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Research Paper

# DNA Methylation-Dependent Epigenetic Regulation of Gene Expression in MCF-7 Breast Cancer Cells

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## KEY WORDS

epigenetics, epigenomics, DNA methylation, MCF-7 cells, breast cancer, CpG island, 5-aza-2'-deoxycytidine, trichostatin A

## ABBREVIATIONS

| | |
|---|---|
| 5-aza | 5-aza-2'-deoxycytidine |
| C8orf4 | chromosome 8 reading frame 4 |
| CST6 | cystatin E/M |
| IFI27 | interferon α-inducible protein 27 |
| TSA | trichostatin A |

## ACKNOWLEDGEMENTS

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## ABSTRACT

To identify epigenetically-regulated genes in breast cancer, MCF-7 cells were exposed to 250 nM 5-aza or 5-aza + 50 nM TSA for three weeks followed by a five week recovery period after treatment withdrawal and gene expression patterns were examined by microarray analysis. We identified 20 genes that are associated with a ≥ 2-fold increase in expression in response to the demethylating treatment but returned to control levels after treatment withdrawal. RT-PCR verified that the genes identified were expressed at low or undetectable levels in control MCF-7 cells, but increased expression in treated cells. Most of these putative epigentically-regulated genes in MCF-7 cells do not contain CpG islands. In fact, these genes could be classified based upon their promoter CpG features, including genes with: (1) typical CpG features (CpG islands), (2) intermediate CpG features (weak CpG islands), and (3) atypical CpG features (no CpG islands). Prototype genes from each class (including CpG-deficient genes) were shown to be methylation-sensitive (subject to CpG methylation and responsive to demethylating agents), suggesting that not all gene targets of DNA methylation in breast cancer will contain a CpG island. Based upon the results of the current study and observations from the literature, we propose expansion of the current model for methylation-dependent regulation of gene expression to include genes lacking typical CpG islands. The expanded model we propose recognizes that all promoter CpG dinucleotides represent legitimate targets for DNA methylation and that the methylation of specific CpG dinucleotides in critical domains of regulatory regions can result in gene silencing.

## INTRODUCTION

Cancer cells of many different human neoplasms exhibit aberrant DNA methylation patterns, with global hypomethylation and gene-specific hypermethylation.[1] DNA methylation occurs predominately on cytosines within CpG dinucleotides and frequently in regions of CpG density termed CpG islands.[2,3] CpG islands occur in the promoter sequences of many genes,[4] and a strong inverse correlation between promoter methylation status and gene expression levels have been documented.[5,6] Hypermethylation of promoter CpG islands leading to gene silencing represents an important mutation-independent mechanism for inactivation of tumor suppressor genes in cancer cells,[1,7,8] many of which contribute to the hallmarks of cancer.[9] These observations combine to strongly suggest that epigenetic events, and particularly those involving DNA methylation, represent fundamental aspects of cancer, and play key roles in neoplastic transformation and tumor progression.

Numerous investigations have focused on methylation events that occur in CpG islands to characterize epigenetic changes in cancer. However, most of these studies acknowledge that a significant percentage of putative epigenetically-regulated genes lack CpG islands.[10-12] Furthermore, it has been recognized that genes lacking CpG islands are frequently induced in response to demethylating drugs.[13-15] Thus, it is probable that novel CpG targets for methylation are present in putative epigenetically-regulated genes that do not contain CpG islands. There is limited evidence indicating that methylation events in promoters lacking CpG islands can result in downregulation of gene expression.[2] Nonetheless, evidence for the epigenetic silencing of gene expression in response to the methylation of novel CpG targets has emerged.[16] Combined, these findings from the literature suggest that targets for CpG methylation will include typical CpG islands, as well as novel methylation targets.

A variety of different genes have been shown to be inactivated in breast cancer through methylation-dependent gene silencing, including cell cycle control genes, steroid receptor

genes, genes associated with cancer metastasis, and others.[17] However, methylation-sensitive genes in breast cancer have not been comprehensively catalogued or characterized. The goal of this study was to identify genes that are epigenetically regulated in a human breast cancer cell model system, using an experimental approach similar to several studies in the literature.[10-12] In the present investigation, MCF-7 cells were exposed to low levels of 5-aza-2'-deoxycytidine (5-aza) or 5-aza + trichostatin A (TSA) for three weeks, followed by a five week recovery period in the absence of these drugs, and gene expression signatures were generated through microarray analysis. The resulting gene expression data were analyzed to identify putative epigenetically-regulated genes based upon the supposition that these genes will demonstrate significantly increased expression in response to treatment (possibly resulting from a demethylation event), followed by a return to control expression levels after the withdrawal of treatment (possibly reflecting a remethylation event). DNA sequence analysis of the promoter and associated sequences (including exon 1) of these putative epigenetically-regulated genes identified some interesting features that may influence their regulation by DNA methylation. Based upon the CpG features of their promoter and proximal sequences (exon 1), the genes identified in this study can be grouped into three distinct classes: (1) genes with typical CpG features (typical CpG islands within the promoter or exon 1), (2) genes with intermediate CpG features (weak CpG islands within the promoter or exon 1), and (3) genes with atypical CpG features (no CpG islands). Bisulfite sequencing produced direct evidence for the methylation-dependent regulation of transcription of prototype genes representing each of these proposed classes of genes, suggesting that methylation-dependent silencing not only occurs in genes that contain CpG islands, but through methylation of novel CpG targets. Based upon these observations, we propose expansion of the model for DNA methylation-dependent epigenetic regulation of gene expression to include genes lacking typical CpG islands. The expanded model that we propose recognizes that all promoter CpG dinucleotides represent legitimate targets for DNA methylation and that the methylation of specific CpGs in critical domains of regulatory regions, representing either CpG islands or novel methylation targets, can result in silencing of gene expression. To further our understanding of the contributions of epigenetics to breast carcinogenesis, it is essential to identify methylation-sensitive genes and to characterize the CpG targets of methylation contained within the sequence of their promoters. The expanded model for DNA methylation-dependent epigenetic regulation of gene expression that we propose will provide a framework for investigation of epigenetic mechanisms in breast carcinogenesis, as well as molecular mechanisms responsible for aberrant DNA methylation-mediated silencing of genes in breast cancer.

## MATERIALS AND METHODS

**MCF-7 cell culture.** MCF-7 human breast cancer cells (ATCC #HTB-22) were obtained from the Tissue Culture Core Facility of the UNC Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. These cells have been shown to display an aberrant methylation phenotype of intermediate severity.[18] MCF-7 cells were propagated in minimal essential medium (MEM) with Earle's salts, containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 μg/ml insulin (GIBCO/Invitrogen Life Technologies, Carlsbad, CA), and 10% fetal bovine serum (Hyclone, Logan, UT). Three MCF-7 cell treatment groups were established from a single founding MCF-7 cell population: (1) control medium, (2) medium containing 250 nM 5-aza-2'-deoxycytidine (5-aza), and (3) medium containing 250 nM 5-aza and 50 nM trichostatin A (TSA). 5-aza and TSA were obtained from Sigma Chemical Company (St. Louis, MO). Cells were plated at 5,000 cells/cm$^2$ in 150 mm polystyrene dishes (Corning Inc., Corning, NY). MCF-7 cells in the treatment groups were exposed to 5-aza or 5-aza + TSA for three weeks, with weekly subcultivation, followed by a five week recovery period in control growth medium, with weekly subcultivations during the last three weeks. Control MCF-7 cells were subcultivated once per week during the eight week cell culture period. Cell cultures were fed fresh growth medium three times weekly. Control MCF-7 cells were harvested weekly for RNA preparation over the 8 week period, whereas cells treated with 5-aza or 5-aza + TSA were harvested for RNA preparation at three weeks and eight weeks. Cells were counted at the end of each week using a Model Z1 Coulter Cell and Particle Counter (Beckman Coulter Inc., Fullerton, CA). RNA was isolated from cultured cells using a modification of the method of Chomczynski and Sacchi[19] utilizing TRIzol Reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. Isolated total RNA was stored in -20°C as an ethanol precipitate until being used for microarray analysis or RT-PCR. RNA samples intended for microarray analysis were processed at Expression Analysis (Durham, NC). Prior to target production, the quality and quantity of each RNA sample was assessed using an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Palo Alto, CA).

