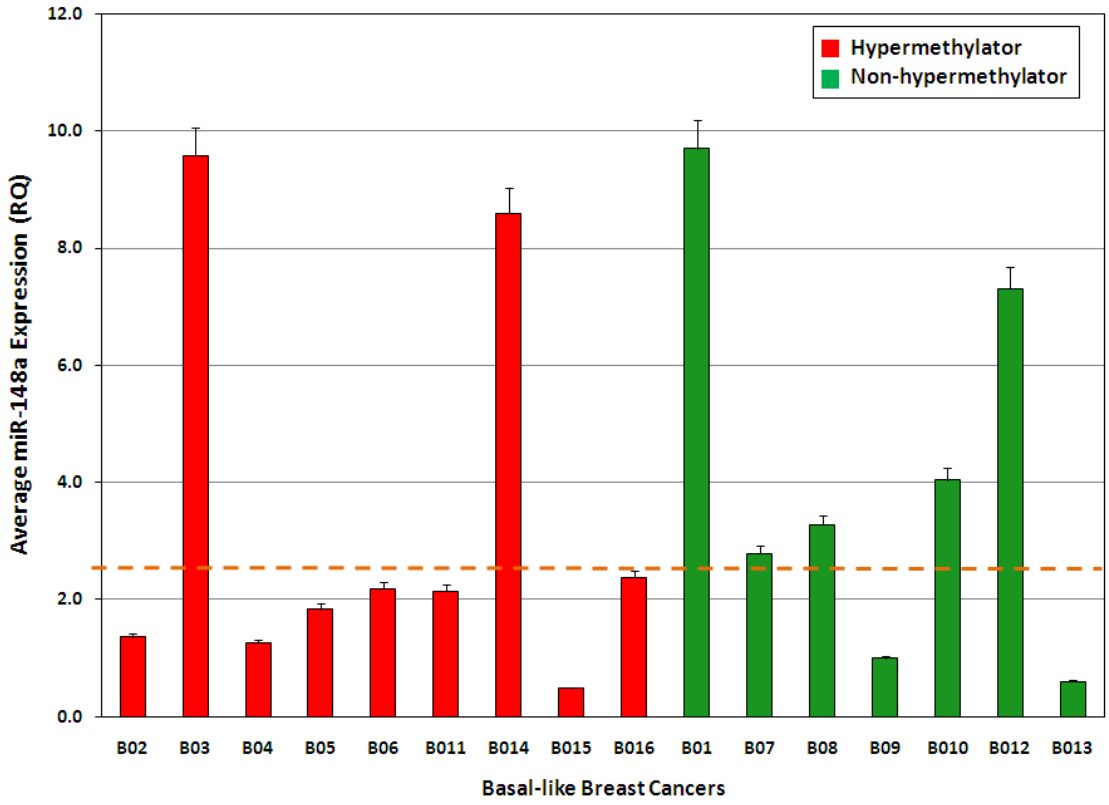

Hypermethylator
Non-hypermethylator
Average mir-29b Expression (RQ)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers


d
Hypermethylator
Non-hypermethylator
Average miR-26b Expression (RQ)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

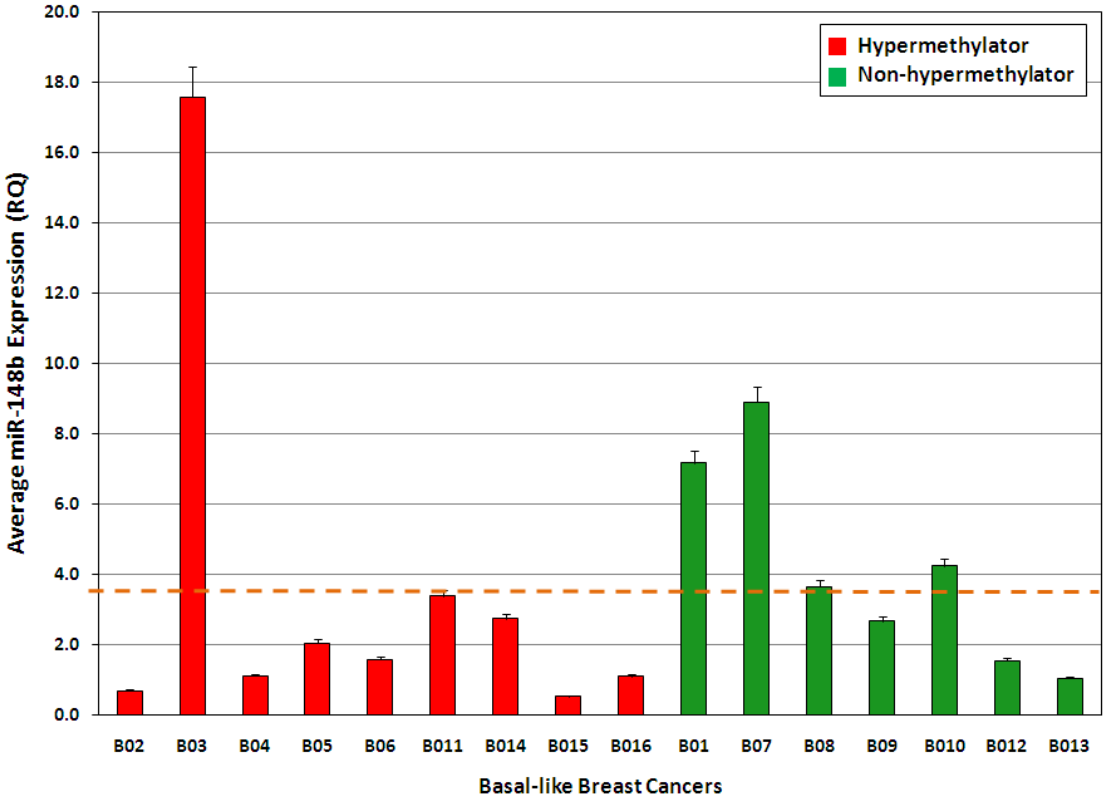
e


Hypermethylator
Non-hypermethylator
Average miR-29c Expression (RQ)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

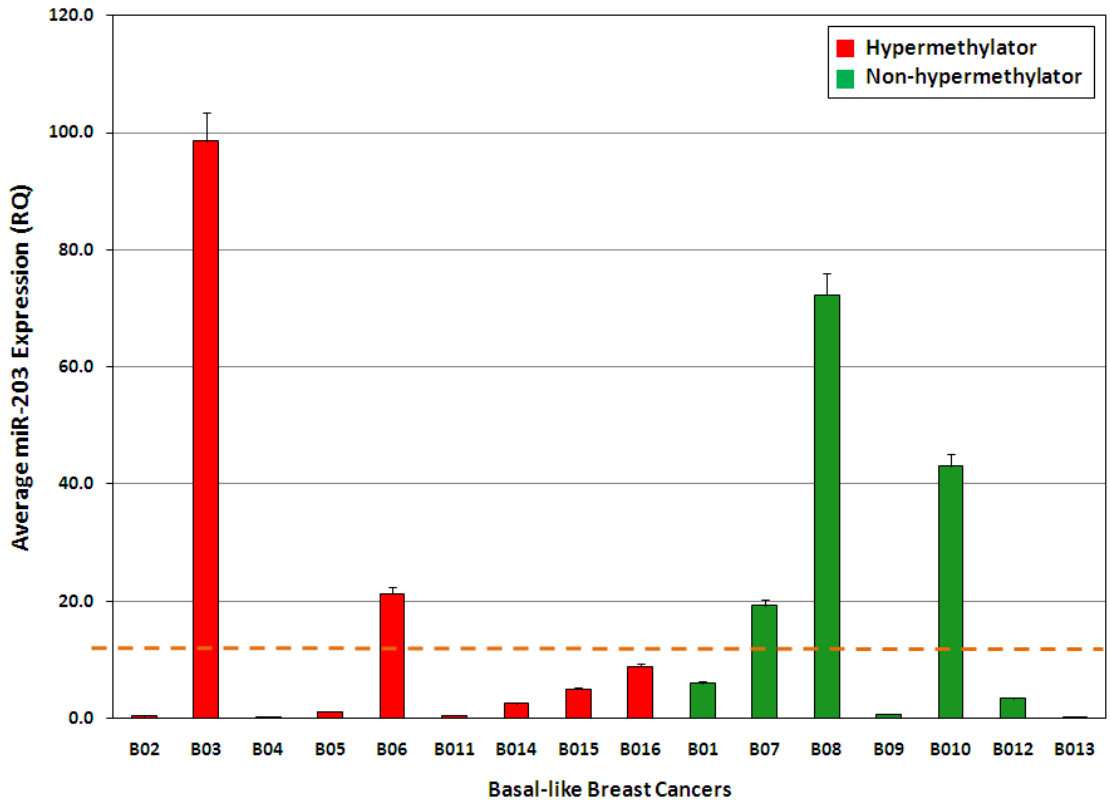
f


Hypermethylator
Non-hypermethylator
Average miR-148a Expression (RQ)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

g


Hypermethylator
Non-hypermethylator
Average miR-148b Expression (RQ)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

h


Hypermethylator
Non-hypermethylator
Average miR-203 Expression (RQ)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancers

i

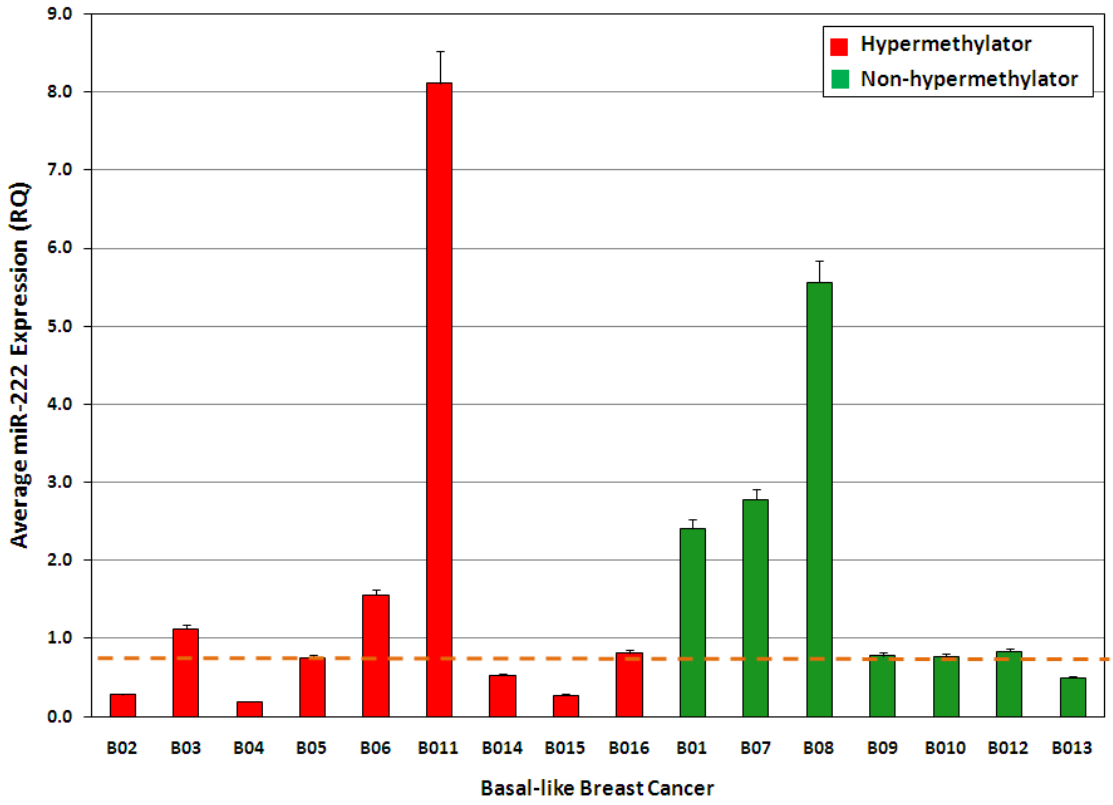

Hypermethylator
Non-hypermethylator
Average miR-222 Expression (RQ)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
B02
B03
B04
B05
B06
B011
B014
B015
B016
B01
B07
B08
B09
B010
B012
B013
Basal-like Breast Cancer

Figure 3.22. *Analysis of miR Expression Among Hypermethylator and Non-Hypermethylator Basal-like Breast Cancers*. Hypermethylator cancers are represented by red bars and non-hypermethylator cancers are represented by green bars. The orange dashed line represents the optimal threshold value determined by Bayesian analysis for correct assignments related to methylation status of individual cell lines. Each real-time assay was performed in triplicate and error bars represent S.E.M. (**a**) miR-29a expression, (**b**) miR-26a expression, (**c**) miR-29b expression, (**d**) miR-26b expression, (**e**) miR-29c expression, (**f**) miR-148a expression, (**g**) miR-148b expression, (**h**) miR-203 expression, and (**i**) miR-222 expression.

