Silencing of the Mammary-derived Growth Inhibitor (MDGI) Gene in Breast Neoplasms Is Associated with Epigenetic Changes[1]

Hung Huynh,[2] Lesley Alpert, and Michael Pollak

Lady Davis Research Institute of the Jewish General Hospital [H. H., L. A., M. P.] and Departments of Medicine [H. H., M. P.], Pathology [L. A.], and Oncology [M. P.], McGill University, Montreal, Quebec H3T 1E2, Canada

Abstract

Recently, we reported that breast cancer cell lines fail to express the gene encoding the fatty acid binding protein mammary derived growth inhibitor (MDGI) and that transfection with an MDGI expression vector results in suppression of the malignant phenotype, suggesting that *MDGI* is a tumor suppressor gene. We also demonstrated that homozygous deletion and point mutation are not common mechanisms for silencing of the *MDGI* gene in human breast neoplasms. We now report that hypermethylation of *Hpa*II and *Hha*I sites upstream of the first exon of the *MDGI* gene, and a *Sac*II site in the first intron, occurs frequently in human breast cancer cell lines. This distinct methylation pattern is associated with loss of transcription and is reversible by treatment with 5-aza-deoxycytidine. Primary breast tumors also exhibited methylation of the *Sac*II site (19 of 35, 54.3%) and the *Hpa*II and *Hha*I sites (21 of 35, 66%). Hypermethylation of these sites was correlated with the absence of MDGI mRNA in these tumors. Our results suggest that epimutation of the *MDGI* gene leads to silencing, which, in turn, may initiate or contribute to progression of breast cancer.

Introduction

Mammary-derived growth inhibitor (also known as heart fatty acid-binding protein) is a member of the fatty acid-binding protein family of lipophilic intracellular proteins, which include retinoic acid-binding proteins (1–5) and related molecules. The human *MDGI*[3] gene has been mapped to chromosome 1p35 (6). This locus was previously identified as a common site of loss of heterozygosity in primary human breast cancer, leading to the conclusion that "chromosome 1p harbors nonidentified tumor suppressor genes" (7, 8). MDGI has certain properties compatible with regulatory or signal transduction functions. These include the presence of an Asn-Phe-Asp-Asp-Tyr consensus site for phosphorylation by tyrosine kinases (9), a differentiation-promoting effect on BLC6 murine pluripotent stem cells (10) and mouse mammary epithelial cells (11), an inhibitory effect on yeast (*Saccharomyces cerevisiae*) proliferation (12), and inhibition of proliferation of Ehrlich mammary ascites cells (13). We recently demonstrated that expression of a cDNA encoding bovine MDGI reverted the transformed phenotype of MCF-7 breast cancer cells, suggesting that the *MDGI* gene has tumor suppressor function (6). However, direct sequencing showed that mutation of the coding region of the *MDGI* gene is not a common mechanism of loss of function of the gene in human breast cancer (14).

Mammalian DNA is heavily methylated at cytosine residues within the CpG dinucleotide (15). The roles of DNA methylation in carcinogenesis have recently been reviewed (16). An overall increase in DNA methyltransferase activity is found in many tumors (17–19) and in the premalignant stages of tumor progression (18, 19). Site-specific hypermethylation, particularly in the 5′ region, has a strong silencing effect on certain genes, whereas hypomethylation activates or enhances gene expression (20–23). In normal tissues and cells, CpG islands are unmethylated, with the exception of transcriptionally silent genes on the inactive X chromosome (24) and some imprinted genes (25–28). Methylation of these islands has been shown to stabilize structural changes in chromatin that prevent transcription (29) or to directly inhibit transcription (30–32). The importance of DNA methylation as a repressor of transcription is emphasized by experiments involving the demethylating agent 5-azacytidine. Treatment of cells with this cytosine analogue often leads to the reactivation of genes that were previously repressed (23, 30–32).

Because the gene encoding MDGI has tumor suppressor activity, understanding the details of the mechanisms and consequences of the loss of function is particularly important. We now report studies of the methylation pattern associated with *MDGI* gene expression in normal human mammary tissues, human breast cancers, and human breast cancer cell lines *in vitro*. Our data suggest that epigenetic mechanisms are responsible for silencing the *MDGI* gene in a significant proportion of human breast cancers and human breast cancer cell lines.