**Affymetrix microarray analysis.** Large-scale gene expression analyses were performed by Expression Analysis (www.expressionanalysis.com), using the Affymetrix Human Genome GeneChip U133A oligonucleotide array (Affymetrix, Santa Clara, CA), which contains 500,000 oligonucleotides corresponding to 22,000 probe sets directed against 18,400 mRNA transcripts and 14,400 well-characterized genes. RNA samples corresponding to control MCF-7 cells (at week three and week eight), MCF-7 cells that were treated with 250 nM 5-aza (week three and week eight), and MCF-7 cells treated with 5-aza + TSA (week three and week eight) were utilized in this analysis. RNA samples from week three were derived from cells harvested after three weeks of exposure to 5-aza or 5-aza + TSA, whereas RNA samples from week eight were derived from cells that were exposed to treatment for three weeks (to 5-aza or 5-aza + TSA) and then allowed to recover in control growth medium for five weeks. Target was prepared and hybridized according to the Affymetrix Technical Manual. Total RNA (10 μg) was converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and a modified oligo(dT)24 primer that contains T7 promoter sequences (GenSet, Evry, France). After first strand synthesis, residual RNA was degraded by the addition of RNaseH and a double-stranded cDNA molecule was generated using DNA Polymerase I and DNA Ligase (Invitrogen Life Technologies). The cDNA was purified and concentrated using a standard phenol:chloroform extraction, followed by ethanol precipitation. Labeled cRNA products were generated from the purified cDNAs by incubation with T7 RNA Polymerase and biotinylated ribonucleotides, using an In Vitro Transcription kit (Enzo Diagnostics, Farmingdale, NY). cRNA products were purified on an RNeasy column (Qiagen Inc., Valencia, CA) and quantified spectrophotometrically. Purified cRNA target (20 μg) was incubated at 94°C for 35 minutes in fragmentation buffer [200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, and 50 mM magnesium acetate], and then diluted into hybridization buffer [100 mM 2-(N-morpholino) ethanesulfonic acid, 20 mM EDTA, and 0.1% Tween 20] containing biotin-labeled OligoB2 and Eukaryotic Hybridization Controls (Affymetrix). The hybridization cocktail was denatured at 99°C for 5 minutes, incubated at 45°C for 5 minutes, and then injected onto a Human Genome U133A GeneChip cartridge. The U133A GeneChip array was incubated at 42°C for at least 16 hours in a rotating oven at 60 rpm. Subsequently, the hybridized GeneChips were washed under nonstringent conditions at 25°C in a buffer consisting of 0.9 M NaCl, 70 mM sodium phosphate (pH 7.4), 6 mM EDTA, and 0.01% Tween 20, and stringent conditions at 50°C in a buffer consisting of 100 mM 2-(N-morpholino) ethanesulfonic acid, 100 mM NaCl, and 0.01% Tween 20. The microarrays were then stained with Streptavidin Phycoerythrin and the fluorescent signal was amplified using a biotinylated antibody solution. Fluorescent images were detected in an

Table 1 **Oligodeoxynucleotide primers for RT-PCR analysis of gene expression**

| Gene Designation | Forward Primer | Reverse Primer | Amplicon Size |
|---|---|---|---|
| *BF* | 5'-GCCAGCAACAAAAGGAAGAG | 5'-GCAAGTATTGGGGTCAGCAT | 242 bp |
| *C8orf4* | 5'-TTTCAAACAGGTTGCACAAAA | 5'-GTTGCATGACATTTGCCAGT | 229 bp |
| *CEACAM5* | 5'-AGATTGCAGTGAGCCCAGAT | 5'-CTGCTTGATCTTGGTGGACA | 200 bp |
| *CEACAM6* | 5'-TGAGCCAGTGGTGCTAAATG | 5'-TGGAACAAGGAAACAGAACCA | 235 bp |
| *CST6* | 5'-AAGACCAGGGTCACTGGAGA | 5'-CGGGGACTTATCACATCTGC | 163 bp |
| *CYP1B1* | 5'-CCCTCATTGTGTTTCTACCG | 5'-GGCTAAGTTCTGGGACATGAA | 222 bp |
| *FLJ10134* | 5'-GGAGAACAGCTGGCTAAGGA | 5'-TTCATAGTGTGGGCATCCAA | 203 bp |
| *G1P2* | 5'-CACCTGAAGCAGCAAGTGAG | 5'-CTTTATTTCCGGCCCTTGAT | 228 bp |
| *G1P3* | 5'-CTCGCTGATGAGCTGGTCT | 5'-TGCTGGCTACTCCTCATCCT | 181 bp |
| *IFI27* | 5'-TCCTCCATAGCAGCCAAGAT | 5'-CCTGGCATGGTTCTCTTCTC | 221 bp |
| *IGFBP5* | 5'-TTCACAGACTCTGGCCTCCT | 5'-TGTGCTATCCATGTGGGCTA | 185 bp |
| *ISGF3G* | 5'-GAGCTCTTCAGAACCGCCTA | 5'-GGCTCTACACCAGGGACAGA | 226 bp |
| *KRTHB1* | 5'-TAGGCACCCCAACTCAAGTC | 5'-AAGTGGGGGATCACACAGAG | 162 bp |
| *LCN2* | 5'-ACGCTGGGCAACATTAAGAG | 5'-CGAAGTCAGCTCCTTGGTTC | 162 bp |
| *LGALS3BP* | 5'-ACCAACAGCTCGAAGAGCAC | 5'-GGTCATTGCAGAGAGGAAGG | 202 bp |
| *SAT* | 5'-ACGGGGTAAGAAGGTTCAGC | 5'-TGTCTGGCGAGTGTGAGTGT | 161 bp |
| *SCNN1A* | 5'-GCCCCCTTTGTTACTTAGGC | 5'-AAAGACACAGGGCAGAGGTG | 153 bp |
| *ZC3HDC1* | 5'-CTTATTGGCACCAGGGACAG | 5'-GTGTCAGAGCAACAGGCAGA | 191 bp |

Agilent GeneArray Scanner (Agilent Technologies Inc.). After probe-level data was extracted from the MicroArray Suite-derived CEL files, the probes were normalized using quantile probe normalization.[20] Signal was computed using the Positional Dependent Nearest Neighbor (PDNN) method,[21] and scaled by Expression Analysis proprietary methods to mitigate bias in fold-change underestimation. Microarray hybridizations were performed in duplicate (for each treatment group and time point) and the final values for (log) signal for all graphs were averages of the duplicates (equivalent to geometric averages of signal). The one exception is the control average, which was an average of the control at two different time points (week three and week eight).

**Semiquantitative RT-PCR.** Total RNA (2 μg) from control MCF-7 cells, or MCF-7 cells treated with 5-aza or 5-aza + TSA was reverse-transcribed into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dT) as the primer, 60 minutes at 42°C, according to standard methodology. Gene-specific oligodeoxynucleotide primers were generated by the UNC Oligodeoxynucleotide Synthesis Core Facility (Chapel Hill, NC) for selected mRNAs based upon their known cDNA sequence (Genbank, www.ncbi.nih.gov). The sequences of gene-specific primers are given in Table 1. Verification of equal template concentration between samples was accomplished using primers that amplify a portion of β-actin mRNA (5'-AGAGATGGCCACGGCTGCTT-3' and 5'-ATTTG-CGGTGGACGATGGAG-3'). PCR reactions were performed in a 50 μl total volume of buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP (EasyStart Micro 50 PCR-mix-in-a-tube, Molecular BioProducts, San Diego, CA), 0.4 μM of each primer, and 2.5 units AmpliTaq enzyme (Perkin Elmer/Cetus, Foster City, CA). Amplifications were carried out in a Perkin Elmer 9700 Thermocycler using a step-cycle program consisting of 25–30 cycles of 94°C for denaturing (one minute), 58°C for annealing (one minute) and 72°C for extension (two minutes).

**Promoter and 5'-upstream sequence analysis.** Genomic sequences corresponding to the promoter and 5'-upstream regions of select genes were identified using the Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway) contained in the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/). GenBank accession numbers were utilized to identify RefSeq records corresponding to each gene, and then the promoter and 5'-upstream sequences were identified using the Genomic Sequence Near Gene tool. For each gene of interest, 3000 bp of sequence 5'-upstream of exon 1 (containing the putative transcriptional promoter and associated elements) were identified. CpG islands were identified within promoter and exon 1 sequences using the CpGPLOT program from the European Bioinformatics Institute website (http://www.ebi.ac.uk/emboss/cpgplot/). Typical CpG islands were defined as ≥200 bp of sequence with ≥50% C + G content and ≥0.6 CpG observed/CpG expected.[22] Weak CpG islands exhibit the same features (with ≥50% G + C content and ≥0.6 CpG observed/CpG expected), but over a shorter sequence segment (>50 bp but <200 bp). Alu repetitive elements were identified using the RepeatMasker Web Server (http://repeatmasker.genome.washington.edu) from the Institute for Systems Biology at the University of Washington (Seattle, WA).

**Bisulfite modification of genomic DNA, cloning and sequencing.** Bisulfite modification of genomic DNA was performed by a procedure adapted from Grunau et al.[23] 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 min; 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 3M NaOH and incubating for 20 min 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 Tris-Cl and desulfonated by addition of 5.5 μl 3 M NaOH and incubation at 37°C for 20 min. 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 min. 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 amplified using primers directed to specific segments within the promoter regions and exon 1 of selected genes (Table 2). PCR amplification was accomplished using a step-cycle program consisting of 40 cycles of 94°C for denaturing (one minute), 55°C for annealing (1.5 minute), and 72°C for extension (two minutes). PCR products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. A portion of the PCR products (1 to 5 μl) was cloned into pGEM-T Easy Vector (Promega, Madison, WI). Five to eight colonies were selected per gene segment and expanded in liquid culture. Plasmid DNA was purified using the Wizard Plus Miniprep DNA purification kit (Promega, Madison, WI), prior to digestion with NcoI and NdeI (New England Biolabs, Beverly, MA) to confirm the presence and size of the cloned insert. Validated clones were sequenced using the universal M13R3 primer with an Applied Biosystems automated sequencer at the UNC Genome Analysis Facility (Chapel Hill, NC). Individual CpG dinucleotides were considered methylated when > 60% of sequenced clones demonstrated conservation of the cytosine. Likewise, individual CpG dinucleotides were considered unmethylated when < 40% of sequenced clones exhibited conservation of the cytosine. The bisulfite conversion efficiency was calculated for each sequenced clone based upon the ratio of converted Cs (non-CpG) to