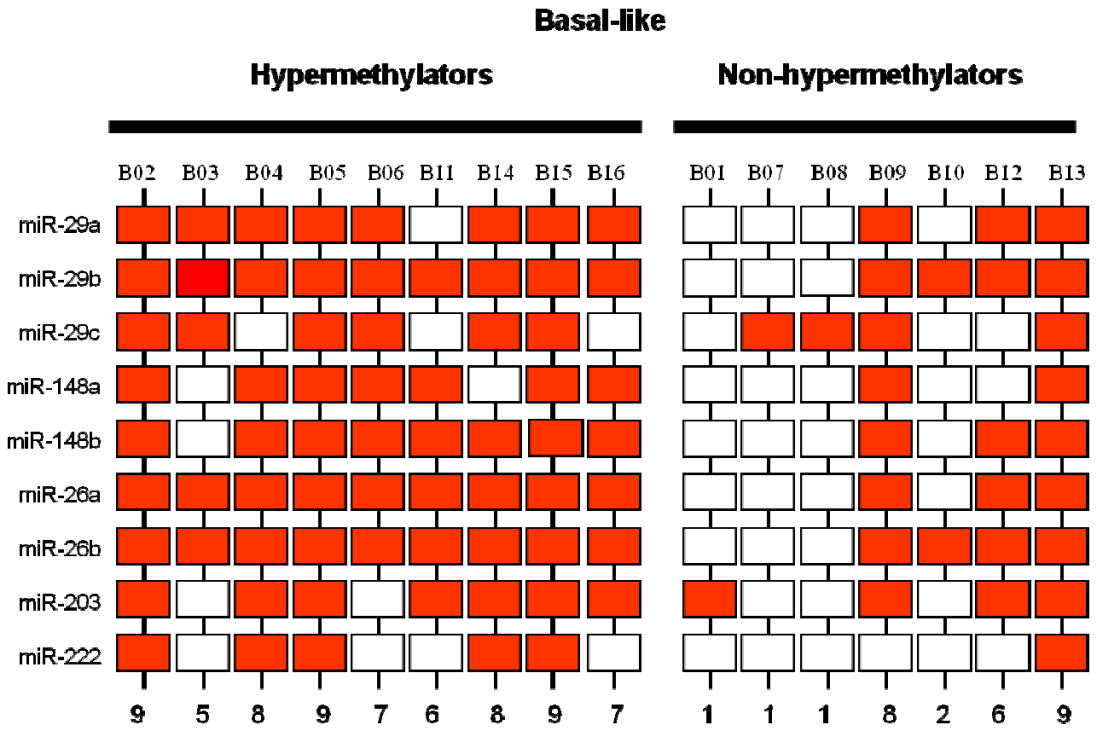

Basal-like
Hypermethylators
Non-hypermethylators
B02 B03 B04 B05 B06 B11 B14 B15 B16
B01 B07 B08 B09 B10 B12 B13
miR-29a
miR-29b
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
miR-222
9 5 8 9 7 6 8 9 7
1 1 1 8 2 6 9

Figure 3.23. *miR Expression Patterns and miR Scores for Hypermethylator and Non-Hypermethylator Basal-Like Breast Cancers.* Red boxes indicate a measured level of expression for an individual miR that is below the threshold value established through Bayesian analysis, and white boxes indicate a measured level of expression of an individual miR that is above the threshold value established through Bayesian analysis. The numbers at the bottom of each column indicate the miR score which represents a measure of the number of miRs expressed at diminished levels in an individual breast cancer.

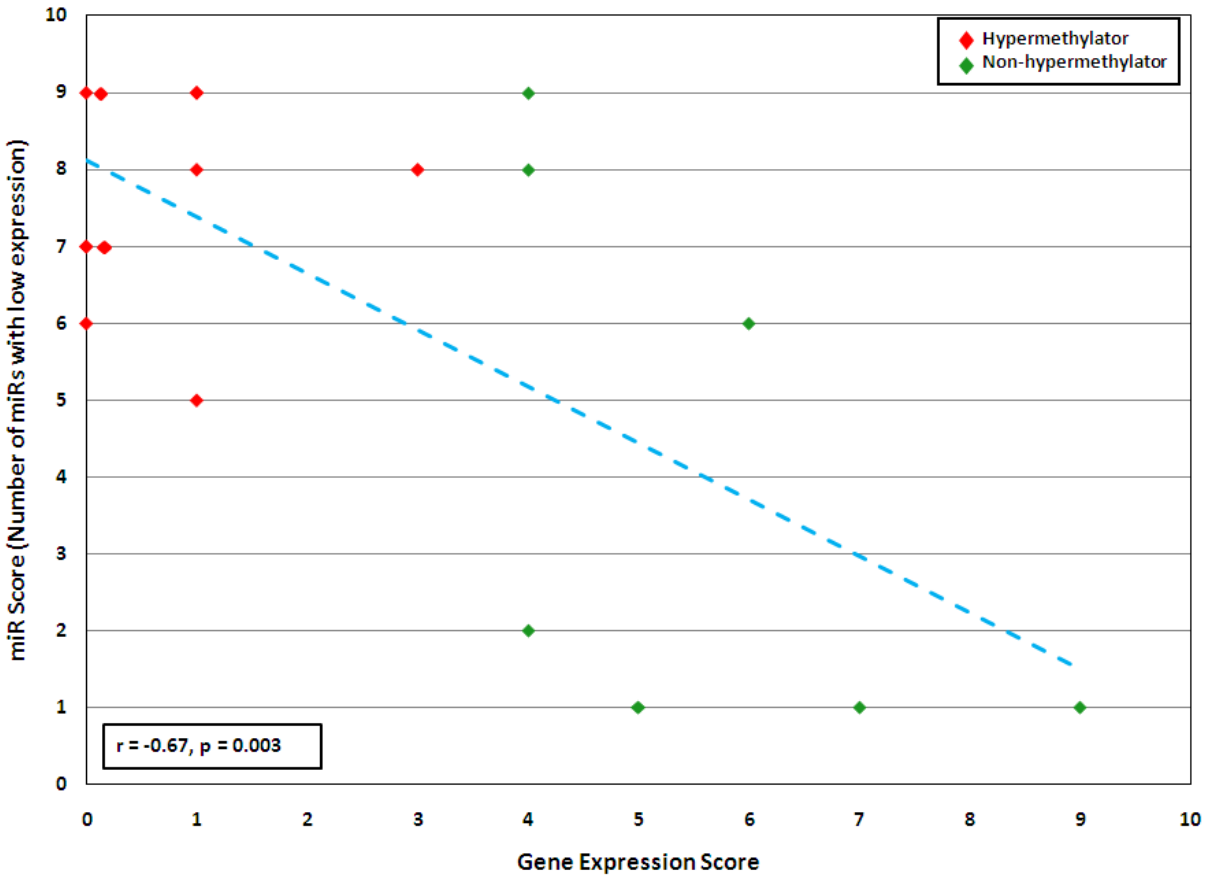

Hypermethylator
Non-hypermethylator
miR Score (Number of miRs with low expression)
Gene Expression Score
r = -0.67, p = 0.003
0
1
2
3
4
5
6
7
8
9
10

Figure 3.24. *miR Expression Patterns Correlate with Promoter Methylation Status among Basal-Like Breast Cancers.* Correlation of miR expression patterns (miR score) with gene expression levels (based on RT-PCR) for methylation-sensitive genes among hypermethylator and non-hypermethylator basal-like breast cancers. Hypermethylator cancers are represented as red diamonds and non-hypermethylator cancers are represented as green diamonds.

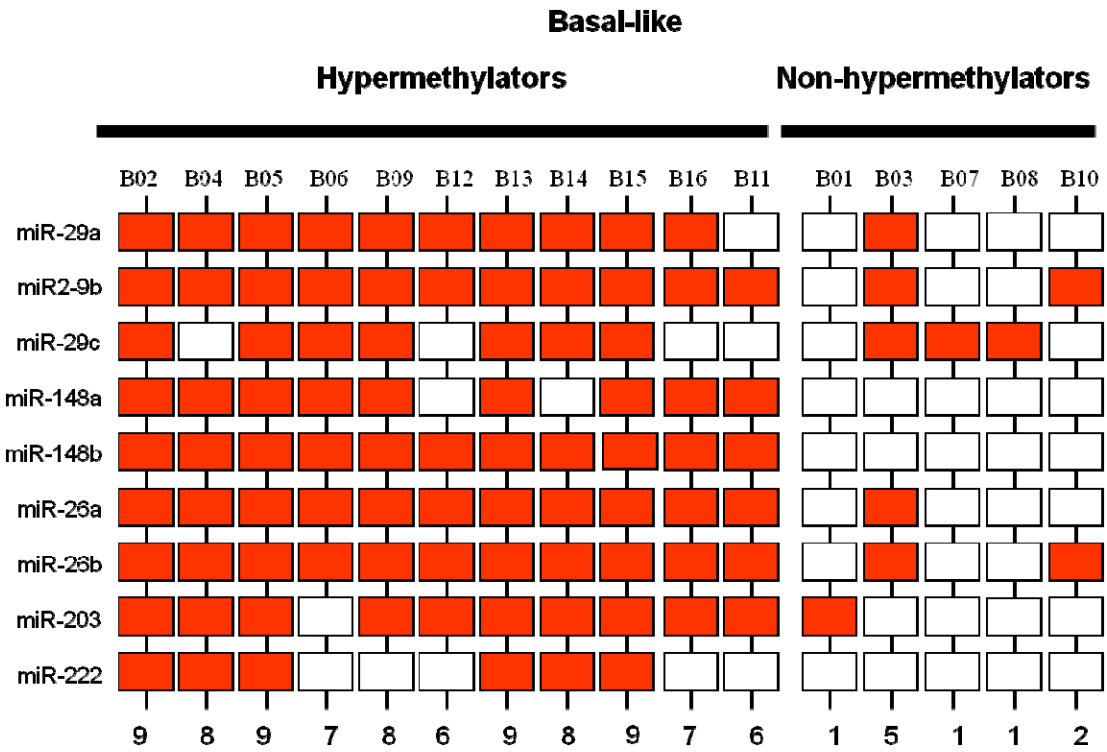

Basal-like
Hypermethylators
Non-hypermethylators
B02 B04 B05 B06 B09 B12 B13 B14 B15 B16 B11
B01 B03 B07 B08 B10
miR-29a
miR2-9b
miR-29c
miR-148a
miR-148b
miR-26a
miR-26b
miR-203
miR-222
9 8 9 7 8 6 9 8 9 7 6
1 5 1 1 2

Figure 3.25. *miR Expression Patterns Classify Basal-Like Breast Cancers into Low Expresser and High Expresser Subsets.* Low-expresser subset had low levels of expression of at least 6/9 miRs of interest for an individual breast cancer. High expresser subset includes the cancers with normal/high expression of at least 4/9 miRs of interest. Red boxes indicate a measured level of expression for an individual miR that is below the threshold value established through Bayesian analysis, and white boxes indicate a measured level of expression of an individual miR that is above the threshold value established through Bayesian analysis. The numbers at the bottom of each column indicate the miR score which represents a measure of the number of miRs expressed at diminished levels in an individual breast cancer.

DISCUSSION

Enhancement of Chemotherapeutic Efficacy in Hypermethylator Breast Cancer Cells by Targeting The Epigenome

The initiation, development, and progression of breast cancer reflects a multistep process that involves genetic and epigenetic changes resulting in activation of oncogenes and inactivation of tumor suppressor genes [77, 239-241]. Frequently observed genetic abnormalities in breast cancer include large-scale chromosomal deletions, sequence mutations (frameshift and point mutations), copy-number changes (including gene amplifications), and gene rearrangements (translocations). The understanding of these specific genetic alterations in breast cancer led to the development of targeted therapeutic approaches. Identification of HER2 amplification as a treatment target in approximately 30% of breast cancers led to the development of human monoclonal antibody, Herceptin (trastuzumab) [242-246]. Treatment with Herceptin significantly improves outcomes in HER2-amplified breast cancer patients [72, 247-249]. In contrast to gain-of-function oncogenic mutations, correction of loss-of-function tumor suppressor gene mutations through gene therapy has been largely unsuccessful. Epigenetic alterations also make substantial contributions to the regulation of gene expression [88, 92, 93] and have been established as an important mechanism contributing to breast carcinogenesis. A number of genes have been shown to be inactivated in breast cancer through methylation-dependent gene silencing, including cell cycle control genes (*APC, RASSF1, RB, TFAP2A*), tumor suppressor genes

(*CST6, BRCA1, PRDM2*), metastasis-associated genes (*CDH1, CEACAM6, LGALS3BP*), steroid receptor genes (*ESR1, PGR, RARα*), and many others [119, 120, 150, 250, 251]. The number of genes silenced by methylation in breast cancer, in association with the cellular activities in which they participate, suggests strongly that aberrant DNA methylation contributes to the biological and clinical behaviors of breast cancer. Aberrant DNA methylation and epigenetic silencing of gene expression are now well recognized as frequent and reversible (unlike genetic mutation) hallmarks of cancer [74, 79, 80, 92, 252], leading numerous investigators to suggest that cancer should be treated with "epigenetic therapy" [253-257]. 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 [239, 258]. In addition, by targeting faulty epigenetic modification patterns, one can concurrently target multiple genes. The goal of such therapy is to effect changes in gene expression, including re-expression of silenced genes (like tumor suppressor genes), that alter the clinical behavior of the tumor or response of the tumor to chemotherapy.

In the current investigation, we tested the hypothesis that epigenetic therapy using very low (non-cytotoxic) doses of 5-aza would sensitize hypermethylator breast cancer cell lines (MDA-MB-453, BT549, and Hs578T) to the cytotoxic effects of standard chemotherapeutic drugs. The results clearly show increased efficacy of chemotherapeutic drugs against these cell lines following epigenetic therapy and the increase in efficacy was a function of dose and duration of exposure to the demethylating drug. To provide further evidence that the improved efficacy was a consequence of modulating the DNA methylation

machinery, we knocked down DNMT3b, which drives expression of hypermethylation defect in breast cancer cells [150]. Targeted inhibition of DNMT3b produced trends of enhanced chemosensitivity in each cell line comparable to that observed with pharmacological inhibition utilizing 5-aza. We employed three different cell lines and three chemotherapeutic drugs to eliminate the possibility that the effects were cell line or drug-specific. 5-aza-2'-deoxycytidine (Decitabine) is FDA approved for clinical use in patients with myelodysplastic syndromes [258-260]. Thus, the possible use of sensitizing doses of 5-aza in conjunction with established chemotherapeutic regimens is very exciting.

In previous studies, mining of microarray-based expression data identified the hypermethylation defect-associated gene expression signature in primary sporadic invasive breast cancers [150]. We observed strong correspondence between expression of the hypermethylation defect and the basal-like sub-group of breast cancers [150]. Many basal-like breast cancers express a hypermethylation defect characterized by silencing of numerous genes associated with DNMT3b protein overexpression. This observation strongly suggests that the unique characteristics of basal-like breast cancers (poor clinical outcomes, variable response to chemotherapy, and recurrence following chemotherapy) may be a direct consequence of methylation-dependent gene silencing associated with DNMT3b overexpression. This fundamental observation related to the basal-like breast cancers identifies the DNA methylation machinery (and specifically DNMT3b) as a novel target for development of new treatment regimens for these deadly breast cancers. Neoplasms that express this hypermethylation defect may exhibit poor response to chemotherapeutic treatment if the targets of methylation-dependent gene silencing encode proteins that function in DNA repair, apoptosis, or other pathways required for drug response. Therefore,

demethylating treatment of basal-like breast cancers may sensitize these neoplasms to standard drug regimens, resulting in increased chemotherapeutic efficacy. Since these tumors lack the expression of hormone receptors and HER2 amplification, patients with these tumors do not derive benefit from targeted therapies like tamoxifen (targeting ER) and trastuzumab (targeting HER2). The poor long-term outcomes for basal-like breast cancers are likely due to high relapse rate [173, 174, 261, 262]. It has been shown that prognosis correlates with pathological complete response and that if initially patients do not achieve complete response, they are more likely to have an early relapse, frequently leading to death of the patient [173, 174]. Thus, enhancement in the efficacy of chemotherapeutics to achieve higher response rate by making these neoplasms more sensitive may lead to higher pathological complete response and consequently better long-term outcomes. Lack of identification of druggable targets in basal-like breast cancers, poor prognosis, and association of positive clinical outcome with pathological complete response in response to chemotherapy makes the assessment of new therapeutic strategies to be of utmost significance. The results of the current study suggest strongly that combined epigenetic and cytotoxic chemotherapy should be evaluated for treatment of basal-like breast cancer.