Materials and Methods

Cell Culture. Human breast cancer cell lines MCF-7, T47D, Hs578T, MDA231, BT-20, and HBL-100 were maintained in media containing 10% FCS (Life Technologies, Inc., Grand Island, NY). For studies of reactivation of the *MDGI* gene, cells were cultured in the presence of 0.5 μmol 5-aza-deoxycytidine (Sigma Chemical Co., St. Louis, MO) for 3 days. Fresh media containing the drug was changed daily.

Purification of Primary Human Mammary Epithelial Cells. Mammary epithelial cells and fibroblasts were isolated from surgically excised fresh human breast tissue as described (33). Briefly, mammary glands were minced and digested in DMEM containing 5% FCS, 0.05% collagenase A (Boehringer Mannhein, Montreal, Quebec, Canada), 100 μg/ml garamycin, and 5 μg/ml fungizone at 37°C in a 5% CO_2 incubator for 16 h. Single cells were obtained by pipetting up and down several times. Cells were washed twice in DMEM containing 10% FCS and pelleted by centrifugation. The epithelial cells and fibroblasts were purified by Percoll gradient centrifugation.

Human Breast Cancer Specimens. To determine whether hypermethylation of the human *MDGI* gene was involved in silencing this gene, we examined 35 breast cancer specimens. For some of these cancers, we also had paired normal breast tissue. These specimens were obtained from surgically biopsied breast tissue, which was frozen in liquid nitrogen or cooled isopentane within 30–45 min of excision and stored at −80°C until extraction procedures were performed. DNA specimens were digested with methylation-sensitive enzymes and analyzed by Southern blot. Digested patterns of tumor DNA were compared with the paired normal breast DNA.

DNA Isolation and Southern Blotting. Genomic DNA was prepared from mammary tissue or cells as described (34). For the analysis of the *MDGI* gene