Table 2 **Oligodeoxynucleotide primers for bisulfite sequencing analysis of selected genes.**

| Gene Designation | Gene Segment | Forward Primer | Reverse Primer | Amplicon Size |
|---|---|---|---|---|
| *CST6* | Segment A (-636 to -206) | 5′-GGTTGGAATGTTGTAGTGGT | 5′-CCCCAACAACAAATACCAA | 413 bp |
| | Segment B (-228 to +10) | 5′-TTGTATTGGTATTTGTTGTTGG | 5′-TTACGACCGCGCAACTTTTA | 238 bp |
| *C8orf4* | Segment A (-1090 to -763) | 5′-GAATTAAAATATAAGGAGAGTTTT | 5′-AACATTACCCAAACATAAAACAA | 328 bp |
| | Segment B (-786 to -278) | 5′-TTGTTTTATGTTTGGGTAATGTT | 5′-CCCCAACAACAAATACCAA | 529 bp |
| | Exon 1 (+23 to +453) | 5′-TTTGGAAAGTTTGGGAGTTG | 5′-ATCTCACAAACTCACTTAATC | 430 bp |
| *IFI27* | Segment A (-1287 to -883) | 5′-GGTGGTGGTAGTATTTTATAA | 5′-AACACAAATCCTAAAATATAC | 404 bp |
| | Segment B (-905 to -463) | 5′-GTATATTTTAGGATTTGGTGTT | 5′-CCTCACAAAATTTACCAAC | 442 bp |
| | Segment C (-399 to -16) | 5′-GTGGGTTTTTAGTTGAGATT | 5′-CCAAACCTTTTAAACAACTC | 385 bp |

total number of Cs (nonCpG) in a given gene segment. Only clones determined to have a conversion efficiency of > 90% were included in the present study.

**Statistical analysis.** Values included in the text represent the mean ± S.E.M. for CpG content (observed CpG dinucleotides/total dinucleotides x 100). The values for the mean and S.E.M. were calculated using the statistical function of KaleidaGraph Version 3.5 (Synergy Software, Essex Junction, VT). Statistical significance was determined using an unpaired t-test (KaleidaGraph).

## RESULTS

**Identification of putative epigenetically-regulated genes in MCF-7 breast cancer cells through microarray analysis of gene expression.** Microarray analysis of gene expression was performed using RNA samples from control MCF-7 cells harvested at the week three and week eight time points of the cell culture period. The gene expression profiles of these control cultures were found to be remarkably consistent when the levels of expression of individual transcripts were compared between the two time points (data not shown). Linear regression analysis of the week three versus week eight control expression data produced a correlation coefficient sufficiently close to 1 ($r^2 = 0.92$). Based upon this result, the expression data for the two control time points were averaged and utilized for normalization of the expression data obtained with RNA samples from treated cells. Treatment of MCF-7 cells with 250 nM 5-aza for three weeks resulted in ≥2-fold increased expression of 79 genes (Fig. 1A). Likewise, treatment of MCF-7 cells with 250 nM 5-aza + 50 nM TSA for three weeks produced ≥2-fold increased expression of 107 genes (Fig. 1C). To reduce the numbers of genes for analysis, and to enrich for genes that are putatively epigenetically regulated, we analyzed the microarray data to identify genes that were modified by treatment (increased expression levels) but then returned to control expression levels following the withdrawal of treatment. This analysis identified 37 genes in 5-aza-treated MCF-7 cells and 70 genes in 5-aza + TSA treated MCF-7 cells that increased ≥ 2-fold at three weeks and returned to control level after eight weeks (Fig. 1B and D). Comparison of these gene lists identified 20 genes in common between the 5-aza and 5-aza + TSA treatment groups (Table 3). Most of these genes (16/20, 80%) have not been shown previously to be subject to methylation-dependent silencing in cancer cells. However, there is evidence for epigenetic regulation of *C8orf4*, *CYP1B1*, *PSG6*, and *SAT*.[10,24,25] Suzuki and colleagues identified *C8orf4* and *PSG6* among genes that are upregulated in human RKO colorectal carcinoma cells in response to 5-aza + TSA treatment.[10] *CYP1B1* has been shown to be methylated in primary breast cancers,[25] and *SAT* is subject to silencing through X-chromosome inactivation.[24] Genes that responded to demethylating treatment with either 5-aza (n = 17) or 5-aza + TSA (n = 50), but not both, are given in Table 4. Most of these putative epigentically-regulated genes were induced with both demethylating treatments, but failed to make the 2-fold control level of expression threshold for one of the treatments. For example, *CYP1A1* was increased 2-fold in response to 5-aza treatment (Table 4), but only 1.9-fold in response to 5-aza + TSA. Likewise, *SYNGR3* was increased 2.3-fold in response to 5-aza + TSA (Table 4), but only 1.9-fold in response to 5-aza.

**Validation of treatment-related changes in gene expression by RT-PCR.** RT-PCR was employed to validate the changes in gene expression identified by microarray analysis that occur in MCF-7 cells with 5-aza or 5-aza + TSA treatment. RT-PCR analysis of RNA samples prepared from control MCF-7 cells at 1, 2, 3, 6, 7 and 8 weeks of cell culture revealed no significant variations in gene expression level for *C8orf4* or *ZC3HDC1* across all time points (data not shown). *C8orf4* was not expressed at any time point and *ZC3HDC1* was expressed at low (but detectable) levels at all time points. β-actin RNA was also expressed evenly across all time points in control MCF-7 cells (data not shown). These results indicate that MCF-7 cells propagated in control growth medium produce consistent patterns of gene expression over time in cell culture. Subsequent analyses focused on the differential expression of putative epigenetically-regulated genes (n = 20) in response to 5-aza and 5-aza + TSA treatment. In total, 15/20 (75%) of these genes were shown by RT-PCR to be increased in response to 5-aza + TSA (including *BF*, *C8orf4*, *CEACAM5*, *CEACAM6*, *CST6*, *CYP1B1*, *FLJ10134*, *G1P2*, *G1P3*, *IFI27*, *ISGF3G*, *KRTHB1*, *LCN2, SCNN1A* and *ZC3HDC1*), and 18/20 (90%) of these genes were shown by RT-PCR to be increased in response to 5-aza treatment alone (those listed above and *IGFBP5*, *LGALS3BP*, and *SAT*). The remaining two genes were not examined (*CRIP1* and *PSG6*). Figure 2 shows representative RT-PCR reactions for seven genes (*C8orf4*, *CEACAM5*, *CEACAM6*, *IFI27*, *ISGF3G*, *SCNN1A*, and *ZC3HDC1*). *C8orf4*, *IFI27*, and *ZC3HDC1* were expressed at low or undetectable levels in control MCF-7 cells, but demonstrated significantly increased expression three weeks following treatment with either 5-aza or 5-aza + TSA (Fig. 2B–D). Likewise, *BF, CST6*, *CYP1B1*, *FLJ10134*, *G1P2*, *G1P3*, *KRTHB1*, *IGFBP5*, *LCN2*, *LGALS3BP* and *SAT* were expressed at very low levels in control MCF-7 cells followed by an increase in expression with 5-aza and/or 5-aza + TSA treatment (data not shown). *CEACAM5*, *CEACAM6*, *ISGF3G* and *SCNN1A* were expressed at moderate levels in control MCF-7 cells, and each of these genes showed significantly increased levels of expression three weeks after treatment (Fig. 2A and E–G).

**Promoter sequence features of putative epigenetically-regulated genes.** An analysis of the promoters and 5'-upstream sequences (3000 bp) for each of the 20 genes identified in MCF-7 cells that responded to both 5-aza and 5-aza + TSA treatment was performed to identify common sequence features that may be associated with methylation-dependent epigenetic regulation, with emphasis on CpG dinucleotide frequency and distribution. This analysis revealed a tremendous variation in promoter CpG content and organization among these putative epigenetically-regulated genes (Table 3). Based upon a comparative analysis of the CpG features of their promoter and proximal sequences (exon 1), we grouped the putative epigenetically-regulated genes identified in this study into three distinct classes, including: (1) genes with typical CpG features (typical CpG islands within the promoter or exon 1), (2) genes with intermediate CpG features (weak CpG islands within the promoter or exon 1), and (3) genes with atypical CpG features (no CpG islands).