In these studies, we provide evidence that targeted and pharmacological inhibition of DNMT3b augments the efficacy of chemotherapeutic drugs. This observation has the potential of becoming a useful therapeutic modality. Increasing the efficacy can be employed to benefit the patients in at least two different ways. First, increasing the efficacy of a certain fixed dose may increase the benefits of chemotherapy without associated increases in toxic side-effects. Secondly, a lower dose of chemotherapeutic drug may be used to achieve a certain fixed therapeutic effect, but with diminished side effects. These studies provide proof-

of-concept that sensitizing pretreatment followed by a standard chemotherapeutic regimen improves cell killing in breast cancer. Since cancer results from a combination of genetic and epigenetic alterations, it can be anticipated that a combination of epigenetic and genetic/chemotherapeutic therapy will be beneficial. However, these results also suggest the combination therapy employing other cytotoxic drugs/classes e.g., cyclophosphamide, carboplatin, methotrexate may also sensitize the cancers to chemotherapy.

Our studies provide proof-of-principle for utilizing epigenetic therapy as a chemosensitizer in treatment of breast cancer. Nevertheless, further laboratory studies and clinical trials are needed to fully establish the therapeutic efficacy of agents that can modify DNA methylation. 5-aza-2'deoxycytidine and its parent compound, 5-azacytidine demonstrate demethylating activity [258, 263, 264] and have been widely used in cell culture models to reverse DNA hypermethylation leading to restoration of silenced gene expression [265, 266]. Being cytidine analogs, these agents become incorporated into DNA during DNA replication, are trapped and inactivated as covalent protein-DNA adducts. Sequestration of these agents leads to depletion of cellular DNA methyltransferase activity during DNA synthesis resulting in reduced methylation (demethylation) of newly synthesized DNA [265, 267]. 5-aza-2'deoxycytidine exhibits favorable properties in comparison with 5-azacytidine that includes greater DNA methylation inhibition and greater anti-cancer activity at equivalent doses in experimental models [263] but the side effects and potential risks hinder its clinical application. These risks include effects relating to bone marrow suppression like neutropenia [268], mutagenesis [269], and tumorigenesis [270]. It is also highly cytotoxic at higher doses and has a short half-life owing to degradation by hydrolytic cleavage and deamination by cytidine deaminase. For 5-aza to progress as a chemosensitizer, these side

effects and toxicity issues have to be addressed. The neutropenia induced by demethylating agent could render a patient unfit for chemotherapy, contradicting the purpose of administering a demethylating agent. The experimental design utilizing low doses of 5-aza in our studies was employed to keep the toxic and cytotoxic effects to minimum. In fact, we did not see any cytotoxic effects with 5-aza in our cell culture model. Further studies need to be conducted focusing on pharmacodynamic endpoints like optimal biologic dose rather than maximal tolerated dose. 5-aza is not very stable and cannot be orally administered, therefore it may need to be given as daily treatments. The resulting frequent visits by the patient to hospital will impact quality of life and have cost implications in addition to patient inconvenience. Therefore, the nucleoside analogues that have longer half-life and/or can be orally administered may offer some advantages over 5-aza. A less toxic derivative of 5-azacytidine, Zebularine has been recently developed [258, 267]. Zebularine can be given orally, but it has variable bioavailability resulting in significant demethylation relative to dose in mice [271] and relatively low demethylation in monkeys [272].

The nucleoside analogues require incorporation into DNA and are S-phase specific. Hence, the majority of cancer cells must pass through S-phase while the drug is present for it to effectively demethylate the DNA. The majority of toxic effects associated with these agents have been associated with the formation of covalent adducts between DNA and trapped DNA methyltransferases [258]. The inherent toxic effects of nucleoside analogues resulted in identification and development of non-nucleoside compounds that target DNA methyltransferases without getting incorporated into DNA and are mostly catalytic site inhibitors [267]. Since these substances directly block DNA methyltransferase activity, they do not display toxicity caused by the covalent trapping of the enzyme seen with nucleoside

analogues. One of these non-nucleoside compounds with DNMT inhibitor activity is epigallocatechin-3-gallate (EGCG), the main polyphenol compound in green tea [273]. EGCG inhibits DNA methyltransferase activity in human cancer cell lines by blocking the active site of human DNMT1 [274]. However, EGCG also generates significant amount of hydrogen peroxide resulting in toxicity due to the effects of hydrogen peroxide as an oxidizing agent [275]. Other compounds include the antiarrythmic drug procainamide and local anesthetic procaine that exhibit demethylating activity. Procainamide reverses hypermethylation in LNCaP (prostate cancer cell line) *in vitro* as well as in LNCaP xenograft tumors *in vivo* [276]. Procaine directly binds to CpG-rich sequences in DNA, inhibiting the interaction between DNA methyltransferases and their target CpGs [277]. However, Procaine exhibits demethylating activity at very high doses and the activity is highly variable across a panel of cell lines [258]. Since non-nucleoside analogues are not incorporated into DNA, it may suggest that these compounds are less toxic. However, it has been reported that non-nucleoside DNMT inhibitors are not any less toxic than nucleoside inhibitors, but are also less potent and less effective as demethylating agents compared to nucleoside inhibitors [265]. Other approaches to target DNA methylation machinery include development of small molecule inhibitor to target DNA methyltransferases. RG108, a small molecule inhibitor of human DNA methyltransferases have been developed that blocks the active site of the enzymes and thus inhibits their catalytic activity [278]. The inhibitory mechanism of RG108 is specific for DNA methyltransferases; therefore it targets DNMT1, DNMT3a, and DNMT3b. Hence, RG108 emerges as an attractive target for functioning as a lead compound for developing inhibitors specific to DNMT1, DNMT3a, and DNMT3b [258]. The current studies also clearly identify DNMT3b as a potential therapeutic target and our RNAi

experiments strongly suggests the potential value of development of true targeted therapy using a small molecule inhibitor of DNMT3b. Small molecule inhibitors may be orally available and express greater target specificity eliminating unwanted side effects associated with broad-spectrum DNMT inhibitors like 5-aza.

The observations made in our studies also suggest that this therapeutic strategy (combining epigenetic treatment with conventional chemotherapy) may find utility in malignancies from other tissue types with well-defined or not so well-defined methylator phenotypes. In our studies, we employed a period of demethylation treatment and used this period as a window for sensitization to chemotherapy. Published investigations show reversal of resistance to chemotherapeutic drugs in human xenograft models as a result of pretreatment with low doses of 5-aza 6-12 days prior to administration of chemotherapeutic drugs [279]. This reversal was associated with epigenetic re-activation of pro-apoptotic genes [258, 279]. More interestingly, this effect was abrogated if 5-aza was given concurrently with the cytotoxic drugs or after the cytotoxic therapy [279]. In another example, a randomized phase II study of the combination of 5-aza and carboplatin in relapsed ovarian cancer is currently underway [267, 280]. Based on results of the phase I trial, a dose/schedule for the phase II trial reflects demethylating treatment with 5-aza (day1) followed by carboplatin (day 8) and the repetition of this cycle every 28 days is recommended [280]. These observations strongly support our conclusion that demethylating pre-treatment sensitizes cancer cells to chemotherapeutic drugs. While additional investigations are needed to fully comprehend the possibilities associated with targeting DNMT3b in basal-like breast cancer (and other cancers), this observation of increased sensitivity to chemotherapeutics as a result of

modulation of DNA methylation machinery (more specifically DNMT3b) may present new options and targets to develop new treatment strategies.

Loss of Post-Transcriptional Regulation of *DNMT3b* by Regulatory miRs Drives The DNMT3b-mediated Hypermethylation Defect in Breast Cancer

Epigenetic changes significantly contribute to the normal regulation of gene expression and when dysregulated can significantly contribute to carcinogenesis [75, 76]. Aberrant epigenetic silencing of tumor suppressor genes and other negative mediators of cell proliferation have been documented in the development and progression of breast cancer [74, 118, 129]. The CpG island methylator phenotype (or CIMP) represents a major epigenetic mechanism of colorectal carcinogenesis that has also been recognized in cancers affecting other tissues [134, 140, 143]. We have identified a hypermethylation defect in a subset of human breast cancer cell lines and primary breast cancers that is characterized by DNMT hyperactivity, overexpression of DNMT3b, and concurrent methylation-dependent silencing of numerous genes (including *CDH1, CEACAM6, CST6, ESR1, GNA11, MYB, MUC1, SCNN1A,* and *TFF*) [150]. Mining of microarray-based expression data identified a strong cluster of primary breast cancers that display a gene expression signature associated with the hypermethylation defect [150]. A strong association was established between the expression of the hypermethylation defect signature and the basal-like molecular subtype of breast cancers [150]. Basal-like breast cancers are typically classified as triple-negative, reflecting lack expression of estrogen and progesterone receptors (ER-/PR-), and absence of HER2 gene amplification (HER2-) [281, 282]. Hence, patients with basal-like breast cancer are not responsive to targeted therapies like tamoxifen (targets ER) and trastuzumab (targets HER2)

[244, 283]. The poor prognosis associated with basal-like breast cancer and lack of druggable targets makes the fundamental observation of the co-segregation of the hypermethylation defect with basal-like breast cancer to be of utmost significance. Our observations suggest strongly that the DNA methylation machinery (and specifically DNMT3b) represent new/novel molecular targets for development of new drugs and treatment strategies for basal-like breast cancer.

In these studies, our goal was to elucidate the molecular mechanism accounting for overexpression of DNMT3b in hypermethylator breast cancer cell lines and hypermethylator breast cancers. Recent studies link miRs to the post-transcriptional regulation of *DNMT3b* expression in various tissues. Loss of expression of members of miR-29 family and overexpression of DNMT3b has been shown in lung cancer [223] and acute myeloid leukemia [224]. Likewise, there is evidence supporting the negative regulation of *DNMT3b* by miR-148a and miR-148b in cell lines of multiple origins [225]. The results of the present study strongly suggest that loss of regulatory miR expression contributes to *DNMT3b* overexpression that characterizes the hypermethylation defect seen in breast cancer. In the breast cancer cell line model, this evidence includes: (i) differential expression of regulatory miRs between hypermethylator and non-hypermethylator cell lines, (ii) significantly diminished expression of miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203 among hypermethylator breast cancer cell lines, (iii) pre-miR-mediated re-expression of miR-148b, miR-26b, or miR-29c in hypermethylator breast cancer cell lines (Hs578T, HCC1937, and SUM185) produces reduced *DNMT3b* mRNA levels, and (iv) antagomir-mediated knockdown of miR-148b, miR-26b, or miR-29c in non-hypermethylator breast cancer cell lines (MDA-MB-468, MDA-MB-415, and BT20) leads to increased *DNMT3b*

mRNA levels. In primary tumors, we observed that (a) significantly reduced expression of miR-29c distinguished basal-like cancers from other subtypes of the primary breast tumors, (b) miR expression patterns revealed two groups among the basal-like breast cancers corresponding to diminished expression and normal levels of expression, (c) 7/9 hypermethylators among basal-like tumors correspond to the group that has diminished expression of regulatory miRs. These findings strongly suggest that: (i) post-transcriptional regulation of *DNMT3b* is combinatorial, involving multiple miR species, (ii) diminished expression of regulatory miRs contributes to *DNMT3b* overexpression, (iii) re-expression of regulatory miRs results in reduced *DNMT3b* mRNA levels in hypermethylator breast cancer cell lines, and (iv) down-regulation of regulatory miRs results in increased *DNMT3b* mRNA levels in non-hypermethylator breast cancer cell lines. The observed loss of regulatory miRs in expression of the pro-cancerogenic hypermethylation defect suggests that these miRs possess a tumor suppressor-like function in breast, similar to other tissues [223-225].

miRs are predicted to post-transcriptionally regulate more that 60% of all protein-encoding genes in mammals and contribute to almost every cellular process, normal and pathological [188]. miRs have been recently been established as key players in carcinogenesis, with functions that can be oncogenic or tumor suppressor-like [187]. Our results suggest loss of combinations of miR-29c, miR-148a, miR-148b, miR-26a, miR-26b, and miR-203 is associated with expression of the hypermethylation defect in breast cancer cell lines, consistent with the idea that these miRs function as negative mediators of the neoplastic phenotype. We have also observed a significant concordance between the hypermethylators among basal-like breast cancers and the group of primary cancers that has diminished expression of regulatory miRs. Diminished levels of these miRs have been

documented in various forms of cancer, supporting the suggestion that these miRs possess tumor suppressor-like function. miR-29a and miR-29b are shown to be down-regulated in chronic lymphocytic leukemia, acute myeloid leukemia, lung cancers, cholangiocarcinoma, and prostate cancer [223, 237, 284-287]. Reduced expression of miR-26a occurs in hepatocellular carcinoma, oral squamous cell carcinoma, bladder cancer, thyroid anaplastic carcinoma, Burkitt's lymphoma, acute myeloid leukemia, papillary carcinoma, prostate cancer, and breast cancer [188, 288, 289]. miR-26b expression is diminished in Hodgkin's lymphoma, oral squamous cell carcinoma, and prostate cancers [289]. miR-29c expression is depressed in nasopharyngeal carcinomas, bladder tumors, chronic lymphocytic leukemia, acute myeloid leukemia, lung cancers, cholangiocarcinoma, esophageal squamous cell carcinoma and pancreatic ductal adenocarcinoma [188, 284, 288-290]. miR-148a is down-regulated in breast cancers, papillary thyroid carcinoma, pancreatic ductal adenocarcinoma, prostate cancer, colorectal adenocarcinoma [288, 289]. miR-148b is expressed at reduced levels in oral squamous cell carcinoma, papillary thyroid carcinoma, prostate cancer, colorectal adenocarcinoma, pancreatic ductal adenocarcinoma [289]. miR-203 levels are diminished in oral squamous cell carcinoma, chronic myeloid leukemia, hepatocellular adenomas, esophageal squamous cell carcinoma [288, 289]. These studies from the literature document loss or diminished expression of these regulatory miRs in various forms of cancer, including breast in some cases.