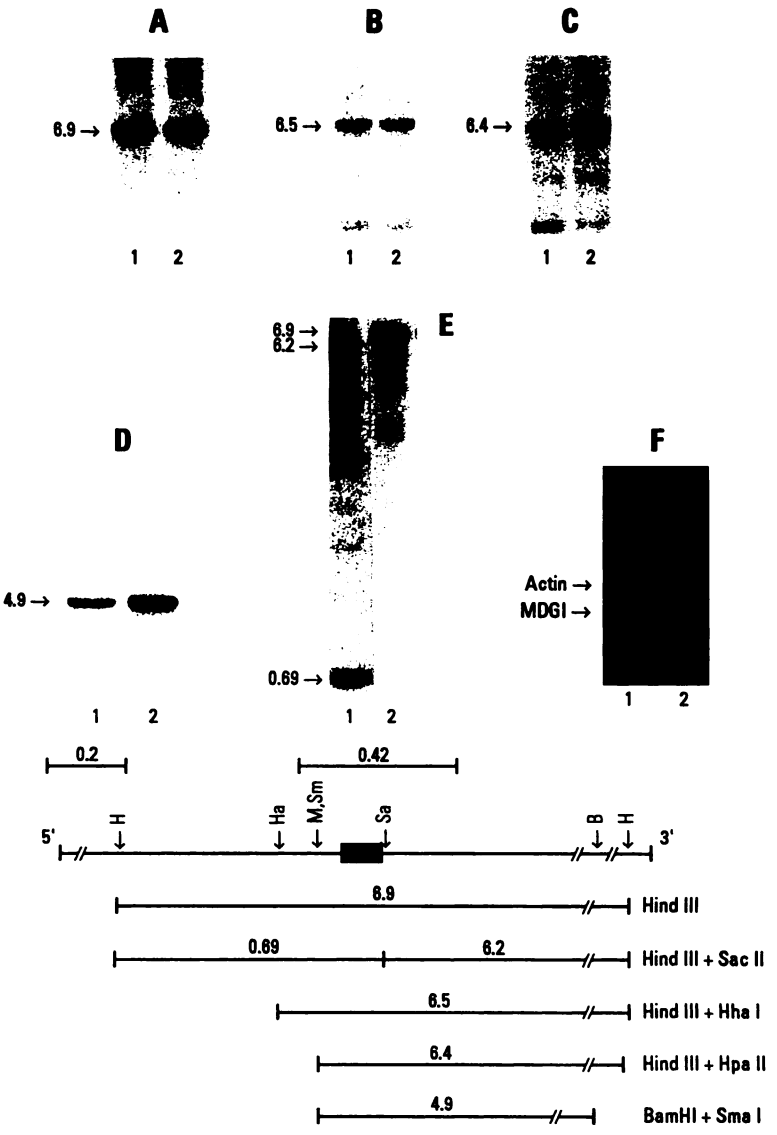

Fig. 1. *MDGI* gene expression and methylation state of *Hha*I, *Hpa*II, and *Sac*II recognition sites of the *MDGI* gene in epithelial and stromal cells from normal human breast. DNA from purified mammary epithelial cells (*Lane 1*) or fibroblasts (*Lane 2*) was cleaved either with *Hin*dIII alone (*A*) or in combination with *Hha*I (*B*), *Hpa*II (*C*), *Sma*I (*D*), and *Sac*II (*E*). *F*, RT studies of MDGI in epithelial and stromal cells from normal human breast tissue. The β-actin RT-PCR product was used as a control for RNA integrity. Molecular sizes in kb were estimated from a λ-*Hin*dIII digest. Restriction sites for *Hin*dIII (*H*), *Msp*I (*M*), *Sma*I (*Sm*), *Bam*HI (*B*), *Hha*I (*Ha*), and *Sac*II (*Sa*) and a 420-bp probe are shown.

methylation status, 10 μg genomic DNA were digested first with 10 units/μg *Hin*dIII, followed by either 10 units/μg *Msp*I, 10 units/μg *Hpa*II, 8 units/μg *Hha*I, 12 units/μg *Sac*II, or 10 units/μg *Sma*I in buffers as recommended by the suppliers (Pharmacia, Montreal, Quebec, Canada) for 16–18 h. To monitor complete cleavage, ΦX174 DNA was added as a standard to each digest, and the digest was examined for complete ΦX174 DNA digestion by gel electrophoresis and ethidium bromide staining. Digested DNA samples were subjected to gel electrophoresis on a 1% agarose gel in Tris-phosphate EDTA buffer (35) at 1.2 V/cm for 20 h. DNA was transferred onto a charged nylon membrane (Zeta-Probe; Bio-Rad) in 0.4 M NaOH according to the supplier's instructions. Prehybridization and hybridization were carried out as described previously (34).

Double-stranded DNA Amplification Using the PCR. Five μg DNase-treated total RNA derived from either cancer cells, normal human breast, or tumors were used for RT reaction using a First-Strand cDNA synthesis kit (Pharmacia), and one tenth of this reaction was used for the PCR. For RT-PCR controls, the reverse transcriptase was omitted from the RT reaction.

The 281-bp exon 4 of the human *MDGI* gene (14) was amplified by PCR with a set of primers (5′-CACTCACCCACGGCACTGC-3′ and 5′-GGCCTTGGCTCTGCTTTATTG-3′). To control for cDNA quality, a set of primers (5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCGGTGGACGAT-3′) was used to amplify 314-bp β-actin. Each reaction consisted of 40 cycles in a PTC-100 programmable thermal controller (M. J. Research, Inc.). The parameters for PCR, temperatures, and times were: denaturation, 94°C for 30 s; annealing, 60°C for 1 min; and elongation, 72°C for 30 s.

To examine the methylation status of the *Hha*I, *Hpa*II, and *Sma*I sites upstream of the first exon and the *Sac*II site in the first intron of the human *MDGI* gene, a set of primers (5′-CCAGCCCCTCCTGGGTGAGCC-3′ and 5′-GCCCCCTCTGAACAGGCCCC-3′) was used to amplify the 420-bp genomic fragment (positions 1100–1520) of the human *MDGI* gene (14). The cycling parameters for amplification of the human genomic DNA were 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min.