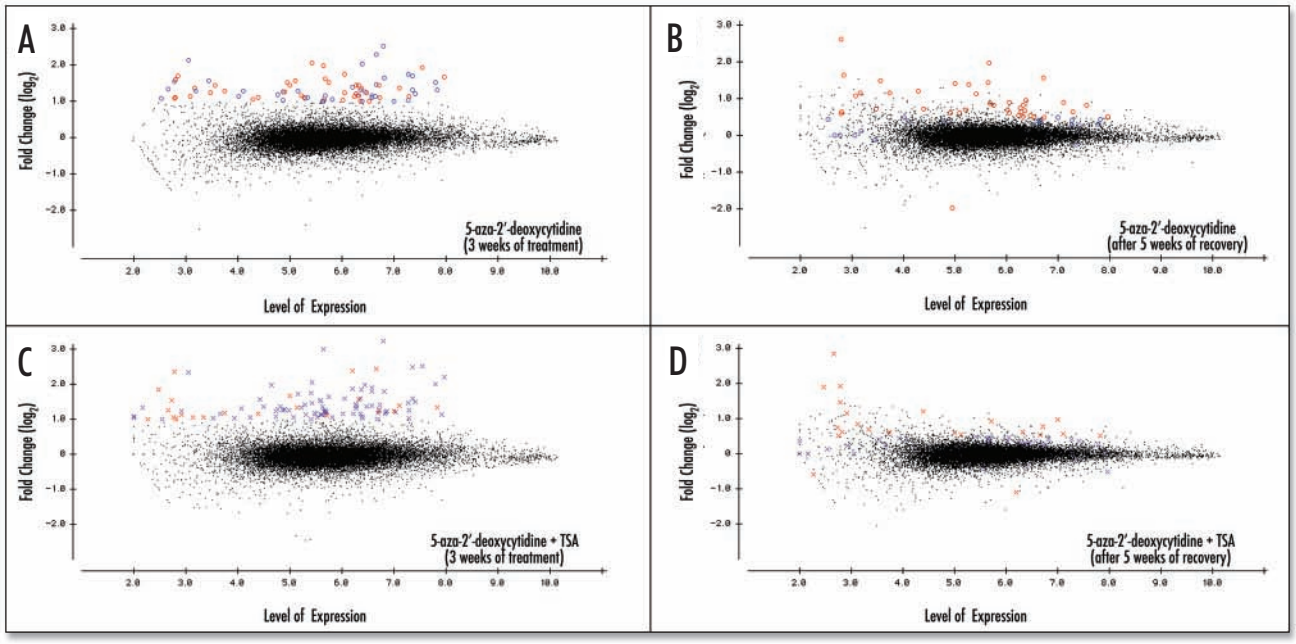


Figure 1. Identification of putative epigenetically-regulated genes in MCF-7 breast cancer cells after exposure to demethylating treatment. M versus A plot of microarray data. The values on the Y-axis reflect log2-scale fold-change (log ratios) for treatment samples relative to control values. The values on the x-axis reflect the average signal intensity for individual control probe sets (transcripts). For (A and B), genes with ≥ 2-fold (log ratio ≥ 1) increased expression in MCF-7 cells after three weeks of 5-aza treatment are shown in red, and genes that returned to control values after a five week recovery period (following withdrawal of 5-aza) are shown in blue (A, week three; B, week eight). This analysis identified 37 genes with increased expression in response to 5-aza treatment in week three that returned to control levels by week eight. For (C and D), genes showing ≥2-fold increased expression in MCF-7 cells after three weeks of 5-aza + TSA treatment are shown in red, and genes that returned to control values after a five week recovery period (following withdrawal of 5-aza + TSA) are shown in blue (C, week three; D, week eight). This analysis identified 70 genes with increased expression in response to 5-aza + TSA treatment in week three that returned to control levels by week eight.

Using the commonly accepted criteria for a typical CpG island,[22] 9/20 (45%) genes were found to contain a CpG island in either their promoter and/or exon 1 (Table 3). This subset of genes exhibits the typical features expected for an epigenetically-regulated gene. Among these genes, 4/9 (44%) contain distinct (typical) CpG islands in both the promoter region and exon 1 (Table 3). The CpG islands found in the promoter and/or exon 1 sequences of *CST6*, *CYP1B1*, *KRTHB1*, *SAT*, and *ZC3HDC1* withstood a more rigorous CpG island analysis (≥200 bp with ≥60% G + C and ≥0.7 CpG observed/CpG expected), which approximates a new standard suggested by Takai and Jones.[3] In 7/9 (78%), genes with typical CpG features, distinct weak CpG islands were detected in the promoter and/or exon 1 (Table 3). As expected, all of the CpG island-containing genes demonstrated significant promoter CpG content, with the highest concentration of CpG dinucleotides in the first 500 bp upstream of the transcription start site in most cases (Table 3). However, CpG islands were detected in several genes with relatively low CpG content (including *G1P3* and *KRTHB1*). In some cases, the CpG content of exon 1 exceeds that of the proximal promoter (like in the case of *ZC3HDC1*), reflecting the presence of a typical CpG island (Table 3). Five genes contain > 10% CpG content in the first 500 bp upstream of the transcription start site, some with much more extensive regions of CpG density (Table 3). Other genes contain more focused regions of CpG density that are confined to the portion of the promoter sequence that is proximal to the transcriptional start site. These include *CST6* (17.6% CpG in proximal 250 bp), *G1P2* (10.4% CpG in proximal 250 bp), *SAT* (18.4% CpG in proximal 250 bp), and *FLJ10134* (20.8% CpG in proximal 250 bp). While 8/9 (89%) genes with typical CpG islands contained Alu repeats (1–6 repeats; average = 3 repeats per promoter), the CpG-rich regions (CpG islands) did not correspond to Alu repetitive elements.

Weak CpG islands were detected in the promoter and/or exon 1 sequences of 8/20 (40%) genes that lacked typical CpG islands (Table 3). We have described this subset of genes as displaying intermediate CpG features based upon the observation that they lack typical CpG islands, but contain smaller regions of CpG density (weak CpG islands). These weak CpG islands occur most often in gene promoter sequences (6/8, 75%), rarely in exon 1 alone (2/8, 25%), or in both the promoter and exon 1 (1/8, 13%) (Table 3). Genes with weak CpG islands display lower promoter and 5'-upstream sequence CpG content than genes containing typical CpG islands (3.0 ± 0.3% versus 5.0 ± 1.0%, N.S.), but this difference was most pronounced when the first 500 bp upstream from the transcription start site was examined (2.5 ± 0.3% versus 9.1 ± 1.8%, p = 0.0066). Alu repeats were detected in 5/8 (63%) genes with intermediate CpG features, but with fewer repeats than genes with typical CpG features (1-4 repeats; average = 2 per promoter).

Three genes (*IFI27*, *LGALS3BP* and *SCNN1A*) contain no CpG islands (typical or weak) in their promoter or exon 1 sequences (Table 3). These genes are CpG-deficient, with no regions of CpG density and no clustering of CpG dinucleotides. Based upon these CpG characteristics, we have described this subset of genes as exhibiting atypical CpG features. Similar to genes with intermediate CpG features, genes with atypical CpG features display significantly lower promoter and 5'-upstream sequence CpG content when compared to genes with typical CpG features. The average CpG content of the atypical features genes was 2.9% when 3000 bp of sequence was examined and 2.7% when the first 500 bp proximal to the transcriptional start site was evaluated (Table 3). Single Alu repeats were detected in the promoters of each of these genes.

A similar analysis of promoter and 5'-upstream CpG sequence features was performed for genes that respond to 5-aza (n = 17) or 5-aza + TSA treatment (n = 50), but not both. *FLJ12055* and *DKFZp761G1812* were omitted from this analysis due to a lack of known promoter sequence. Genes with CpG sequence characteristics corresponding to each of the proposed classes of putative epigenetically-regulated genes were identified in these groups of genes. Among those responding to 5-aza alone (n = 16), 4/16

Table 3 **Putative epigenetically-regulated genes identified in MCF-7 breast cancer cells after exposure to demethylating treatment.**

| | | | | Promoter and Exon 1 Sequence Features | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Genbank | Relative | Typical CpG Island[b] | | Weak CpG Island[c] | | CpG Content | | |
| Gene Designation | Gene Name | Accession Number | Expression Level[a] | Promoter | Exon 1 | Promoter | Exon 1 | Entire Promoter[d] | Proximal Promoter[e] | Promoter +Exon1[f] |
| *Genes with Typical CpG Features* | | | | | | | | | | |
| *CRIP1* | Cysteine-rich protein 1 (intestinal) | NM_001311.1 | 3.1 | Yes | No | Yes | No | 8.1% | 13.2% | 8.4% |
| *CST6* | Cystatin E/M | NM_001323.1 | 2.4 | Yes | Yes | Yes | No | 4.9% | 11.6% | 6.6% |
| *CYP1B1* | Cytochrome P450, family 1, subfamily B, polypeptide 1 | NM_000104.2 | 2.1 | Yes | Yes | Yes[g] | No | 10.9% | 16.8% | 11.2% |
| *FLJ10134* | Hypothetical protein FLJ10134 | NM_018004 | 2.9 | Yes | Yes | Yes | No | 4.1% | 14.8% | 4.6% |
| *G1P2* | Interferon α-inducible protein clone IFI-6-16 | NM_005101.1 | 3.6 | Yes | No | Yes[g] | No | 5.5% | 6.4% | 5.5% |
| *G1P3* | Interferon α-inducible protein clone IFI-15K | NM_022873.1 | 3.2 | Yes | No | No | No | 2.7% | 2.4% | 2.9% |
| *KRTHB1* | Keratin, hair basic type 1 | NM_002281.1 | 3.1 | No | Yes | No | No | 1.4% | 2.0% | 3.5% |
| *SAT* | Spermidine/spermine N1-acetyltransferase | NM_002970.1 | 2.5 | Yes | Yes | No | Yes | 4.9% | 10.4% | 5.5% |
| *ZC3HDC1* | Zinc finger CCCH-type domain containing protein type 1 | NM_022750 | 2.4 | No | Yes | Yes[g] | Yes[g] | 2.8% | 4.4% | 8.2% |
| *Genes with Intermediate CpG Features* | | | | | | | | | | |
| *BF* | B Factor, properdin | NM_001710.1 | 2.8 | No | No | Yes | Yes | 2.7% | 2.0% | 2.7% |
| *C8orf4* | Chromosome 8 reading frame 4 | NM_020130 | 4.8 | No | No | No | Yes | 1.9% | 2.0% | 2.1% |
| *CEACAM5* | Carcinoembryonic antigen-related cell adhesion molecule 5 | NM_004363.1 | 3.2 | No | No | Yes | No | 3.5% | 3.0% | 3.5% |
| *CEACAM6* | Carcinoembryonic antigen-related cell adhesion molecule 6 | NM_002483.3 | 5.2 | No | No | Yes[g] | No | 2.8% | 2.4% | 2.7% |
| *IGFBP5* | Insulin-like growth factor binding protein 5 | NM_000599.2 | 2.1 | No | No | No | Yes[g] | 2.1% | 4.0% | 4.4% |
| *ISGF3G* | Interferon-stimulated transcription factor 3 | NM_006084.1 | 2.8 | No | No | Yes[g] | No | 3.5% | 2.4% | 3.5% |
| *LCN2* | Lipocalin 2 | NM_005564.1 | 4.4 | No | No | Yes | No | 4.1% | 2.8% | 4.0% |
| *PSG6* | Pregnancy specific β-1 glycoprotein 6 | NM_002782 | 2.3 | No | No | Yes[g] | No | 3.3% | 1.2% | 3.2% |
| *Genes with Atypical CpG Features* | | | | | | | | | | |
| *IFI27* | Interferon α-inducible protein 27 | NM_005532.1 | 7.6 | No | No | No | No | 2.3% | 2.0% | 2.3% |
| *LGALS3BP* | Lectin galactoside-binding soluble 3 binding protein | NM_005567.2 | 2.8 | No | No | No | No | 3.5% | 2.4% | 3.7% |
| *SCNN1A* | Sodium channel, nonvoltage-gated type 1α | NM_001038.1 | 2.3 | No | No | No | No | 2.9% | 3.6% | 3.1% |