Several molecular mechanisms contribute to miR dysregulation in cancer, including genetic abnormalities (such as chromosomal rearrangement, deletion, amplification, or sequence mutations) and epigenetic changes (methylation-dependent silencing of miR expression or alterations in the miRNA biogenesis machinery) [188]. Numerous miR genes

(>50%) are positioned within or close to chromosomal fragile sites and other genomic regions associated with cancer [188]. Genetic alterations involving these chromosomal regions result in dramatic alteration of miR expression levels [188]. Likewise, numerous studies report promoter hypermethylation as an important mechanism leading to loss of miR expression in cancer [187]. Loss of miR-203 expression is associated with fragile site on chromosome 14q32 [291], as well as through promoter hypermethylation in hematopoietic malignancies [291, 292]. miR-148a and miR148b are also susceptible to methylation-dependent silencing in cancer [187]. We found miR-203 to be significantly co-regulated with miR148a and miR-148b, suggesting the possibility of a common epigenetic mechanism accounting for their diminished expression in hypermethylator cell lines. These examples from the literature suggest that loss of regulatory miR expression leading to *DNMT3b* dysregulation could be the result of genetic or epigenetic mechanisms.

Numerous studies have established that miRs exhibit a unique and different expression in cancer tissues compared to normal tissues suggesting that miRs have a role in defining the molecular and pathological profiles of cancers including breast cancer [214, 216, 238]. miRs expression profiles have shown that miRs are more efficient in differentiating between normal and cancer tissues, and are better at classifying poorly differentiated tissues [188, 293]. miRs are relatively stable and are resistant to RNAse degradation, most likely due to their small size [294-296]. They are highly stable in tissue sections and can be isolated and quantified from FFPE tissues. In addition, investigations have found that real-time PCR data and microarray data obtained from routinely processed FFPE tissues and/or frozen for 10 years were reliable, reproducible, and consistent with data from fresh frozen samples [297, 298]. These features and observations make miRs excellent biomarkers for cancer detection

and classification in terms of tissue of origin, stage, and other pathological features. Furthermore, the techniques of miR detection are now sufficiently sensitive to analyze miRs in a few nanograms of total RNA making miRs even more attractive candidates as biomarkers. Our studies indicate that loss of regulatory miRs is associated with expression of hypermethylation defect. We also observed that there exists substantial overlap between the expression of hypermethylation defect and basal-like subtype of breast cancers. These observations suggest that miRs can be used as potential biomarkers for the detection of the subset of primary breast cancers that express the hypermethylation defect. We have also shown that use of epigenetic treatment to alter the methylation status of breast cancers makes these cancers more sensitive to standard chemotherapeutic treatment. In current studies, we observed changes in *DNMT3b* levels with transient transfection of breast cancer cell lines with pre-miRs or with antagomirs. These findings suggest that modulating (re-expressing) levels of miRNAs that are deregulated resulting in DNMT3b overexpression and expression of hypermethylation defect in breast cancer may be significant to the outcomes of these cancers. These regulatory miRs represent potentially useful therapeutic 'agents' to amend the methylation machinery in hypermethylator breast cancers. Pre-treatment with these miRs may sensitize hypermethylator cancers to standard chemotherapy similar to our findings that demonstrate sensitization of hypermethylator breast cancer following treatment with 5-aza and/or DNMT3b knockdown. Several methods exist that can be employed to increase the expression of the miRs whose loss is associated with expression of the hypermethylation defect. This can be achieved by introducing a short synthetic duplex RNA molecule that is loaded onto RISC or by viral or liposomal delivery methods aimed at inducing the expression of pre-miRs [214, 299, 300]. Synthetic miRNAs include siRNA-like oligonucleotides as well

as chemically modified oligonucleotides. Studies aimed at facilitating alterations in the levels of miRs have the advantage that a lot of fundamental work in the field of delivery of siRNA and antisense molecules can be directly exploited. Although siRNA delivery has paved the way for potential use of miRs as therapeutic agents, it has its own challenges, such as developing ways to steer delivery to cancer cells, (possibly by antibody-mediated targeting of cancer-specific antigens), short life of the oligonucleotides necessitating repetitive administration and so on [214]. Nevertheless, miRs exhibit great potential to contribute to the future management of breast cancers among other cancers.

Targeting the Epigenome in Basal-like Breast Cancer: Implications and Potential Impact in Prevention and Treatment

The natural history of breast cancer development has long been recognized to progress from atypical ductal hyperplasia to ductal carcinoma *in situ* (DCIS), and then evolution of this pre-invasive lesion into invasive breast cancer that can ultimately develop into metastatic disease [301]. All of these stages represent a complex and multidimensional process of initiation, development, and progression of breast cancer. Intervention at any of these stages has the potential to significantly disrupt the natural history of disease and affect the outcome. For example, DCIS represents a commonly diagnosed breast lesion that accounts for 25% of breast neoplasms diagnosed in the United States and ~55,000 new cases each year [1, 302]. It is by definition non-invasive, but can vary from low-grade (and not life threatening) to high-grade lesions that may contain invasive elements and represents a risk factor for development of invasive breast cancer [303]. Consistent with natural history of disease, the incidence of DCIS increases with age in parallel with the incidence of invasive

breast cancer and many invasive breast cancers are associated with adjacent DCIS lesions. In the last decade, invasive breast cancers have been characterized using gene expression analysis and classified into several molecular subtypes that have implications for treatment and long-term survival [7, 58, 59, 304, 305]. More recently, analyses of gene expression patterns in pre-invasive breast cancers has identified similar molecular subtypes [306-311]. There exists a strong correspondence between molecular subtypes of pre-invasive and invasive cancers and this overlap is supported by the observation of basal-like DCIS in patients with basal-like breast cancer [309]. Numerous lines of evidence also suggest that breast cancers may develop through field cancerization, including significant observations made in patients with pre-invasive breast neoplasms. Several studies have noted that the width of the surgical margin is directly associated with the risk of local recurrence (or development of invasive breast cancer) following breast conserving surgery for DCIS [302, 303]. Likewise, whole-breast radiation therapy has been shown to significantly reduce the risk of development of invasive breast cancer following breast conserving surgery for DCIS [302]. Molecular evidence for field cancerization in the breast includes the observation of both genetic and epigenetic alterations in otherwise normal appearing breast epithelium. Genomic instability in the form of allelic imbalances have been characterized in histologically normal breast tissue adjacent to invasive breast cancers, suggesting that these genetic alterations occur early in breast carcinogenesis [312]. Likewise, numerous epigenetic alterations have been identified in histologically normal breast epithelium adjacent to invasive breast cancers, including promoter methylation-dependent silencing of *CDH1*, *RASSF1A*, *RARα*, *APC*, and others [313-316]. Likewise, epigenetic silencing of various genes has been shown in pre-neoplastic (atypical ductular hyperplasia) and pre-invasive

DCIS lesions [128, 317]. Given the linkage between basal-like breast cancers and expression of the hypermethylation defect, loss of regulatory miR expression leading to *DNMT3b* overexpression may represent a very early and significant molecular alteration during the natural history of breast carcinogenesis. This observation paves the way for the potential role of miRs as diagnostic markers for detecting hypermethylation in very early/pre-invasive breast cancers. The loss of regulatory miRs in pre-invasive lesions leading to the establishment of the hypermethylation defect (with *DNMT3b* overexpression) may suggest that these alterations determine the basal-like molecular subtype of breast cancer. On the other hand, loss of miRs that regulate DNMT3b detected at a relatively advanced stage of breast cancer indicate that these miRs drive the basal-like molecular subtype of breast cancer. Early detection of these lesions creates the potential to identify patients for epigenetic therapies. Presence of epigenetic changes before cancer appears/becomes invasive not only highlights epigenetic changes as seminal events in breast cancer initiation but also represents a potential target for chemoprevention. Treatment with demethylating agents and/or DNMT3b small molecule inhibitor can potentially halt the progression of disease and positively influence the outcome. Though in its infancy, the emerging concept of circulatory miRs is a great area of interest. Once, the high risk patients are identified (and/or treated with epigenetic therapy), circulating miRs provide non-invasive biomarkers for surveillance to determine if there is a need for continuing the prophylactic treatment or not. Likewise, after treatment, miR expression patterns associated with DNMT3b overexpression and hypermethylation defect can be used as surrogate endpoints for prognostic purposes to detect relapse and to monitor disease activity and response to therapy. Once the dose and schedule of epigenetic treatments is optimized so as to minimize toxicity, there is a possibility of using

these agents as maintenance therapies to prevent relapse following chemotherapy or even radiotherapy.

Restoration of the regulatory miRs by themselves is another attractive approach to correct the miR dysregulation that results in DNMT3b overexpression and hypermethylation defect. The expression patterns as well as promoter methylation status of methylation-sensitive genes that are established targets of DNMT3b can be used as endpoints to regulate the therapy aimed at re-expression of the regulatory miRs. However, development of the miRs as diagnostic, therapeutic, and prognostic targets comes with lots of challenges and obstacles. Development of *in vivo* organ-specific delivery systems is very important. Since, the miRs are functionally very different based on the target organ, it will be imperative to develop a tissue-specific delivery system to prevent off-target effects and toxicity. Chemotherapy continues to be the mainstay of treatment for most of the breast cancers, especially in basal-like breast cancers. It is now known that miRs regulate multiple biological processes, it is conceivable that re-expressing regulatory miRs may alter drug response in multiple ways and not just by altering the epigenetic signature. Therefore, further studies are required before miR biology can move to clinical application in cancer. The translation of the observations made in our studies from bench to bedside remains a work in progress. In addition, further independent studies of miRs and their role in establishing hypermethylation effects in breast cancer will be helpful to validate and identify the most relevant miRs for this purpose. To fully understand and characterize the linkage between miR dysregulation and DNMT3b overexpression, it is imperative that all human miRs are known, identified and characterized fully including the features like functional targets of miRs and the phenotypic changes associated with their manipulation. In addition, the standardization of techniques for

miR analysis is necessary. However, miRs have shown a great potential in a relatively short time since their discovery, and seem to provide a promising and exciting basis for future studies.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E: **Cancer statistics, 2010**. *CA Cancer J Clin* 2010, **60**(5):277-300.

2. Jemal A, Ward E, Thun MJ: **Recent trends in breast cancer incidence rates by age and tumor characteristics among U.S. women**. *Breast Cancer Res* 2007, **9**(3):R28.

3. Breen N, K AC, Meissner HI, Taplin SH, Tangka FK, Tiro JA, McNeel TS: **Reported drop in mammography : is this cause for concern?** *Cancer* 2007, **109**(12):2405-2409.

4. Chlebowski RT, Kuller LH, Prentice RL, Stefanick ML, Manson JE, Gass M, Aragaki AK, Ockene JK, Lane DS, Sarto GE *et al*: **Breast cancer after use of estrogen plus progestin in postmenopausal women**. *N Engl J Med* 2009, **360**(6):573-587.

5. Ravdin PM, Cronin KA, Howlader N, Berg CD, Chlebowski RT, Feuer EJ, Edwards BK, Berry DA: **The decrease in breast-cancer incidence in 2003 in the United States**. *N Engl J Med* 2007, **356**(16):1670-1674.

6. Ryerson AB, Miller JW, Eheman CR, Leadbetter S, White MC: **Recent trends in U.S. mammography use from 2000-2006: a population-based analysis**. *Prev Med* 2008, **47**(5):477-482.

7. Jeffrey S Ross GNH: **Molecular Oncology of Breast Cancer**: Jones and Bartlett Publishers; 2005.

8. Pike MC, Pearce CL, Wu AH: **Prevention of cancers of the breast, endometrium and ovary**. *Oncogene* 2004, **23**(38):6379-6391.

9. Kolonel LN, Altshuler D, Henderson BE: **The multiethnic cohort study: exploring genes, lifestyle and cancer risk**. *Nat Rev Cancer* 2004, **4**(7):519-527.

10. McTiernan A, Kooperberg C, White E, Wilcox S, Coates R, Adams-Campbell LL, Woods N, Ockene J: **Recreational physical activity and the risk of breast cancer in postmenopausal women: the Women's Health Initiative Cohort Study**. *Jama* 2003, **290**(10):1331-1336.

11. Hankinson SE, Colditz GA, Willett WC: **Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones**. *Breast Cancer Res* 2004, **6**(5):213-218.

12. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: **Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults**. *N Engl J Med* 2003, **348**(17):1625-1638.

13. Terry P, Jain M, Miller AB, Howe GR, Rohan TE: **No association among total dietary fiber, fiber fractions, and risk of breast cancer**. *Cancer Epidemiol Biomarkers Prev* 2002, **11**(11):1507-1508.

14. Song M, Lee KM, Kang D: **Breast cancer prevention based on gene-environment interaction**. *Mol Carcinog*, **50**(4):280-290.

15. Kelsey JL, Bernstein L: **Epidemiology and prevention of breast cancer**. *Annu Rev Public Health* 1996, **17**:47-67.

16. Fraumeni JF, Jr., Lloyd JW, Smith EM, Wagoner JK: **Cancer mortality among nuns: role of marital status in etiology of neoplastic disease in women**. *J Natl Cancer Inst* 1969, **42**(3):455-468.

17. van den Brandt PA, Spiegelman D, Yaun SS, Adami HO, Beeson L, Folsom AR, Fraser G, Goldbohm RA, Graham S, Kushi L *et al*: **Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk**. *Am J Epidemiol* 2000, **152**(6):514-527.

18. Key TJ, Verkasalo PK, Banks E: **Epidemiology of breast cancer**. *Lancet Oncol* 2001, **2**(3):133-140.

19. Ballard-Barbash R, Schatzkin A, Taylor PR, Kahle LL: **Association of change in body mass with breast cancer**. *Cancer Res* 1990, **50**(7):2152-2155.