The PCR products were purified by low melting point agarose gel electrophoresis. The bands were isolated from the gel and diluted in water. Probes were randomly labeled with ^{32}P using a T-7 Quick-Prime kit (Pharmacia) according to the instructions of the supplier.

Results

***MDGI* Gene Expression and Methylation Analysis of the *MDGI* Gene in Normal Human Breast Epithelial and Stromal Cells.** Southern blots were carried out to examine methylation of the *MDGI* gene. Because stromal and epithelial cell populations of breast tissue clearly differ with respect to *MDGI* expression (6), it was necessary to compare first the methylation pattern of stromal cells to that of normal mammary epithelial cells.

As shown in Fig. 1, the *Hha*I, *Hpa*II, *Sma*I, and *Sac*II restriction sites are located within a 6.9-kb fragment that is flanked by two *Hin*dIII recognition sites. Due to *Alu*I repetitive sequences within the

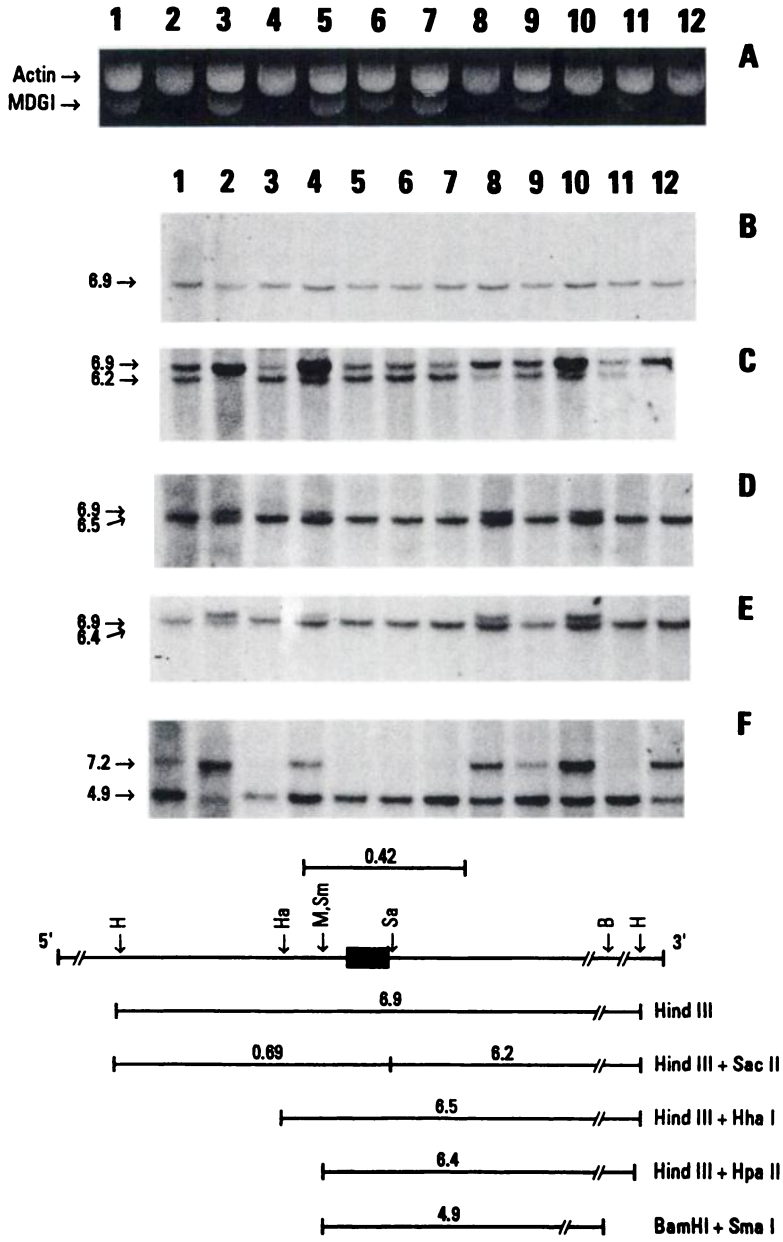

Fig. 2. Expression of the *MDGI* gene in paired normal and neoplastic human breast tissue and methylation state of *Sac*II, *Hha*I, *Hpa*II, and *Sma*I recognition sites as assessed by Southern hybridization with the 420-bp MDGI probe. *Lanes 1, 3, 5, 7, 9,* and *11,* results from normal human breast tissues; *Lanes 2, 4, 6, 8, 10,* and *12,* results from breast cancers. These samples were paired as followed: *1* and *2, 3* and *4, 5* and *6, 7* and *8, 9* and *10,* and *11* and *12. A,* RT studies of MDGI in these normal and neoplastic human breast tissues. The β-actin RT-PCR product was used as a control for RNA integrity. DNA from the same breast samples was subjected to Southern blot analysis. DNA was cleaved with *Hin*dIII (*B*), *Hin*dIII and *Sac*II (copy) or *Hin*dIII and *Hha*I (*D*), *Hin*dIII and *Hpa*II (*E*), or *Bam*HI and *Sma*I (*F*). Molecular sizes in kb were estimated from a λ-*Hin*dIII digest. Restriction sites for *Hin*dIII (*H*), *Bam*HI (*H*), *Msp*I and *Hpa*II (*M*), *Hha*I (*Ha*), *Sma*I (*Sm*), and *Sac*II (*Sa*) and a 420-bp probe are shown.