[a]Relative expression levels are expressed as average fold control levels of expression at the end of 3 weeks of demethylating treatment (5-aza and 5-aza + TSA). [b]Typical CpG islands were defined using the conventionally accepted criteria for these promoter elements, consisting of a region of ≥200 bp with ≥50% C + G, and ≥0.6 CpG observed/CpG expected (Gardiner-Garden and Frommer, 1987, ref. 22). [c]Weak CpG islands were defined as a region of >50 bp but <200 bp with ≥50% C + G and ≥0.6 CpG observed/CpG expected. [d]% CpG dinucleotides in the putative gene promoter, defined as 3000 bp upstream of exon 1 for the purpose of this analysis (observed CpG dinucleotides/total dinucleotides × 100). [e]%CpG dinucleotides in the proximal promoter region, defined as 0 to -500 bp upstream of exon 1 (observed CpG dinucleotides/total dinucleotides × 100). [f]% CpG dinucleotides in the putative gene promoter (3000 bp) and exon 1 (observed CpG dinucleotides/total dinucleotides × 100). [g]These genes contained multiple distinct weak CpG islands.

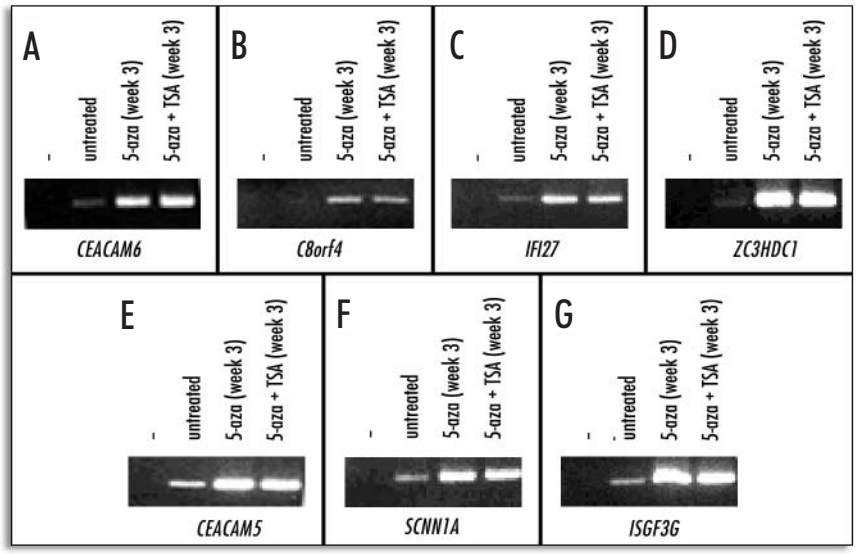


Figure 2. Expression of putative epigenetically-regulated genes in response to demethylating treatment in MCF-7 breast cancer cells. Representative agarose gels of RT-PCR products are shown. In each panel, lane 1 corresponds to a no cDNA template control, lane 2 corresponds to cDNA from untreated (control) MCF-7 cells, and lanes 3 and 4 correspond to MCF-7 cells after three weeks of treatment with 5-aza or 5-aza + TSA, respectively. (A) *CEACAM6*; (B) *C8orf4*; (C) *IFI27*; (D) *ZC3HDC1*; (E) *CEACAM5*; (F) *SCNN1A*; (G) *ISGF3G*.

(25%) genes exhibit typical CpG features, 8/16 (50%) exhibit intermediate CpG features, and 4/16 (25%) display atypical CpG features (Table 4). Likewise, among genes responding to 5-aza + TSA (n = 49), 26/49 (53%) exhibit typical CpG features, 13/49 (27%) exhibit intermediate CpG features, and 10/49 (20%) display atypical CpG features (Table 4).

**Bisulfite sequencing demonstrates that putative epigenetically-regulated genes are subject to methylation-dependent regulation.** Methylation analysis by bisulfite sequencing[23] was performed on the promoter regions of prototype genes from each proposed class of putative epigenetically-regulated gene: *CST6* (typical CpG features), *C8orf4* (intermediate CpG features) and *IFI27* (atypical CpG features). This analysis produced evidence for the regulation of genes in each proposed class by CpG methylation in MCF-7 breast cancer cells, and began to identify CpG methylation events that are critical for gene silencing.

To directly address whether *CST6* is methylated in MCF-7 cells, we analyzed 33 CpG dinucleotides from a segment of the promoter region (+10 to -636) that contains a typical CpG island (Fig. 3). *CST6* is not expressed in untreated MCF-7 cells, but expression is significantly increased with 5-aza and 5-aza + TSA treatment (Fig. 3). Furthermore, the treatment-related increase in gene expression is reversible, and treatment withdrawal results in significant reduction of *CST6* mRNA levels (Fig. 3). In untreated MCF-7 cells, 29/33 (89%) CpG dinucleotides were methylated. Treatment of MCF-7 cells with 5-aza or 5-aza + TSA resulted in demethylation of 30/33 (91%) and 20/33 (61%) CpGs respectively, and *CST6* was expressed at detectable levels (Fig. 3). Treatment of MCF-7 cells with 5-aza resulted in a relatively higher level of expression for *CST6*, which appears to correlate with the degree of promoter demethylation in this region (Fig. 3). Withdrawal of 5-aza or 5-aza + TSA treatment resulted in silencing of *CST6* gene expression concurrent with remethylation of 14/33 (42%) and 28/33 (85%) of CpG dinucleotides respectively (Fig. 3).

The promoter of *C8orf4* is CpG-deficient, but does contain a weak CpG island in exon 1. *C8orf4* is not expressed at detectable levels in MCF-7 cells. We analyzed an 812 bp segment (-278 to -1090) of the *C8orf4* promoter containing 12 CpG dinucleotides (Fig. 4). In untreated MCF-7 cells, 8/12 (67%) of these CpGs were methylated, with the greatest concentration of methylated CpGs in a 103 bp region (-926 to -1029) containing 5 CpG dinucleotides (100% methylated; n = 5) (Fig. 4). Treatment of MCF-7 cells with 5-aza or 5-aza + TSA resulted in demethylation of 10/12 (83%) and 8/12 (67%) CpG dinucleotides respectively, and coordinate expression of *C8orf4* (Fig. 4). Treatment of MCF-7 cells with 5-aza resulted in a relatively higher level of expression for *C8orf4*, which appeared to correlate with the degree of promoter demethylation in this region (Fig. 4). However, treatment withdrawal resulted in silencing or significantly lower expression of *C8orf4* and coordinate remethylation of the majority of these CpGs (Fig. 4). We also examined the methylation status of *C8orf4* exon 1 (between +23 and +453) which contains 14 CpGs, forming a weak CpG island. In control MCF-7 cells, 9/14 (64%) CpG dinucleotides are methylated. Following treatment with 5-aza or 5-aza + TSA, 11/14 (79%) CpGs become demethylated concurrent with reexpression of the gene. Withdrawal of the treatment resulted in silencing of gene expression, but without significant remethylation of this weak CpG island (data not shown). These results suggest that CpG methylation events occurring within the promoter region rather than exon 1 may be most important for the silencing of *C8orf4* in MCF-7 cells.

We examined the methylation status of 15 CpGs within a 1271 bp segment (-16 to -1287) of the *IFI27* promoter, which lacks typical or weak CpG islands. Untreated MCF-7 cells do not express *IFI27* and 14/15 (93%) CpG dinucleotides within the promoter region are methylated (Fig. 5). Treatment of MCF-7 cells with 5-aza or 5-aza + TSA resulted in expression of *IFI27* and demethylation of the majority of CpG dinucleotides: 12/15 (80%) CpGs were demethylated following 5-aza treatment and 9/15 (60%) CpGs were demethylated after 5-aza + TSA treatment (Fig. 5). Withdrawal of 5-aza treatment leads to remethylation of the majority of CpG dinucleotides (13/15, 87%). Likewise, withdrawal of 5-aza + TSA treatment resulted in remethylation of 12/15 (80%) of CpGs and concurrent loss of gene expression (Fig. 5).