20. Barnes-Josiah D, Potter JD, Sellers TA, Himes JH: **Early body size and subsequent weight gain as predictors of breast cancer incidence (Iowa, United States)**. *Cancer Causes Control* 1995, **6**(2):112-118.

21. Huang Z, Hankinson SE, Colditz GA, Stampfer MJ, Hunter DJ, Manson JE, Hennekens CH, Rosner B, Speizer FE, Willett WC: **Dual effects of weight and weight gain on breast cancer risk**. *Jama* 1997, **278**(17):1407-1411.

22. Berkey CS, Frazier AL, Gardner JD, Colditz GA: **Adolescence and breast carcinoma risk**. *Cancer* 1999, **85**(11):2400-2409.

23. Le Marchand L, Kolonel LN, Earle ME, Mi MP: **Body size at different periods of life and breast cancer risk**. *Am J Epidemiol* 1988, **128**(1):137-152.

24. Huang Z, Willett WC, Colditz GA, Hunter DJ, Manson JE, Rosner B, Speizer FE, Hankinson SE: **Waist circumference, waist:hip ratio, and risk of breast cancer in the Nurses' Health Study**. *Am J Epidemiol* 1999, **150**(12):1316-1324.

25. Kaaks R, Van Noord PA, Den Tonkelaar I, Peeters PH, Riboli E, Grobbee DE: **Breast-cancer incidence in relation to height, weight and body-fat distribution in the Dutch "DOM" cohort**. *Int J Cancer* 1998, **76**(5):647-651.

26. Morimoto LM, White E, Chen Z, Chlebowski RT, Hays J, Kuller L, Lopez AM, Manson J, Margolis KL, Muti PC *et al*: **Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States)**. *Cancer Causes Control* 2002, **13**(8):741-751.

27. Boyd NF, Byng JW, Jong RA, Fishell EK, Little LE, Miller AB, Lockwood GA, Tritchler DL, Yaffe MJ: **Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study**. *J Natl Cancer Inst* 1995, **87**(9):670-675.

28. Brisson J, Merletti F, Sadowsky NL, Twaddle JA, Morrison AS, Cole P: **Mammographic features of the breast and breast cancer risk**. *Am J Epidemiol* 1982, **115**(3):428-437.

29. Byrne C, Schairer C, Wolfe J, Parekh N, Salane M, Brinton LA, Hoover R, Haile R: **Mammographic features and breast cancer risk: effects with time, age, and menopause status**. *J Natl Cancer Inst* 1995, **87**(21):1622-1629.

30. Armstrong B, Doll R: **Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices**. *Int J Cancer* 1975, **15**(4):617-631.

31. Willett WC: **Diet and cancer**. *Oncologist* 2000, **5**(5):393-404.

32. Hanf V, Gonder U: **Nutrition and primary prevention of breast cancer: foods, nutrients and breast cancer risk**. *Eur J Obstet Gynecol Reprod Biol* 2005, **123**(2):139-149.

33. Terry PD, Miller AB, Rohan TE: **Cigarette smoking and breast cancer risk: a long latency period?** *Int J Cancer* 2002, **100**(6):723-728.

34. Smith-Warner SA, Spiegelman D, Yaun SS, van den Brandt PA, Folsom AR, Goldbohm RA, Graham S, Holmberg L, Howe GR, Marshall JR *et al*: **Alcohol and breast cancer in women: a pooled analysis of cohort studies**. *Jama* 1998, **279**(7):535-540.

35. Smith-Warner SA, Spiegelman D, Yaun SS, Adami HO, Beeson WL, van den Brandt PA, Folsom AR, Fraser GE, Freudenheim JL, Goldbohm RA *et al*: **Intake of fruits and vegetables and risk of breast cancer: a pooled analysis of cohort studies**. *Jama* 2001, **285**(6):769-776.

36. Marcus PM, Newman B, Millikan RC, Moorman PG, Baird DD, Qaqish B: **The associations of adolescent cigarette smoking, alcoholic beverage consumption, environmental tobacco smoke, and ionizing radiation with subsequent breast cancer risk (United States)**. *Cancer Causes Control* 2000, **11**(3):271-278.

37. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW, Wu-Williams AH, Kolonel LN, Horn-Ross PL, Rosenthal JF *et al*: **Migration patterns and breast cancer risk in Asian-American women**. *J Natl Cancer Inst* 1993, **85**(22):1819-1827.

38. Cui X, Dai Q, Tseng M, Shu XO, Gao YT, Zheng W: **Dietary patterns and breast cancer risk in the shanghai breast cancer study**. *Cancer Epidemiol Biomarkers Prev* 2007, **16**(7):1443-1448.

39. Mavaddat N, Antoniou AC, Easton DF, Garcia-Closas M: **Genetic susceptibility to breast cancer**. *Mol Oncol* 2010, **4**(3):174-191.

40. Thompson D, Easton D: **The genetic epidemiology of breast cancer genes**. *J Mammary Gland Biol Neoplasia* 2004, **9**(3):221-236.

41. Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J *et al*: **Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium**. *Am J Hum Genet* 1998, **62**(3):676-689.

42. Narod SA: **Modifiers of risk of hereditary breast and ovarian cancer**. *Nat Rev Cancer* 2002, **2**(2):113-123.

43. Nelen MR, Padberg GW, Peeters EA, Lin AY, van den Helm B, Frants RR, Coulon V, Goldstein AM, van Reen MM, Easton DF *et al*: **Localization of the gene for Cowden disease to chromosome 10q22-23**. *Nat Genet* 1996, **13**(1):114-116.

44. Garber JE, Goldstein AM, Kantor AF, Dreyfus MG, Fraumeni JF, Jr., Li FP: **Follow-up study of twenty-four families with Li-Fraumeni syndrome**. *Cancer Res* 1991, **51**(22):6094-6097.

45. Malkin D: **Germline p53 mutations and heritable cancer**. *Annu Rev Genet* 1994, **28**:443-465.

46. Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P *et al*: **A serine/threonine kinase gene defective in Peutz-Jeghers syndrome**. *Nature* 1998, **391**(6663):184-187.

47. Meijers-Heijboer H, van den Ouweland A, Klijn J, Wasielewski M, de Snoo A, Oldenburg R, Hollestelle A, Houben M, Crepin E, van Veghel-Plandsoen M *et al*:

Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet* 2002, **31**(1):55-59.

48. Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, Reid S, Spanova K, Barfoot R, Chagtai T *et al*: **PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene**. *Nat Genet* 2007, **39**(2):165-167.

49. Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, Chagtai T, Jayatilake H, Ahmed M, Spanova K *et al*: **Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles**. *Nat Genet* 2006, **38**(11):1239-1241.

50. Renwick A, Thompson D, Seal S, Kelly P, Chagtai T, Ahmed M, North B, Jayatilake H, Barfoot R, Spanova K *et al*: **ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles**. *Nat Genet* 2006, **38**(8):873-875.

51. Bartkova J, Tommiska J, Oplustilova L, Aaltonen K, Tamminen A, Heikkinen T, Mistrik M, Aittomaki K, Blomqvist C, Heikkila P *et al*: **Aberrations of the MRE11-RAD50-NBS1 DNA damage sensor complex in human breast cancer: MRE11 as a candidate familial cancer-predisposing gene**. *Mol Oncol* 2008, **2**(4):296-316.

52. Antoniou AC, Easton DF: **Models of genetic susceptibility to breast cancer**. *Oncogene* 2006, **25**(43):5898-5905.

53. Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, Ballinger DG, Struewing JP, Morrison J, Field H, Luben R *et al*: **Genome-wide association study identifies novel breast cancer susceptibility loci**. *Nature* 2007, **447**(7148):1087-1093.

54. Thomas G, Jacobs KB, Kraft P, Yeager M, Wacholder S, Cox DG, Hankinson SE, Hutchinson A, Wang Z, Yu K *et al*: **A multistage genome-wide association study in breast cancer identifies two new risk alleles at 1p11.2 and 14q24.1 (RAD51L1)**. *Nat Genet* 2009, **41**(5):579-584.

55. Milne RL, Benitez J, Nevanlinna H, Heikkinen T, Aittomaki K, Blomqvist C, Arias JI, Zamora MP, Burwinkel B, Bartram CR *et al*: **Risk of estrogen receptor-positive and -negative breast cancer and single-nucleotide polymorphism 2q35-rs13387042**. *J Natl Cancer Inst* 2009, **101**(14):1012-1018.

56. Stacey SN, Manolescu A, Sulem P, Rafnar T, Gudmundsson J, Gudjonsson SA, Masson G, Jakobsdottir M, Thorlacius S, Helgason A *et al*: **Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer**. *Nat Genet* 2007, **39**(7):865-869.

57. Sotiriou C, Powles TJ, Dowsett M, Jazaeri AA, Feldman AL, Assersohn L, Gadisetti C, Libutti SK, Liu ET: **Gene expression profiles derived from fine needle**

aspiration correlate with response to systemic chemotherapy in breast cancer. *Breast Cancer Res* 2002, **4**(3):R3.

58. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al*: **Molecular portraits of human breast tumours**. *Nature* 2000, **406**(6797):747-752.

59. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al*: **Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications**. *Proc Natl Acad Sci U S A* 2001, **98**(19):10869-10874.

60. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S *et al*: **Repeated observation of breast tumor subtypes in independent gene expression data sets**. *Proc Natl Acad Sci U S A* 2003, **100**(14):8418-8423.

61. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET: **Breast cancer classification and prognosis based on gene expression profiles from a population-based study**. *Proc Natl Acad Sci U S A* 2003, **100**(18):10393-10398.

62. Ma XJ, Wang Z, Ryan PD, Isakoff SJ, Barmettler A, Fuller A, Muir B, Mohapatra G, Salunga R, Tuggle JT *et al*: **A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen**. *Cancer Cell* 2004, **5**(6):607-616.

63. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ *et al*: **A gene-expression signature as a predictor of survival in breast cancer**. *N Engl J Med* 2002, **347**(25):1999-2009.

64. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT *et al*: **Gene expression profiling predicts clinical outcome of breast cancer**. *Nature* 2002, **415**(6871):530-536.

65. Huang E, Cheng SH, Dressman H, Pittman J, Tsou MH, Horng CF, Bild A, Iversen ES, Liao M, Chen CM *et al*: **Gene expression predictors of breast cancer outcomes**. *Lancet* 2003, **361**(9369):1590-1596.

66. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S *et al*: **Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study**. *Jama* 2006, **295**(21):2492-2502.

67. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, van't Veer LJ, Perou CM: **Concordance among gene-expression-based predictors for breast cancer**. *N Engl J Med* 2006, **355**(6):560-569.

68. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, Nooij MA, Voest EE, van der Wall E, Hupperets P *et al*: **Molecular subtypes of breast cancer and amplification of topoisomerase II alpha: predictive role in dose intensive adjuvant chemotherapy**. *Br J Cancer* 2006, **95**(10):1334-1341.

69. Potemski P, Kusinska R, Watala C, Pluciennik E, Bednarek AK, Kordek R: **Prognostic relevance of basal cytokeratin expression in operable breast cancer**. *Oncology* 2005, **69**(6):478-485.

70. Kapp AV, Jeffrey SS, Langerod A, Borresen-Dale AL, Han W, Noh DY, Bukholm IR, Nicolau M, Brown PO, Tibshirani R: **Discovery and validation of breast cancer subtypes**. *BMC Genomics* 2006, **7**:231.

71. Brenton JD, Carey LA, Ahmed AA, Caldas C: **Molecular classification and molecular forecasting of breast cancer: ready for clinical application?** *J Clin Oncol* 2005, **23**(29):7350-7360.

72. Baselga J, Perez EA, Pienkowski T, Bell R: **Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer**. *Oncologist* 2006, **11 Suppl 1**:4-12.

73. Pal SK, Mortimer J: **Triple-negative breast cancer: novel therapies and new directions**. *Maturitas* 2009, **63**(4):269-274.

74. Yang X, Yan L, Davidson NE: **DNA methylation in breast cancer**. *Endocr Relat Cancer* 2001, **8**(2):115-127.

75. Esteller M: **Epigenetics in cancer**. *N Engl J Med* 2008, **358**(11):1148-1159.

76. Feinberg AP, Tycko B: **The history of cancer epigenetics**. *Nat Rev Cancer* 2004, **4**(2):143-153.

77. Widschwendter M, Jones PA: **DNA methylation and breast carcinogenesis**. *Oncogene* 2002, **21**(35):5462-5482.

78. Gardiner-Garden M, Frommer M: **CpG islands in vertebrate genomes**. *J Mol Biol* 1987, **196**(2):261-282.

79. Baylin S: **DNA methylation and epigenetic mechanisms of carcinogenesis**. *Dev Biol (Basel)* 2001, **106**:85-87; discussion 143-160.

80. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP: **Alterations in DNA methylation: a fundamental aspect of neoplasia**. *Adv Cancer Res* 1998, **72**:141-196.

81. Herman JG, Baylin SB: **Gene silencing in cancer in association with promoter hypermethylation**. *N Engl J Med* 2003, **349**(21):2042-2054.

82. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D: **Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells**. *Nat Genet* 2005, **37**(8):853-862.

83. Feinberg AP, Vogelstein B: **Hypomethylation of ras oncogenes in primary human cancers**. *Biochem Biophys Res Commun* 1983, **111**(1):47-54.

84. Eden A, Gaudet F, Waghmare A, Jaenisch R: **Chromosomal instability and tumors promoted by DNA hypomethylation**. *Science* 2003, **300**(5618):455.

85. Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, Ehrlich M: **Hypomethylation of pericentromeric DNA in breast adenocarcinomas**. *Int J Cancer* 1998, **77**(6):833-838.

86. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R: **Induction of tumors in mice by genomic hypomethylation**. *Science* 2003, **300**(5618):489-492.

87. Momparler RL: **Cancer epigenetics**. *Oncogene* 2003, **22**(42):6479-6483.

88. Jones PA, Laird PW: **Cancer epigenetics comes of age**. *Nat Genet* 1999, **21**(2):163-167.

89. Rivenbark AG, Jones WD, Risher JD, Coleman WB: **DNA methylation-dependent epigenetic regulation of gene expression in MCF-7 breast cancer cells**. *Epigenetics* 2006, **1**(1):32-44.

90. Hanahan D, Weinberg RA: **The hallmarks of cancer**. *Cell* 2000, **100**(1):57-70.

91. Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA: **DNA methylation analysis: a powerful new tool for lung cancer diagnosis**. *Oncogene* 2002, **21**(35):5450-5461.

92. Jones PA, Baylin SB: **The fundamental role of epigenetic events in cancer**. *Nat Rev Genet* 2002, **3**(6):415-428.

93. Herman JG: **Hypermethylation of tumor suppressor genes in cancer**. *Semin Cancer Biol* 1999, **9**(5):359-367.

94. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D: **5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers**. *Nat Med* 1995, **1**(7):686-692.

95. Okano M, Xie S, Li E: **Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells**. *Nucleic Acids Res* 1998, **26**(11):2536-2540.

96. Baylin SB: **DNA methylation and gene silencing in cancer**. *Nat Clin Pract Oncol* 2005, **2 Suppl 1**:S4-11.

97. Bestor TH: **The DNA methyltransferases of mammals**. *Hum Mol Genet* 2000, **9**(16):2395-2402.

98. Kim GD, Ni J, Kelesoglu N, Roberts RJ, Pradhan S: **Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases**. *Embo J* 2002, **21**(15):4183-4195.

99. Okano M, Bell DW, Haber DA, Li E: **DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development**. *Cell* 1999, **99**(3):247-257.

100. Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW, Jones PA: **Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements**. *Mol Cell Biol* 2002, **22**(2):480-491.

101. Chen T, Ueda Y, Dodge JE, Wang Z, Li E: **Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b**. *Mol Cell Biol* 2003, **23**(16):5594-5605.

102. Kautiainen TL, Jones PA: **DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture**. *J Biol Chem* 1986, **261**(4):1594-1598.

103. Li S, Chiang TC, Richard-Davis G, Barrett JC, McLachlan JA: **DNA hypomethylation and imbalanced expression of DNA methyltransferases (DNMT1, 3A, and 3B) in human uterine leiomyoma**. *Gynecol Oncol* 2003, **90**(1):123-130.

104. Ahluwalia A, Yan P, Hurteau JA, Bigsby RM, Jung SH, Huang TH, Nephew KP: **DNA methylation and ovarian cancer. I. Analysis of CpG island hypermethylation in human ovarian cancer using differential methylation hybridization**. *Gynecol Oncol* 2001, **82**(2):261-268.

105. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA: **The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors**. *Nucleic Acids Res* 1999, **27**(11):2291-2298.

106. Fournel M, Sapieha P, Beaulieu N, Besterman JM, MacLeod AR: **Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators**

p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. *J Biol Chem* 1999, **274**(34):24250-24256.

107. Suzuki M, Sunaga N, Shames DS, Toyooka S, Gazdar AF, Minna JD: **RNA interference-mediated knockdown of DNA methyltransferase 1 leads to promoter demethylation and gene re-expression in human lung and breast cancer cells**. *Cancer Res* 2004, **64**(9):3137-3143.

108. Yan L, Nass SJ, Smith D, Nelson WG, Herman JG, Davidson NE: **Specific inhibition of DNMT1 by antisense oligonucleotides induces re-expression of estrogen receptor-alpha (ER) in ER-negative human breast cancer cell lines**. *Cancer Biol Ther* 2003, **2**(5):552-556.

109. Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR: **DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells**. *Nat Genet* 2003, **33**(1):61-65.

110. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW *et al*: **DNMT1 and DNMT3b cooperate to silence genes in human cancer cells**. *Nature* 2002, **416**(6880):552-556.

111. Leu YW, Rahmatpanah F, Shi H, Wei SH, Liu JC, Yan PS, Huang TH: **Double RNA interference of DNMT3b and DNMT1 enhances DNA demethylation and gene reactivation**. *Cancer Res* 2003, **63**(19):6110-6115.

112. Ting AH, Jair KW, Suzuki H, Yen RW, Baylin SB, Schuebel KE: **CpG island hypermethylation is maintained in human colorectal cancer cells after RNAi-mediated depletion of DNMT1**. *Nat Genet* 2004, **36**(6):582-584.

113. Girault I, Tozlu S, Lidereau R, Bieche I: **Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas**. *Clin Cancer Res* 2003, **9**(12):4415-4422.

114. Kanai Y, Hirohashi S: **Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state**. *Carcinogenesis* 2007, **28**(12):2434-2442.

115. Ting AH, Jair KW, Schuebel KE, Baylin SB: **Differential requirement for DNA methyltransferase 1 in maintaining human cancer cell gene promoter hypermethylation**. *Cancer Res* 2006, **66**(2):729-735.

116. James SR, Link PA, Karpf AR: **Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b**. *Oncogene* 2006, **25**(52):6975-6985.

117. Melnikov AA, Gartenhaus RB, Levenson AS, Motchoulskaia NA, Levenson Chernokhvostov VV: **MSRE-PCR for analysis of gene-specific DNA methylation**. *Nucleic Acids Res* 2005, **33**(10):e93.

118. Esteller M: **CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future**. *Oncogene* 2002, **21**(35):5427-5440.

119. Esteller M: **Dormant hypermethylated tumour suppressor genes: questions and answers**. *J Pathol* 2005, **205**(2):172-180.

120. Parrella P, Poeta ML, Gallo AP, Prencipe M, Scintu M, Apicella A, Rossiello R, Liguoro G, Seripa D, Gravina C *et al*: **Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors**. *Clin Cancer Res* 2004, **10**(16):5349-5354.

121. Lapidus RG, Nass SJ, Davidson NE: **The loss of estrogen and progesterone receptor gene expression in human breast cancer**. *J Mammary Gland Biol Neoplasia* 1998, **3**(1):85-94.

122. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, Weitzman SA, Baylin SB, Issa JP, Davidson NE: **Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors**. *Clin Cancer Res* 1996, **2**(5):805-810.

123. Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE: **Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells**. *Cancer Res* 1994, **54**(10):2552-2555.

124. Jin Z, Tamura G, Tsuchiya T, Sakata K, Kashiwaba M, Osakabe M, Motoyama T: **Adenomatous polyposis coli (APC) gene promoter hypermethylation in primary breast cancers**. *Br J Cancer* 2001, **85**(1):69-73.

125. Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, Taback B: **Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis**. *Clin Cancer Res* 2005, **11**(6):2156-2162.

126. Virmani AK, Rathi A, Sathyanarayana UG, Padar A, Huang CX, Cunnigham HT, Farinas AJ, Milchgrub S, Euhus DM, Gilcrease M *et al*: **Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas**. *Clin Cancer Res* 2001, **7**(7):1998-2004.

127. Muller HM, Fiegl H, Widschwendter A, Widschwendter M: **Prognostic DNA methylation marker in serum of cancer patients**. *Ann N Y Acad Sci* 2004, **1022**:44-49.

128. Lewis CM, Cler LR, Bu DW, Zochbauer-Muller S, Milchgrub S, Naftalis EZ, Leitch AM, Minna JD, Euhus DM: **Promoter hypermethylation in benign breast epithelium in relation to predicted breast cancer risk**. *Clin Cancer Res* 2005, **11**(1):166-172.

129. Ai L, Kim WJ, Kim TY, Fields CR, Massoll NA, Robertson KD, Brown KD: **Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression**. *Cancer Res* 2006, **66**(16):7899-7909.

130. Rivenbark AG, Jones WD, Coleman WB: **DNA methylation-dependent silencing of CST6 in human breast cancer cell lines**. *Lab Invest* 2006, **86**(12):1233-1242.

131. Hazan RB, Qiao R, Keren R, Badano I, Suyama K: **Cadherin switch in tumor progression**. *Ann N Y Acad Sci* 2004, **1014**:155-163.

132. Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR: **CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer**. *BMC Cancer* 2006, **6**:48.

133. Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM, Schalken JA: **Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors**. *Cancer Res* 1993, **53**(14):3241-3245.

134. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP: **CpG island methylator phenotype in colorectal cancer**. *Proc Natl Acad Sci U S A* 1999, **96**(15):8681-8686.

135. Strathdee G, Appleton K, Illand M, Millan DW, Sargent J, Paul J, Brown R: **Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes**. *Am J Pathol* 2001, **158**(3):1121-1127.

136. Liang G, Salem CE, Yu MC, Nguyen HD, Gonzales FA, Nguyen TT, Nichols PW, Jones PA: **DNA methylation differences associated with tumor tissues identified by genome scanning analysis**. *Genomics* 1998, **53**(3):260-268

.

137. Marsit CJ, Houseman EA, Christensen BC, Eddy K, Bueno R, Sugarbaker DJ, Nelson HH, Karagas MR, Kelsey KT: **Examination of a CpG island methylator phenotype and implications of methylation profiles in solid tumors**. *Cancer Res* 2006, **66**(21):10621-10629.

138. Suzuki M, Shigematsu H, Iizasa T, Hiroshima K, Nakatani Y, Minna JD, Gazdar AF, Fujisawa T: **Exclusive mutation in epidermal growth factor receptor gene, HER-2, and KRAS, and synchronous methylation of nonsmall cell lung cancer**. *Cancer* 2006, **106**(10):2200-2207.

139. An C, Choi IS, Yao JC, Worah S, Xie K, Mansfield PF, Ajani JA, Rashid A, Hamilton SR, Wu TT: **Prognostic significance of CpG island methylator phenotype and microsatellite instability in gastric carcinoma**. *Clin Cancer Res* 2005, **11**(2 Pt 1):656-663.

140. Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa JP: **Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype**. *Cancer Res* 1999, **59**(21):5438-5442.

141. Kim H, Kim YH, Kim SE, Kim NG, Noh SH, Kim H: **Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability**. *J Pathol* 2003, **200**(1):23-31.

142. Kusano M, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, Hosokawa M, Shinomura Y, Imai K, Tokino T: **Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus**. *Cancer* 2006, **106**(7):1467-1479.

143. Shen L, Ahuja N, Shen Y, Habib NA, Toyota M, Rashid A, Issa JP: **DNA methylation and environmental exposures in human hepatocellular carcinoma**. *J Natl Cancer Inst* 2002, **94**(10):755-761.

144. Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JP, Hruban RH, Goggins M: **Hypermethylation of multiple genes in pancreatic adenocarcinoma**. *Cancer Res* 2000, **60**(7):1835-1839.

145. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV *et al*: **Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma**. *Cancer Res* 2000, **60**(18):5021-5026.

146. Dulaimi E, Ibanez de Caceres I, Uzzo RG, Al-Saleem T, Greenberg RE, Polascik TJ, Babb JS, Grizzle WE, Cairns P: **Promoter hypermethylation profile of kidney cancer**. *Clin Cancer Res* 2004, **10**(12 Pt 1):3972-3979.

147. Abe M, Ohira M, Kaneda A, Yagi Y, Yamamoto S, Kitano Y, Takato T, Nakagawara A, Ushijima T: **CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas**. *Cancer Res* 2005, **65**(3):828-834.

148. Melki JR, Vincent PC, Brown RD, Clark SJ: **Hypermethylation of E-cadherin in leukemia**. *Blood* 2000, **95**(10):3208-3213.

149. Kaneko Y, Sakurai S, Hironaka M, Sato S, Oguni S, Sakuma Y, Sato K, Sugano K, Saito K: **Distinct methylated profiles in Helicobacter pylori dependent and independent gastric MALT lymphomas**. *Gut* 2003, **52**(5):641-646.

150. Roll JD, Rivenbark AG, Jones WD, Coleman WB: **DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines**. *Mol Cancer* 2008, **7**:15.

151. Bae YK, Brown A, Garrett E, Bornman D, Fackler MJ, Sukumar S, Herman JG, Gabrielson E: **Hypermethylation in histologically distinct classes of breast cancer**. *Clin Cancer Res* 2004, **10**(18 Pt 1):5998-6005.

152. Hess KR, Anderson K, Symmans WF, Valero V, Ibrahim N, Mejia JA, Booser D, Theriault RL, Buzdar AU, Dempsey PJ *et al*: **Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer**. *J Clin Oncol* 2006, **24**(26):4236-4244.

153. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J *et al*: **Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer**. *Lancet* 2005, **365**(9460):671-679.

154. Wetzels RH, Holland R, van Haelst UJ, Lane EB, Leigh IM, Ramaekers FC: **Detection of basement membrane components and basal cell keratin 14 in noninvasive and invasive carcinomas of the breast**. *Am J Pathol* 1989, **134**(3):571-579.

155. Dairkee SH, Ljung BM, Smith H, Hackett A: **Immunolocalization of a human basal epithelium specific keratin in benign and malignant breast disease**. *Breast Cancer Res Treat* 1987, **10**(1):11-20.

156. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: **The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells**. *Cell* 1982, **31**(1):11-24.

157. Moll R, Krepler R, Franke WW: **Complex cytokeratin polypeptide patterns observed in certain human carcinomas**. *Differentiation* 1983, **23**(3):256-269.

158. Kim MJ, Ro JY, Ahn SH, Kim HH, Kim SB, Gong G: **Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes**. *Hum Pathol* 2006, **37**(9):1217-1226.

159. O'Hare MJ, Ormerod MG, Monaghan P, Lane EB, Gusterson BA: **Characterization in vitro of luminal and myoepithelial cells isolated from the human mammary gland by cell sorting**. *Differentiation* 1991, **46**(3):209-221.

160. Tsubura A, Okada H, Senzaki H, Hatano T, Morii S: **Keratin expression in the normal breast and in breast carcinoma**. *Histopathology* 1991, **18**(6):517-522.

161. Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Smith LV, Labbok MH, Geradts J, Bensen JT, Jackson S *et al*: **Epidemiology of basal-like breast cancer**. *Breast Cancer Res Treat* 2008, **109**(1):123-139.

162. Reis-Filho JS, Tutt AN: **Triple negative tumours: a critical review**. *Histopathology* 2008, **52**(1):108-118.

163. Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, Wong N, Trudel M, Akslen LA: **Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer**. *J Natl Cancer Inst* 2003, **95**(19):1482-1485.

164. Tsuda H, Takarabe T, Hasegawa F, Fukutomi T, Hirohashi S: **Large, central acellular zones indicating myoepithelial tumor differentiation in high-grade invasive ductal carcinomas as markers of predisposition to lung and brain metastases**. *Am J Surg Pathol* 2000, **24**(2):197-202.

165. Tsuda H, Takarabe T, Hasegawa T, Murata T, Hirohashi S: **Myoepithelial differentiation in high-grade invasive ductal carcinomas with large central acellular zones**. *Hum Pathol* 1999, **30**(10):1134-1139.