## DISCUSSION

**Identification of putative epigenetically-regulated genes in MCF-7 breast cancer cells.** Recent evidence suggests that epigenetic mechanisms play a major role in breast carcinogenesis, contributing to genetic instability in breast cancer, as well as to the silencing of specific genes.[26,27] A number of different genes have been shown to be inactivated in breast cancer through methylation-dependent gene silencing, including cell cycle control genes (*p16$^{INK4a}$*), steroid receptor genes (*ERα, PR, RARβ2*), tumor suppressor genes (*BRCA1*), genes associated with cancer metastasis (*E-cadherin, TIMP-3*), and others.[26,28-31] In an attempt to more comprehensively catalogue methylation-sensitive genes in breast cancer, several recent studies have employed treatment of cells in culture with demethylating drugs and microarray analysis of gene expression.[10-12] We utilized a similar strategy to identify epigenetically-regulated genes in human MCF-7 breast cancer cells treated with a low concentration of 5-aza or 5-aza + TSA for 3 weeks, followed by a 5 week recovery period after treatment withdrawal. The concentration of 5-aza and TSA, utilized in this study was 4-fold to 6-fold lower than traditional methods,[18,32-34] eliminating the typically encountered cytotoxic effects[10,35] and allowing prolonged exposure of MCF-7 cells to the demethylating drugs. Treatment of MCF-7 cells resulted in increased and decreased expression of numerous genes (Fig. 1), many of which may not be directly regulated by DNA methylation. Therefore, to enrich for genes that are putatively epigenetically regulated, we identified subsets of genes that demonstrated a significant increase in expression level in response to demethylating treatment, but then returned to steady-state levels of expression following a recovery

Table 4 **Putative epigenetically-regulated genes identified in MCF7 cells after demethylating treatment with either 5-aza or 5-aza + TSA[a]**

| Gene Designation | Gene Name | GenBank Accession Number | Relative Expression Level[b] |
|---|---|---|---|
| **Genes Responding to 5-aza Treatment (n = 17)[c]** | | | |
| *Genes with Typical CpG Features*[d] | | | |
| *CENTB2* | Centaurin, beta 2 | NM_012287.3 | 2.2 |
| *CYP1A1* | Cytochrome P450, family 1, subfamily A, polypeptide 1 | NM_000499.2 | 2.0 |
| *ID1* | Inhibitor of DNA binding 1 | NM_002165.2 | 2.0 |
| *MAP3K8* | Mitogen-activated protein kinase kinase kinase 8 | NM_005204.2 | 2.4 |
| *Genes with Intermediate CpG Features*[d] | | | |
| *COL4A6* | Collagen type IV alpha 6 | NM_001847 | 2.2 |
| *GDF-15* | Growth differentiation factor 15 | NM_004864.1 | 2.5 |
| *LCP2* | Lymphocyte cytosolic protein 2 | NM_005565.3 | 3.0 |
| *LXN* | Latexin protein | NM_020169.2 | 2.0 |
| *NOX1* | NADPH oxidase 1 | NM_007052.3 | 2.8 |
| *SLICK* | Potassium channel, subfamily T, member 2 | NM_198503.2 | 2.5 |
| *WISP2* | WNT1 inducible signaling pathway protein 2 | NM_003881.2 | 2.2 |
| *ZFHX1B* | Zinc finger homeobox 1b | NM_014795.2 | 2.1 |
| *Genes with Atypical CpG Features*[d] | | | |
| *CTAGE-1* | CTAGE-1 protein | NM_022663.1 | 2.2 |
| *GH2* | Growth hormone 2 | NM_022558 | 2.5 |
| *GNRH1* | Gonadotropin-releasing hormone 1 | NM_000825.2 | 3.0 |
| *RARRES3* | Retinoic acid receptor responder | NM_004585.2 | 2.2 |
| **Genes Responding to 5-aza + TSA Treatment (n = 50)[c]** | | | |
| *Genes with Typical CpG Features*[d] | | | |
| *ABCG2* | ATP-binding cassette, subfamily G, member 2 | NM_004827.2 | 3.6 |
| *AQP3* | Aquaporin 3 | NM_004925.3 | 2.7 |
| *BUB1* | Budding uninhibited by benzimidazoles 1 | NM_004336 | 2.4 |
| *FLJ90013* | Cytomegalovirus partial fusion receptor | NM_153365.1 | 2.1 |
| *GREM1* | Gremlin 1, cysteine knot superfamily | NM_013372.4 | 2.1 |
| *HAPLN1* | Homo sapiens hyaluronan and proteogylcan link protein 1 | NM_001884.2 | 2.2 |
| *HLA-B* | Major histocompatibility complex, class 1, B | NM_000885.3 | 2.2 |
| *HLA-C* | Major histocompatibility complex, class 1, C | NM_002117 | 2.4 |
| *IFITM1* | Interferon-inducible protein 9-27 | NM_003641 | 2.0 |
| *IGFBP3* | Insulin-like growth factor binding protein 3 | NM_000598.4 | 3.1 |
| *INHA* | Inhibin alpha | NM_002191.2 | 2.6 |
| *ITGA4* | Integrin, alpha 4 (antigen CD49D) | NM_000885.4 | 2.1 |
| *KRTHB6* | Keratin, hair, basic, 6 (monilethrix) | NM_002284.2 | 2.5 |
| *L1CAM* | L1 cell adhesion molecule | NM_000425.2 | 2.6 |
| *LOXL2* | Lysyl oxidase-like 2 | NM_002318.1 | 2.5 |
| *PLSCR1* | Phospholipid scramblase 1 | NM_021105.1 | 2.5 |
| *PSMB9* | Proteosome (prosome, macropain), subunit beta, type 9 | NM_002800.3 | 2.3 |
| *RAFTLIN* | Raft-linking protein | NM_015150.1 | 3.1 |
| *RGS16* | Regulator of G-protein signaling 16 | NM_002928.2 | 2.3 |
| *RIG-I* | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide | NM_014314.2 | 2.1 |
| *STAT1* | Signal transducer and activator of transcription 1 | NM_007315.2 | 2.0 |
| *SYNGR3* | Synaptogyrin 3 | NM_004209.4 | 2.3 |
| *TAP1* | Transporter 1, ATP-binding cassette, subfamily B | NM_00593.5 | 2.8 |
| *TUBB* | Tubulin, beta polypeptide | NM_178014.2 | 2.2 |
| *TXNRD1* | Thioredoxin reductase 1 | NM_003330.2 | 2.4 |
| *UBE2L6* | Ubiquitin-conjugating enzyme E2L 6 | NM_004223.3 | 2.3 |
| *Genes with Intermediate CpG Features*[d] | | | |
| *BST2* | Bone marrow stromal cell antigen 2 | NM_004335.2 | 2.7 |
| *C3* | Complement component 3 | NM_000064.1 | 2.3 |
| *CGB* | Chorionic gonadotropin, beta polypeptide | NM_000737.2 | 3.8 |
| *DIO2* | Deiodinase, iodothyronine, type II | NM_013989.2 | 4.2 |
| *FLJ20035* | Hypothetical protein FLJ20035 | NM_017631.3 | 3.0 |
| *GAGE4* | G antigen 4 | NM_001474 | 2.2 |
| *IFIT1* | Interferon-induced protein with tetratricopeptide repeats 1 | NM_001548.1 | 3.4 |
| *LY6D* | Lymphocyte antigen 6 complex, locus D | NM_003695.2 | 2.2 |
| *PDE6C* | Phosphodiesterase 6C | NM_006204.2 | 2.0 |
| *PLAC8* | Placenta-specific 8 | NM_016619 | 2.6 |
| *S100P* | S100 calcium binding protein P | NM_05980.2 | 2.2 |
| *SCGB1A1* | Secretoglobin, family 1A, member 1 (uteroglobin) | NM_003357.3 | 2.6 |

Table 4 **Putative epigenetically-regulated genes identified in MCF7 cells after demethylating treatment with either 5-aza or 5-aza + TSA[a] (continued)**

| | | | |
|---|---|---|---|
| *SP110* | SP110 nuclear body protein | NM_004509.1 | 2.2 |
| *Genes with Atypical CpG Features*[d] | | | |
| *CGA* | Glycoprotein hormone, alpha polypeptide | NM_000735.2 | 8.1 |
| *CYP11A1* | Cytochrome P450, family 11, subfamily A, polypeptide 1 | NM_000781.1 | 4.0 |
| *FLG* | Filaggrin | NM_002016.1 | 2.4 |
| *GJA1* | Gap junction protein, alpha 1 | NM_00165.2 | 2.6 |
| *ITGB6* | Integrin, beta 6 | NM_000888.3 | 2.5 |
| *KYNU* | Kynureninase (L-kynurenine hydrolase) | NM_003937.1 | 2.1 |
| *OAS1* | 2',5'-Oligoadenylate synthetase 1 | NM_016816 | 2.3 |
| *S100A8* | S100 calcium binding protein A8 (calgranulin A) | NM_002964.3 | 2.9 |
| *S100A9* | S100 calcium binding protein A9 (calgranulin B) | NM_002965.2 | 5.8 |
| *S100A12* | S100 calcium binding protein A12 (calgranulin C) | NM_005621.1 | 2.1 |

[a]Genes listed in this table were found to display increased expression in MCF-7 cells in response to treatment with either 5-aza and 5-aza + TSA, but not both. Genes that displayed increased expression in response to both treatments are listed in Table 3. [b]Relative expression levels are expressed as average fold control levels of expression at the end of 3 weeks of demethylating treatment (5-aza or 5-aza + TSA). [c]Genes responding to 5-aza treatment included FLJ12055 (Genbank accession AK022117), and genes responding to 5-aza + TSA included DKFZp761G18121 (Genbank accession BC018100). These genes were omitted from this analysis as no promoter sequence information was available. [d]Genes with typical CpG features contain typical CpG islands (defined as a region of ≥ 200 bp with ≥ 50% C+G and ≥ 0.6 CpG observed/CpG expected; ref. 22). Genes with intermediate CpG features contain weak CpG islands (defined as a region of > 50 bp but < 200 bp with ≥ 50% C + G and ≥ 0.6 CpG observed/CpG expected). Genes with atypical CpG features do not contain CpG islands (typical or weak).

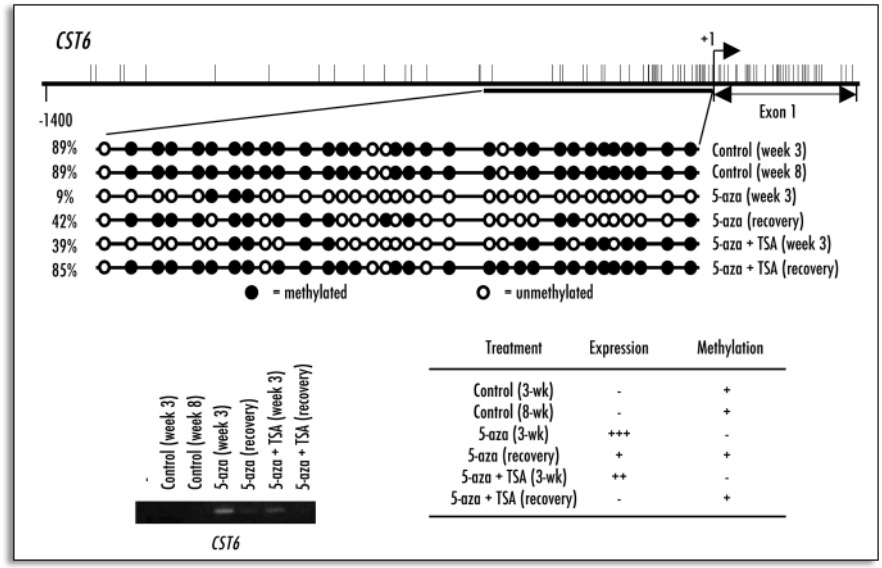


Figure 3. Correlative analysis of promoter methylation and gene expression for *CST6*. The distribution of CpG dinucleotides proximal to the transcription start site in the promoter (0 to -1400 nucleotides) and exon 1 (0 to +294 nucleotides) of *CST6* are depicted schematically (vertical lines indicate the relative position of individual CpG dinucleotides). Methylation analysis was performed on a region of the promoter spanning from +10 to -636 (indicated by a solid horizontal line), which contains 33 CpG dinucleotides and is part of a large CpG island. A summary of results for the methylation analysis is shown for control MCF-7 cells and cells treated with 5-aza and 5-aza + TSA for the week 3 and week 8 time points. Each circle represents 3-5 replicates of bisulfite sequencing. Representative RT-PCR reactions are shown demonstrating the level of *CST6* expression in control and treated MCF-7 cells at each time point. The correlation between *CST6* promoter methylation status and gene expression for all treatments is shown in the inset table.

period. Increased gene expression in response to treatment presumably reflects a demethylation event, resulting in an induction or enhancement of gene expression. Likewise, the return of gene expression to control levels following treatment withdrawal presumably reflects remethylation of the promoter sequence, resulting in gene silencing or downregulation of expression. This analysis identified a group of 20 putative epigenetically-regulated genes for further study, some of which have been suggested to be epigenetically regulated by other investigators.[10,24,25] These observations combine to suggest that our strategy for selection of putative epigenetically-regulated genes was sound.

**Classification of epigenetically-regulated genes based upon promoter CpG features.** It is well known that methylation affecting the promoter and downstream proximal sequences can result in gene silencing, but that methylation elsewhere in a gene will not hinder transcription.[36] Therefore, we expected that many of the putative epigenetically-regulated genes identified in MCF-7 cells would contain CpG islands within their promoter sequences. However, we found that only 45% of putative epigenetically-regulated genes contain typical CpG islands[22] in their promoter and/or exon 1, consistent with other studies reporting that genes lacking CpG islands are frequently induced in response to demethylating drugs.[10-15,37] Some (or all) of these genes lacking CpG islands may respond to demethylating drugs as a result of indirect regulation by DNA methylation. That is, these genes may be regulated directly by the protein products (transcription factors, etc.) of genes containing CpG islands. Alternatively, novel CpG targets of DNA methylation may function to confer methylation-sensitivity to genes lacking CpG islands. In the present study, 40% of putative epigenetically-regulated genes contained weak CpG islands, whereas three genes were identified that lack features expected for epigenetically-regulated genes (typical or weak CpG islands). It is intriguing to suggest that novel CpG target sequences may confer methylation-sensitivity to these genes. Direct evidence for methylation-dependent regulation of genes lacking CpG islands has emerged from a few investigations.[16,38,39] Based upon our observations, we propose that putative epigenetically-regulated genes can be classified based upon their promoter sequence characteristics related to CpG frequency and distribution: (1) genes with typical CpG features (CpG islands within

the promoter or exon 1), (2) genes with intermediate CpG features (weak CpG islands within the promoter or exon 1), and (3) genes with atypical CpG features (no CpG islands).

Putative epigenetically-regulated genes with typical CpG features contain conventionally-defined CpG islands,[22] and in some cases weak CpG islands as well. *CST6* represents the prototype of a gene with typical CpG features, with a large CpG island that spans the promoter and exon 1, encompassing the start site for transcription. *CST6* is a member of a family of proteins that represent physiological inhibitors of lysosomal cysteine proteases that are expressed in normal and premalignant breast epithelium, but not in metastatic breast cancer cell lines.[40] Ectopic expression of *CST6* suppresses the neoplastic phenotype of MDA-MD-435S breast cancer cells, reducing their cell proliferation, migration, and invasion in vitro[41] and delaying tumor growth and reducing metastatic tumor burden in vivo.[42] *CST6* expression is significantly diminished in primary human breast cancers,[42] which is unrelated to gene deletion[40] but may be due to transcriptional silencing through methylation of its CpG island.[43] Our methylation analysis of the *CST6* promoter shows that this gene is subject to DNA methylation in MCF-7 cells, and that there is an inverse correlation between *CST6* expression and methylation of its promoter CpG island (Fig. 3). These results strongly suggest that *CST6*, a putative breast cancer tumor suppressor gene,[42] is sensitive to DNA methylation and that methylation-dependent epigenetic silencing may represent an important mechanism for loss of this gene during breast carcinogenesis and/or tumor progression.

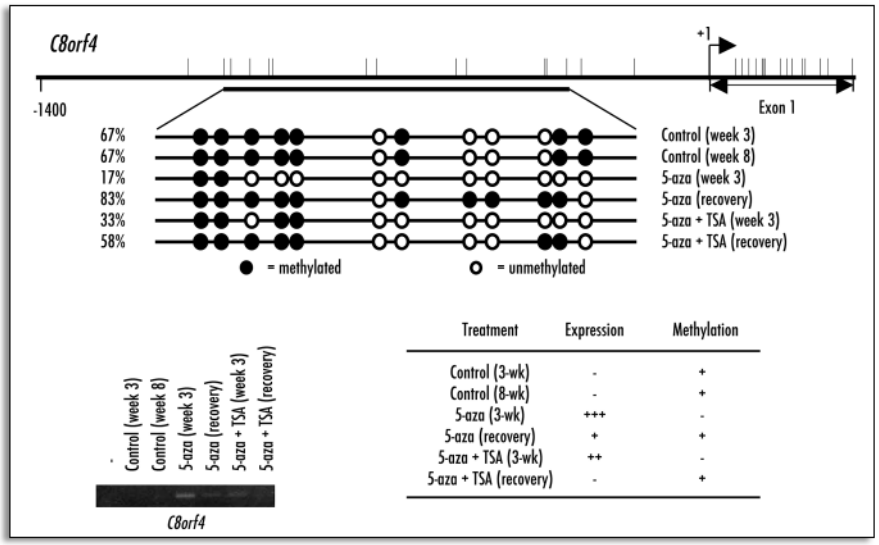


Figure 4. Correlative analysis of promoter methylation and gene expression for *C8orf4*. The distribution of CpG dinucleotides proximal to the transcription start site in the promoter (0 to -1400 nucleotides) and exon 1 (0 to +529 nucleotides) of *C8orf4* are depicted schematically (vertical lines indicate the relative position of individual CpG dinucleotides). *C8orf4* contains no typical CpG islands in the promoter, but does contain a weak CpG island in exon 1 (see results). Methylation analysis was performed on a region of the promoter spanning from -278 to -1090 (indicated by a solid horizontal line), which contains 12 CpG dinucleotides. A summary of results for the methylation analysis is shown for control MCF-7 cells and cells treated with 5-aza and 5-aza + TSA for the week 3 and week 8 time points. Each circle represents 5 replicates of bisulfite sequencing. Methylation analysis of exon 1 was also performed, but is not shown (see results). Representative RT-PCR reactions are shown demonstrating the level of *C8orf4* expression in control and treated MCF-7 cells at each time point. The correlation between *C8orf4* promoter methylation status and gene expression for all treatments is shown in the inset table.