166. Fulford LG, Easton DF, Reis-Filho JS, Sofronis A, Gillett CE, Lakhani SR, Hanby A: **Specific morphological features predictive for the basal phenotype in grade 3 invasive ductal carcinoma of breast**. *Histopathology* 2006, **49**(1):22-34.

167. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, Bishop T, Benitez J, Rivas C, Bignon YJ *et al*: **Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype**. *Clin Cancer Res* 2005, **11**(14):5175-5180.

168. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, Perou CM: **Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma**. *Mod Pathol* 2006, **19**(2):264-271.

169. Jacquemier J, Padovani L, Rabayrol L, Lakhani SR, Penault-Llorca F, Denoux Y, Fiche M, Figueiro P, Maisongrosse V, Ledoussal V *et al*: **Typical medullary breast carcinomas have a basal/myoepithelial phenotype**. *J Pathol* 2005, **207**(3):260-268.

170. Vincent-Salomon A, Gruel N, Lucchesi C, MacGrogan G, Dendale R, Sigal-Zafrani B, Longy M, Raynal V, Pierron G, de Mascarel I *et al*: **Identification of typical medullary breast carcinoma as a genomic sub-group of basal-like carcinomas, a heterogeneous new molecular entity**. *Breast Cancer Res* 2007, **9**(2):R24.

171. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L *et al*: **Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma**. *Clin Cancer Res* 2004, **10**(16):5367-5374.

172. Corkery B, Crown J, Clynes M, O'Donovan N: **Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer**. *Ann Oncol* 2009, **20**(5):862-867.

173. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P *et al*: **Breast cancer molecular subtypes respond differently to preoperative chemotherapy**. *Clin Cancer Res* 2005, **11**(16):5678-5685.

174. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML, Perou CM: **The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes**. *Clin Cancer Res* 2007, **13**(8):2329-2334.

175. Jones C, Nonni AV, Fulford L, Merrett S, Chaggar R, Eusebi V, Lakhani SR: **CGH analysis of ductal carcinoma of the breast with basaloid/myoepithelial cell differentiation**. *Br J Cancer* 2001, **85**(3):422-427.

176. Rakha EA, Putti TC, Abd El-Rehim DM, Paish C, Green AR, Powe DG, Lee AH, Robertson JF, Ellis IO: **Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation**. *J Pathol* 2006, **208**(4):495-506.

177. Laakso M, Tanner M, Nilsson J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, Malmstrom P, Wilking N, Bergh J, Isola J: **Basoluminal carcinoma: a new biologically and prognostically distinct entity between basal and luminal breast cancer**. *Clin Cancer Res* 2006, **12**(14 Pt 1):4185-4191.

178. Fulford LG, Reis-Filho JS, Ryder K, Jones C, Gillett CE, Hanby A, Easton D, Lakhani SR: **Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival**. *Breast Cancer Res* 2007, **9**(1):R4.

179. Hicks DG, Short SM, Prescott NL, Tarr SM, Coleman KA, Yoder BJ, Crowe JP, Choueiri TK, Dawson AE, Budd GT *et al*: **Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR**. *Am J Surg Pathol* 2006, **30**(9):1097-1104.

180. Rodriguez-Pinilla SM, Sarrio D, Honrado E, Hardisson D, Calero F, Benitez J, Palacios J: **Prognostic significance of basal-like phenotype and fascin expression in node-negative invasive breast carcinomas**. *Clin Cancer Res* 2006, **12**(5):1533-1539.

181. Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF, Nicholson RI, Ellis IO: **Expression of luminal and basal cytokeratins in human breast carcinoma**. *J Pathol* 2004, **203**(2):661-671.

182. Banerjee S, Reis-Filho JS, Ashley S, Steele D, Ashworth A, Lakhani SR, Smith IE: **Basal-like breast carcinomas: clinical outcome and response to chemotherapy**. *J Clin Pathol* 2006, **59**(7):729-735.

183. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L *et al*: **The molecular portraits of breast tumors are conserved across microarray platforms**. *BMC Genomics* 2006, **7**:96.

184. Sorlie T, Wang Y, Xiao C, Johnsen H, Naume B, Samaha RR, Borresen-Dale AL: **Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms**. *BMC Genomics* 2006, **7**:127.

185. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, Sasaki H: **Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia**. *Blood* 2001, **97**(5):1172-1179.

186. Jin F, Dowdy SC, Xiong Y, Eberhardt NL, Podratz KC, Jiang SW: **Up-regulation of DNA methyltransferase 3B expression in endometrial cancers**. *Gynecol Oncol* 2005, **96**(2):531-538.

187. Veeck J, Esteller M: **Breast cancer epigenetics: from DNA methylation to microRNAs**. *J Mammary Gland Biol Neoplasia*, **15**(1):5-17.

188. Melo SA, Esteller M: **Dysregulation of microRNAs in cancer: Playing with fire**. *FEBS Lett*, **585**(13):2087-2099.

189. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function**. *Cell* 2004, **116**(2):281-297.

190. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.

191. Nana-Sinkam SP, Croce CM: **MicroRNAs as therapeutic targets in cancer**. *Transl Res*, **157**(4):216-225.

192. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF: **Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs**. *Science* 2006, **312**(5770):75-79.

193. Bartel B: **MicroRNAs directing siRNA biogenesis**. *Nat Struct Mol Biol* 2005, **12**(7):569-571.

194. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE: **Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation**. *Cell* 2005, **122**(4):553-563.

195. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W: **Inhibition of translational initiation by Let-7 MicroRNA in human cells**. *Science* 2005, **309**(5740):1573-1576.

196. Orom UA, Nielsen FC, Lund AH: **MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation**. *Mol Cell* 2008, **30**(4):460-471.

197. Gu S, Jin L, Zhang F, Sarnow P, Kay MA: **Biological basis for restriction of microRNA targets to the 3' untranslated region in mammalian mRNAs**. *Nat Struct Mol Biol* 2009, **16**(2):144-150.

198. Rigoutsos I: **New tricks for animal microRNAS: targeting of amino acid coding regions at conserved and nonconserved sites**. *Cancer Res* 2009, **69**(8):3245-3248.

199. Eiring AM, Harb JG, Neviani P, Garton C, Oaks JJ, Spizzo R, Liu S, Schwind S, Santhanam R, Hickey CJ *et al*: **miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts**. *Cell*, **140**(5):652-665.

200. Sevignani C, Calin GA, Siracusa LD, Croce CM: **Mammalian microRNAs: a small world for fine-tuning gene expression**. *Mamm Genome* 2006, **17**(3):189-202.

201. Miska EA: **How microRNAs control cell division, differentiation and death**. *Curr Opin Genet Dev* 2005, **15**(5):563-568.

202. Carleton M, Cleary MA, Linsley PS: **MicroRNAs and cell cycle regulation**. *Cell Cycle* 2007, **6**(17):2127-2132.

203. Ambros V: **The functions of animal microRNAs**. *Nature* 2004, **431**(7006):350-355.

204. Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J: **Endogenous human microRNAs that suppress breast cancer metastasis**. *Nature* 2008, **451**(7175):147-152.

205. Shi M, Liu D, Duan H, Shen B, Guo N: **Metastasis-related miRNAs, active players in breast cancer invasion, and metastasis**. *Cancer Metastasis Rev*, **29**(4):785-799.

206. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E: **Phylogenetic shadowing and computational identification of human microRNA genes**. *Cell* 2005, **120**(1):21-24.

207. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN: **MicroRNA genes are transcribed by RNA polymerase II**. *Embo J* 2004, **23**(20):4051-4060.

208. Borchert GM, Lanier W, Davidson BL: **RNA polymerase III transcribes human microRNAs**. *Nat Struct Mol Biol* 2006, **13**(12):1097-1101.

209. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S *et al*: **The nuclear RNase III Drosha initiates microRNA processing**. *Nature* 2003, **425**(6956):415-419.

210. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ: **Processing of primary microRNAs by the Microprocessor complex**. *Nature* 2004, **432**(7014):231-235.

211. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R: **The Microprocessor complex mediates the genesis of microRNAs**. *Nature* 2004, **432**(7014):235-240.

212. Yi R, Qin Y, Macara IG, Cullen BR: **Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs**. *Genes Dev* 2003, **17**(24):3011-3016.

213. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD: **A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA**. *Science* 2001, **293**(5531):834-838.

214. Corcoran C, Friel AM, Duffy MJ, Crown J, O'Driscoll L: **Intracellular and extracellular microRNAs in breast cancer**. *Clin Chem*, **57**(1):18-32.

215. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K *et al*: **Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia**. *Proc Natl Acad Sci U S A* 2002, **99**(24):15524-15529.

216. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M *et al*: **MicroRNA gene expression deregulation in human breast cancer**. *Cancer Res* 2005, **65**(16):7065-7070.

217. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ *et al*: **A microRNA DNA methylation signature for human cancer metastasis**. *Proc Natl Acad Sci U S A* 2008, **105**(36):13556-13561.

218. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T: **A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation**. *Cancer Res* 2005, **65**(21):9628-9632.

219. Korpal M, Lee ES, Hu G, Kang Y: **The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2**. *J Biol Chem* 2008, **283**(22):14910-14914.

220. Anand S, Majeti BK, Acevedo LM, Murphy EA, Mukthavaram R, Scheppke L, Huang M, Shields DJ, Lindquist JN, Lapinski PE *et al*: **MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis**. *Nat Med*, **16**(8):909-914.

221. Ma L, Teruya-Feldstein J, Weinberg RA: **Tumour invasion and metastasis initiated by microRNA-10b in breast cancer**. *Nature* 2007, **449**(7163):682-688.

222. Rivenbark AG, Livasy CA, Boyd CE, Keppler D, Coleman WB: **Methylation-dependent silencing of CST6 in primary human breast tumors and metastatic lesions**. *Exp Mol Pathol* 2007, **83**(2):188-197.

223. Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C *et al*: **MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B**. *Proc Natl Acad Sci U S A* 2007, **104**(40):15805-15810.

224. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N *et al*: **MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1**. *Blood* 2009, **113**(25):6411-6418.

225. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R: **miR-148 targets human DNMT3b protein coding region**. *Rna* 2008, **14**(5):872-877.

226. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA: **Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells**. *Cancer Cell* 2006, **9**(6):435-443.

227. Sowinska A, Jagodzinski PP: **RNA interference-mediated knockdown of DNMT1 and DNMT3B induces CXCL12 expression in MCF-7 breast cancer and AsPC1 pancreatic carcinoma cell lines**. *Cancer Lett* 2007, **255**(1):153-159.

228. Puppe J, Drost R, Liu X, Joosse SA, Evers B, Cornelissen-Steijger P, Nederlof P, Yu Q, Jonkers J, van Lohuizen M *et al*: **BRCA1-deficient mammary tumor cells are dependent on EZH2 expression and sensitive to Polycomb Repressive Complex 2-inhibitor 3-deazaneplanocin A**. *Breast Cancer Res* 2009, **11**(4):R63.

229. Chomczynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction**. *Anal Biochem* 1987, **162**(1):156-159.

230. Grunau C, Clark SJ, Rosenthal A: **Bisulfite genomic sequencing: systematic investigation of critical experimental parameters**. *Nucleic Acids Res* 2001, **29**(13):E65-65.

231. Lapidus RG, Nass SJ, Butash KA, Parl FF, Weitzman SA, Graff JG, Herman JG, Davidson NE: **Mapping of ER gene CpG island methylation-specific polymerase chain reaction**. *Cancer Res* 1998, **58**(12):2515-2519.

232. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, Ambros VR, Israel MA: **Characterization of microRNA expression levels and their biological correlates in human cancer cell lines**. *Cancer Res* 2007, **67**(6):2456-2468.

233. Troester MA, Hoadley KA, Parker JS, Perou CM: **Prediction of toxicant-specific gene expression signatures after chemotherapeutic treatment of breast cell lines**. *Environ Health Perspect* 2004, **112**(16):1607-1613.

234. Troester MA, Hoadley KA, Sorlie T, Herbert BS, Borresen-Dale AL, Lonning PE, Shay JW, Kaufmann WK, Perou CM: **Cell-type-specific responses to chemotherapeutics in breast cancer**. *Cancer Res* 2004, **64**(12):4218-4226.

235. Pegram MD, Konecny GE, O'Callaghan C, Beryt M, Pietras R, Slamon DJ: **Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer**. *J Natl Cancer Inst* 2004, **96**(10):739-749.

236. Sandhu R, Rivenbark AG, Coleman WB: **Enhancement of chemotherapeutic efficacy in hypermethylator breast cancer cells through targeted and pharmacologic inhibition of DNMT3b**. *Breast Cancer Res Treat*.

237. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M *et al*: **A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia**. *N Engl J Med* 2005, **353**(17):1793-1801.

238. Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov YE: **MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility**. *J Clin Endocrinol Metab* 2008, **93**(5):1600-1608.

239. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, Taja-Chayeb L, Mariscal I, Chavez A, Acuna C, Salazar AM, Lizano M, Duenas-Gonzalez A: **Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy**. *Clin Cancer Res* 2003, **9**(5):1596-1603.

240. Mielnicki LM, Asch HL, Asch BB: **Genes, chromatin, and breast cancer: an epigenetic tale**. *J Mammary Gland Biol Neoplasia* 2001, **6**(2):169-182.

241. Bergamaschi A, Kim YH, Wang P, Sorlie T, Hernandez-Boussard T, Lonning PE, Tibshirani R, Borresen-Dale AL, Pollack JR: **Distinct patterns of DNA copy number alteration are associated with different clinicopathological features and gene-expression subtypes of breast cancer**. *Genes Chromosomes Cancer* 2006, **45**(11):1033-1040.

242. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M *et al*: **Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2**. *N Engl J Med* 2001, **344**(11):783-792.

243. Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J *et al*: **Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer**. *J Clin Oncol* 1996, **14**(3):737-744.

244. Slamon D, Pegram M: **Rationale for trastuzumab (Herceptin) in adjuvant breast cancer trials**. *Semin Oncol* 2001, **28**(1 Suppl 3):13-19.

245. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM: **Humanization of an anti-p185HER2 antibody for human cancer therapy**. *Proc Natl Acad Sci U S A* 1992, **89**(10):4285-4289.

246. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G *et al*: **Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease**. *J Clin Oncol* 1999, **17**(9):2639-2648.

247. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C *et al*: **Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer**. *N Engl J Med* 2005, **353**(16):1659-1672.

248. Demonty G, Bernard-Marty C, Puglisi F, Mancini I, Piccart M: **Progress and new standards of care in the management of HER-2 positive breast cancer**. *Eur J Cancer* 2007, **43**(3):497-509.

249. Moasser MM: **Targeting the function of the HER2 oncogene in human cancer therapeutics**. *Oncogene* 2007, **26**(46):6577-6592.

250. Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, Sukumar S, Argani P: **DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma**. *Int J Cancer* 2003, **107**(6):970-975.

251. Miyamoto K, Fukutomi T, Akashi-Tanaka S, Hasegawa T, Asahara T, Sugimura T, Ushijima T: **Identification of 20 genes aberrantly methylated in human breast cancers**. *Int J Cancer* 2005, **116**(3):407-414.

252. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG: **Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer**. *Hum Mol Genet* 2001, **10**(7):687-692.

253. Yoo CB, Cheng JC, Jones PA: **Zebularine: a new drug for epigenetic therapy**. *Biochem Soc Trans* 2004, **32**(Pt 6):910-912.

254. Segura-Pacheco B, Perez-Cardenas E, Taja-Chayeb L, Chavez-Blanco A, Revilla-Vazquez A, Benitez-Bribiesca L, Duenas-Gonzalez A: **Global DNA hypermethylation-associated cancer chemotherapy resistance and its reversion with the demethylating agent hydralazine**. *J Transl Med* 2006, **4**:32.

255. Brueckner B, Kuck D, Lyko F: **DNA methyltransferase inhibitors for cancer therapy**. *Cancer J* 2007, **13**(1):17-22.

256. Brueckner B, Lyko F: **DNA methyltransferase inhibitors: old and new drugs for an epigenetic cancer therapy**. *Trends Pharmacol Sci* 2004, **25**(11):551-554.

257. Mirza S, Sharma G, Pandya P, Ralhan R: **Demethylating agent 5-aza-2-deoxycytidine enhances susceptibility of breast cancer cells to anticancer agents**. *Mol Cell Biochem*, **342**(1-2):101-109.

258. Lyko F, Brown R: **DNA methyltransferase inhibitors and the development of epigenetic cancer therapies**. *J Natl Cancer Inst* 2005, **97**(20):1498-1506.

259. Leone G, Voso MT, Teofili L, Lubbert M: **Inhibitors of DNA methylation in the treatment of hematological malignancies and MDS**. *Clin Immunol* 2003, **109**(1):89-102.

260. Mund C, Brueckner B, Lyko F: **Reactivation of epigenetically silenced genes by DNA methyltransferase inhibitors: basic concepts and clinical applications**. *Epigenetics* 2006, **1**(1):7-13.

261. Rakha EA, Reis-Filho JS, Ellis IO: **Basal-like breast cancer: a critical review**. *J Clin Oncol* 2008, **26**(15):2568-2581.

262. Schneider BP, Winer EP, Foulkes WD, Garber J, Perou CM, Richardson A, Sledge GW, Carey LA: **Triple-negative breast cancer: risk factors to potential targets**. *Clin Cancer Res* 2008, **14**(24):8010-8018.

263. Momparler RL, Momparler LF, Samson J: **Comparison of the antileukemic activity of 5-AZA-2'-deoxycytidine, 1-beta-D-arabinofuranosylcytosine and 5-azacytidine against L1210 leukemia**. *Leuk Res* 1984, **8**(6):1043-1049.

264. Jones PA, Taylor SM: **Cellular differentiation, cytidine analogs and DNA methylation**. *Cell* 1980, **20**(1):85-93.

265. Perry AS, Watson RW, Lawler M, Hollywood D: **The epigenome as a therapeutic target in prostate cancer**. *Nat Rev Urol*, **7**(12):668-680.

266. Bender CM, Pao MM, Jones PA: **Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines**. *Cancer Res* 1998, **58**(1):95-101.

267. Graham JS, Kaye SB, Brown R: **The promises and pitfalls of epigenetic therapies in solid tumours**. *Eur J Cancer* 2009, **45**(7):1129-1136.

268. Issa JP, Gharibyan V, Cortes J, Jelinek J, Morris G, Verstovsek S, Talpaz M, Garcia-Manero G, Kantarjian HM: **Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate**. *J Clin Oncol* 2005, **23**(17):3948-3956.

269. Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R: **Mutagenicity of 5-aza-2'-deoxycytidine is mediated by the mammalian DNA methyltransferase**. *Proc Natl Acad Sci U S A* 1997, **94**(9):4681-4685.

270. Walker C, Nettesheim P: **In vitro neoplastic progression of rat tracheal epithelial cells transformed by diverse carcinogens**. *Cancer Res* 1989, **49**(16):4427-4430.

271. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, Jones PA, Selker EU: **Inhibition of DNA methylation and reactivation of silenced genes by zebularine**. *J Natl Cancer Inst* 2003, **95**(5):399-409.

272. Holleran JL, Parise RA, Joseph E, Eiseman JL, Covey JM, Glaze ER, Lyubimov AV, Chen YF, D'Argenio DZ, Egorin MJ: **Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine**. *Clin Cancer Res* 2005, **11**(10):3862-3868.

273. Moyers SB, Kumar NB: **Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials**. *Nutr Rev* 2004, **62**(5):204-211.

274. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS: **Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines**. *Cancer Res* 2003, **63**(22):7563-7570.

275. Nakagawa H, Hasumi K, Woo JT, Nagai K, Wachi M: **Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (-)-epigallocatechin gallate**. *Carcinogenesis* 2004, **25**(9):1567-1574.

276. Lin X, Asgari K, Putzi MJ, Gage WR, Yu X, Cornblatt BS, Kumar A, Piantadosi S, DeWeese TL, De Marzo AM *et al*: **Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide**. *Cancer Res* 2001, **61**(24):8611-8616.

277. Villar-Garea A, Fraga MF, Espada J, Esteller M: **Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells**. *Cancer Res* 2003, **63**(16):4984-4989.

278. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, Suhai S, Wiessler M, Lyko F: **Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases**. *Cancer Res* 2005, **65**(14):6305-6311.

279. Plumb JA, Strathdee G, Sludden J, Kaye SB, Brown R: **Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter**. *Cancer Res* 2000, **60**(21):6039-6044.

280. Appleton K, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, Lee C, Barrett S, Reade S, Jadayel D *et al*: **Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors**. *J Clin Oncol* 2007, **25**(29):4603-4609.

281. Diaz LK, Cryns VL, Symmans WF, Sneige N: **Triple negative breast carcinoma and the basal phenotype: from expression profiling to clinical practice**. *Adv Anat Pathol* 2007, **14**(6):419-430.

282. Korsching E, Jeffrey SS, Meinerz W, Decker T, Boecker W, Buerger H: **Basal carcinoma of the breast revisited: an old entity with new interpretations**. *J Clin Pathol* 2008, **61**(5):553-560.

283. Duffy MJ: **Estrogen receptors: role in breast cancer**. *Crit Rev Clin Lab Sci* 2006, **43**(4):325-347.

284. Garzon R, Calin GA, Croce CM: **MicroRNAs in Cancer**. *Annu Rev Med* 2009, **60**:167-179.

285. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T *et al*: **Unique microRNA molecular profiles in lung cancer diagnosis and prognosis**. *Cancer Cell* 2006, **9**(3):189-198.

286. Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiorri F, Fabbri M, Coombes K, Alder H, Nakamura T *et al*: **MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia**. *Blood* 2008, **111**(6):3183-3189.

287. Mott JL, Kobayashi S, Bronk SF, Gores GJ: **mir-29 regulates Mcl-1 protein expression and apoptosis**. *Oncogene* 2007, **26**(42):6133-6140.

288. Visone R, Croce CM: **MiRNAs and cancer**. *Am J Pathol* 2009, **174**(4):1131-1138.

289. Wang Y, Lee CG: **MicroRNA and cancer--focus on apoptosis**. *J Cell Mol Med* 2009, **13**(1):12-23.

290. Sengupta S, den Boon JA, Chen IH, Newton MA, Stanhope SA, Cheng YJ, Chen CJ, Hildesheim A, Sugden B, Ahlquist P: **MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins**. *Proc Natl Acad Sci U S A* 2008, **105**(15):5874-5878.

291. Bueno MJ, Perez de Castro I, Gomez de Cedron M, Santos J, Calin GA, Cigudosa JC, Croce CM, Fernandez-Piqueras J, Malumbres M: **Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression**. *Cancer Cell* 2008, **13**(6):496-506.

292. Sato F, Tsuchiya S, Meltzer SJ, Shimizu K: **MicroRNAs and epigenetics**. *Febs J*, **278**(10):1598-1609.

293. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA *et al*: **MicroRNA expression profiles classify human cancers**. *Nature* 2005, **435**(7043):834-838.

294. Kim do N, Chae HS, Oh ST, Kang JH, Park CH, Park WS, Takada K, Lee JM, Lee WK, Lee SK: **Expression of viral microRNAs in Epstein-Barr virus-associated gastric carcinoma**. *J Virol* 2007, **81**(2):1033-1036.

295. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM: **Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs**. *Nature* 2005, **433**(7027):769-773.

296. Tang F, Hajkova P, Barton SC, Lao K, Surani MA: **MicroRNA expression profiling of single whole embryonic stem cells**. *Nucleic Acids Res* 2006, **34**(2):e9.

297. Xi Y, Nakajima G, Gavin E, Morris CG, Kudo K, Hayashi K, Ju J: **Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples**. *Rna* 2007, **13**(10):1668-1674.

298. Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, Cattan H, Enver T, Mager R, Boultwood J *et al*: **MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma**. *Int J Cancer* 2007, **121**(5):1156-1161.

299. Liu Z, Sall A, Yang D: **MicroRNA: An emerging therapeutic target and intervention tool**. *Int J Mol Sci* 2008, **9**(6):978-999.

300. Heneghan HM, Miller N, Kerin MJ: **MiRNAs as biomarkers and therapeutic targets in cancer**. *Curr Opin Pharmacol*, **10**(5):543-550.

301. Wellings SR, Jensen HM: **On the origin and progression of ductal carcinoma in the human breast**. *J Natl Cancer Inst* 1973, **50**(5):1111-1118.

302. Virnig BA, Tuttle TM, Shamliyan T, Kane RL: **Ductal carcinoma in situ of the breast: a systematic review of incidence, treatment, and outcomes**. *J Natl Cancer Inst*, **102**(3):170-178.

303. Allegra CJ, Aberle DR, Ganschow P, Hahn SM, Lee CN, Millon-Underwood S, Pike MC, Reed SD, Saftlas AF, Scarvalone SA *et al*: **National Institutes of Health State-of-the-Science Conference statement: Diagnosis and Management of Ductal Carcinoma In Situ September 22-24, 2009**. *J Natl Cancer Inst*, **102**(3):161-169.

304. Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC *et al*: **Distinctive gene expression patterns in human mammary epithelial cells and breast cancers**. *Proc Natl Acad Sci U S A* 1999, **96**(16):9212-9217.

305. Sorlie T: **Molecular portraits of breast cancer: tumour subtypes as distinct disease entities**. *Eur J Cancer* 2004, **40**(18):2667-2675.

306. Dabbs DJ, Chivukula M, Carter G, Bhargava R: **Basal phenotype of ductal carcinoma in situ: recognition and immunohistologic profile**. *Mod Pathol* 2006, **19**(11):1506-1511.

307. Bryan BB, Schnitt SJ, Collins LC: **Ductal carcinoma in situ with basal-like phenotype: a possible precursor to invasive basal-like breast cancer**. *Mod Pathol* 2006, **19**(5):617-621.

308. Hannemann J, Velds A, Halfwerk JB, Kreike B, Peterse JL, van de Vijver MJ: **Classification of ductal carcinoma in situ by gene expression profiling**. *Breast Cancer Res* 2006, **8**(5):R61.

309. Livasy CA, Perou CM, Karaca G, Cowan DW, Maia D, Jackson S, Tse CK, Nyante S, Millikan RC: **Identification of a basal-like subtype of breast ductal carcinoma in situ**. *Hum Pathol* 2007, **38**(2):197-204.

310. Allred DC, Wu Y, Mao S, Nagtegaal ID, Lee S, Perou CM, Mohsin SK, O'Connell P, Tsimelzon A, Medina D: **Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution**. *Clin Cancer Res* 2008, **14**(2):370-378.

311. Tamimi RM, Baer HJ, Marotti J, Galan M, Galaburda L, Fu Y, Deitz AC, Connolly JL, Schnitt SJ, Colditz GA *et al*: **Comparison of molecular phenotypes of ductal carcinoma in situ and invasive breast cancer**. *Breast Cancer Res* 2008, **10**(4):R67

.

312. Heaphy CM, Bisoffi M, Fordyce CA, Haaland CM, Hines WC, Joste NE, Griffith JK: **Telomere DNA content and allelic imbalance demonstrate field cancerization in histologically normal tissue adjacent to breast tumors**. *Int J Cancer* 2006, **119**(1):108-116.

313. Berman H, Zhang J, Crawford YG, Gauthier ML, Fordyce CA, McDermott KM, Sigaroudinia M, Kozakiewicz K, Tlsty TD: **Genetic and epigenetic changes in mammary epithelial cells identify a subpopulation of cells involved in early carcinogenesis**. *Cold Spring Harb Symp Quant Biol* 2005, **70**:317-327.

314. Yan PS, Venkataramu C, Ibrahim A, Liu JC, Shen RZ, Diaz NM, Centeno B, Weber F, Leu YW, Shapiro CL *et al*: **Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue**. *Clin Cancer Res* 2006, **12**(22):6626-6636.

315. Zou D, Yoon HS, Perez D, Weeks RJ, Guilford P, Humar B: **Epigenetic silencing in non-neoplastic epithelia identifies E-cadherin (CDH1) as a target for chemoprevention of lobular neoplasia**. *J Pathol* 2009, **218**(2):265-272.

316. Van der Auwera I, Bovie C, Svensson C, Trinh XB, Limame R, van Dam P, van Laere SJ, van Marck EA, Dirix LY, Vermeulen PB: **Quantitative methylation profiling in tumor and matched morphologically normal tissues from breast cancer patients**. *BMC Cancer*, **10**:97.

317. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S: **Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer**. *Oncogene* 2001, **20**(26):3348-3353.