Putative epigenetically-regulated genes with intermediate CpG features contain small regions of CpG density (weak CpG islands), but lack typical CpG islands. *C8orf4* is an example of a gene with intermediate CpG features. While the specific function of *C8orf4* is not known, its loss of expression in primary tumors, metastases, and cancer cell lines,[44] along with its expression/ involvement in the TGFβ-suppressive pathway, suggest that this gene is a growth suppressive gene associated with negative regulation of cell proliferation. Thus, decreased *C8orf4* expression could contribute to the loss of TGFβ responsiveness in breast cancer.[45] The relative paucity of CpG dinucleotides within the promoter/exon 1 of *C8orf4* argues against its direct regulation by DNA methylation. Nonetheless, *C8orf4* is responsive to demethylating drugs in RKO colorectal carcinoma cells,[10] as well as MCF-7 breast cancer cells (this study), suggesting the possibility that this gene contains novel CpG targets for methylation. The two strongest possibilities for novel methylation targets include the weak CpG island contained in exon 1 and individual CpG dinucleotides contained in the gene promoter. The weak CpG island is substantially methylated when *C8orf4* is silent, and becomes demethylated in response to treatment, coordinate with reexpression of the gene. However, treatment withdrawal results in gene silencing with only partial remethylation of these CpG dinucleotides, suggesting that methylation events in the promoter rather than exon 1 may be responsible for regulation of *C8orf4* in MCF-7 breast cancer cells. In fact, the greatest concentration of methylated CpGs in the *C8orf4* promoter was localized to a 103 bp region containing 5 CpG dinucleotides. Treatment of MCF-7 cells resulted in demethylation of this region and coordinate expression of *C8orf4*, while treatment withdrawal resulted in silencing of *C8orf4* expression and remethylation of these CpGs (Fig. 4). These results suggest that *C8orf4* is subject to methylation-dependent epigenetic silencing in MCF-7 breast cancer cells through discrete promoter methylation events, possibly resulting in loss of TGFβ responsiveness.

The third class of genes identified in this study lack all features expected for epigenetically-regulated genes (including CpG islands). *IFI27* is a prototype for genes with atypical CpG features and an example of an interferon α-inducible gene,[46] which have been implicated in primary breast tumors[47] and breast cancer cell lines[48] suggesting their importance in breast carcinogenesis. Untreated MCF-7 cells lack expression of *IFI27* and most (93%) CpG dinucleotides within the promoter region are methylated. Treatment of MCF-7 cells results in demethylation of the majority of CpG dinucleotides and concurrent expression of the gene, while treatment withdrawal leads to remethylation and loss of gene expression (Fig. 5). These results suggest that *IFI27* is subject to epigenetic regulation in MCF-7 breast cancer cells, related to methylation of individual CpG dinucleotides contained in its promoter.

**An expanded model for methylation-dependent epigenetic regulation of gene expression.** DNA methylation has three major effects on gene promoter sequences: (1) direct interference with the binding of transcription factors, (2) attraction of methylated-DNA

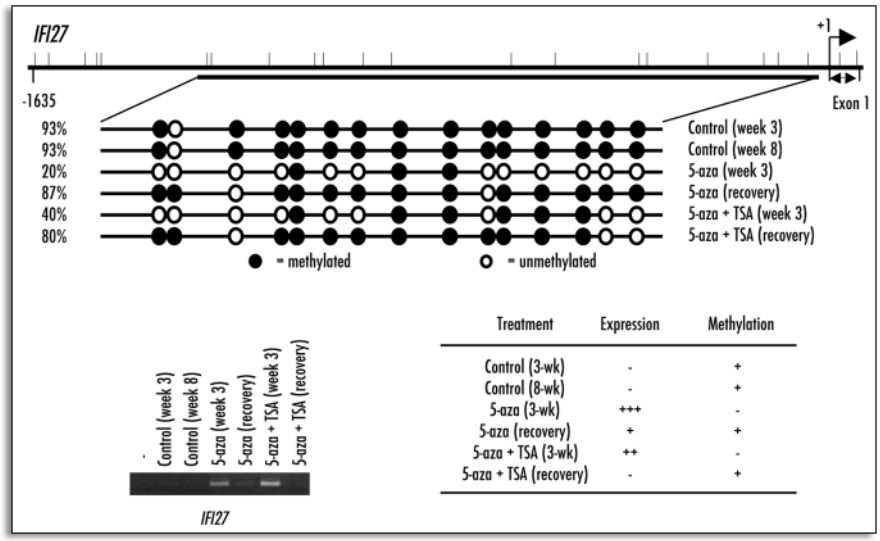


Figure 5. Correlative analysis of promoter methylation and gene expression for *IFI27*. The distribution of CpG dinucleotides proximal to the transcription start site in the promoter (0 to -1635 nucleotides) and exon 1 (0 to +65 nucleotides) of *IFI27* are depicted schematically (vertical lines indicate the relative position of individual CpG dinucleotides). *IFI27* contains no CpG islands (typical or weak) or other regions of CpG density. Methylation analysis was performed on a region of the promoter spanning from -16 to -1287 (indicated by a solid horizontal line), which contains 15 CpG dinucleotides. A summary of results for the methylation analysis is shown for control MCF-7 cells and cells treated with 5-aza and 5-aza + TSA for the week three and week eight time points. Each circle represents 4–5 replicates of bisulfite sequencing. Representative RT-PCR reactions are shown demonstrating the level of *IFI27* expression in control and treated MCF-7 cells at each time point. The correlation between *IFI27* promoter methylation status and gene expression for all treatments is shown in the inset table.

binding proteins and (3) alteration in chromatin packaging.[1,49] Each of these effects results in diminished promoter activity as a consequence of impaired interactions between the transcription machinery and the promoter DNA sequence. To this point in time, it has been thought that a promoter CpG island was necessary to effectively catalyze methylation-dependent gene silencing through one of these mechanisms. However, recent evidence suggests that genes lacking CpG islands can be directly regulated through methylation-dependent mechanisms.[16,38,39] Therefore, based upon our results and studies from the literature, we propose expansion of the current model for DNA methylation-dependent epigenetic regulation of gene expression to include genes lacking typical CpG islands. The expanded model we propose recognizes (a) that all promoter CpG dinucleotides represent legitimate targets for methylation, (b) that sites for methylation may represent regional targets (CpG islands), local CpG density (weak CpG islands), or isolated CpGs, (c) that discrete methylation events occurring within CpG target sequences can contribute to gene silencing, and (d) that CpG methylation can contribute to gene silencing (or diminished expression) through several different mechanisms. This expanded model highlights the importance of the CpG characteristics of individual gene promoters and the targets for methylation that they contain, the nature of specific methylation events, and how these factors combine to regulate gene expression and/or silencing.

Promoter CpG islands represent a recognized target for methylation leading to gene silencing. In most cases, methylation of a CpG island is considered to be a regional methylation event, where methylation of specific CpG dinucleotides is less important than the overall methylation of the CpG-dense region. CpG island methylation can result in gene silencing through several different mechanisms, including recruitment of methylated DNA binding proteins and/or direct interference with transcription factor complex binding to the promoter region. A number of methylated DNA binding proteins have been identified,[50-52] several of which have particular affinity for CpG-rich heterochromatin.[53] The binding of these proteins to methylated target sequences results in inhibition of transcription,[54] possibly through the specific function of transcription repression domains.[55] In addition, several transcription factors have been shown to be sensitive to methylation of their recognition sequence.[56] However, some investigators have suggested that transcriptional silencing requires association of proteins to methylated sequences.[57,58] Nonetheless, there is strong evidence that methylation can directly interfere with the binding of some transcription factors to their recognition site.[59,60] While several mechanisms for inhibition of transcription involving methylation of CpG islands have been established or proposed, DNA methylation-dependent mechanisms of regulation of genes lacking well-defined CpG islands are more elusive. One possibility is that genes lacking CpG islands are not truly epigenetically regulated, but that their expression is governed by indirect methylation-dependent mechanisms, secondary to the epigenetic regulation of CpG island-containing regulatory genes (encoding transcription factors or other regulatory proteins). While in some cases this may be true, in other cases there is direct evidence for methylation-dependent regulation of genes lacking CpG islands.[2] We propose that mechanisms for methylation-dependent gene silencing similar to those suggested for genes with typical CpG features may also apply to genes with intermediate or atypical CpG features. Hence, methylation of weak CpG islands or discrete methylation events affecting specific CpG dinucleotides may (1) recruit methylated DNA binding proteins resulting in a blockade of transcription factor access to crucial recognition sequences, or (2) directly inhibit transcription factor binding to the promoter region. Methylated DNA binding proteins that require very few methylated CpG sites or only a single methylated CpG dinucleotide for binding have been described.[61] Likewise, methylation of specific CpG dinucleotides within or proximal to transcription factor binding sequences can lead to loss of proper transcription factor interaction with its target sequence, negatively impacting on gene transcription.[59,60] There are several examples in the literature of methylation-dependent silencing of genes with intermediate or atypical CpG features. Well-characterized examples of methylation-sensitive genes with intermediate CpG features include *E-cadherin*,[17,62] *RAR-β2* ,[16] and *APC*.[60] Our survey of the literature identified only one example of a well characterized gene with atypical CpG features, *LAMB3*, which is silenced through promoter methylation in cancers of the prostate, breast, lung, and bladder.[63-66] Whereas there are only a few examples of methylation-sensitive genes lacking CpG islands in the current literature, numerous genes with intermediate or atypical CpG features are likely to have been identified in microarray studies aimed at cataloguing cancer-related epigenetically-regulated genes.[10-12] The results from the current study suggest that genes lacking CpG islands from these previous studies should be rigorously evaluated to characterize

their methylation status in breast cancer, and to examine the possible mechanisms through which methylation of weak CpG islands or discrete methylation targets (individual CpG dinucleotides) produce gene silencing.

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