human *MDGI* gene, DNA fragments spanning the entire human *MDGI* gene were not suitable probes, because these gave a smear with no discrete bands (data not shown). Genomic DNA was digested with the methylation-insensitive enzyme *Hin*dIII and one of the four aforementioned methylation sensitive enzymes and examined by Southern blot analysis using the 420-bp DNA fragment as a probe. DNA that was completely methylated at the restriction sites yielded a 6.9-kb DNA fragment. If the DNA had been partially or fully demethylated, smaller DNA fragments would have been expected.

*Hin*dIII-cleaved DNA from both purified human breast epithelial cells and from fibroblasts gave a single band of 6.9 kb (Fig. 1*A*). Double digestion with *Hin*dIII and three other methylation-sensitive enzymes, *Hha*I (Fig. 1*B*), *Hpa*II (Fig. 1*C*), and *Sma*I (Fig. 1*D*), yielded 6.5-, 6.4-, and 4.9-kb fragments, respectively, suggesting that the *Hha*I, *Hpa*II, and *Sma*I sites were not methylated in breast epithelial cells or in fibroblasts. Bands of 6.2 and 0.69 kb were observed in breast epithelial cell DNA, but a 6.9-kb fragment was seen in fibroblasts following *Hin*dIII and *Sac*II double digestion (Fig. 1*D*), suggesting that the *Sac*II site located in the first intron was hypomethylated only in MDGI-expressing cells. RT-PCR confirmed the presence of MDGI transcripts in breast epithelial cells and not in stromal cells (Fig. 1*F*).

***MDGI* Gene Expression and Methylation in Normal and Neoplastic Human Breast Tissues.** The methylation status of the *Hpa*II, *Hha*I, and *Sac*II sites in 35 primary human breast tumors was examined. We observed that 19 of 35 (54%) human breast tumors studied exhibited *Sac*II site hypermethylation, and only a representative autoradiograph of *Hin*dIII and *Sac*II digestion is shown in Fig. 2*C*. Retention of a faint 6.2-kb band reflecting the unmethylated *Sac*II site was seen in tumor DNA specimens. This likely represents contamination of tumors by nonneoplastic cells. The degree of methylation of the *Sac*II site, as determined by the relative intensities of the 6.2- and 6.9-kb fragments, ranged from 30 to 50% for the normal breast tissue and from 75 to 100% for breast tumors. A partial methylation pattern was observed in these DNA samples when digested with *Hin*dIII and *Hha*I (Fig. 2*D*) and *Hin*dIII and *Hpa*II (Fig. 2*E*). Hypermethylation at the *Hha*I and *Hpa*II sites was seen in 21 of 35 (66%) breast tumors. The faint 7.2-kb band was also observed in two normal breast tissue

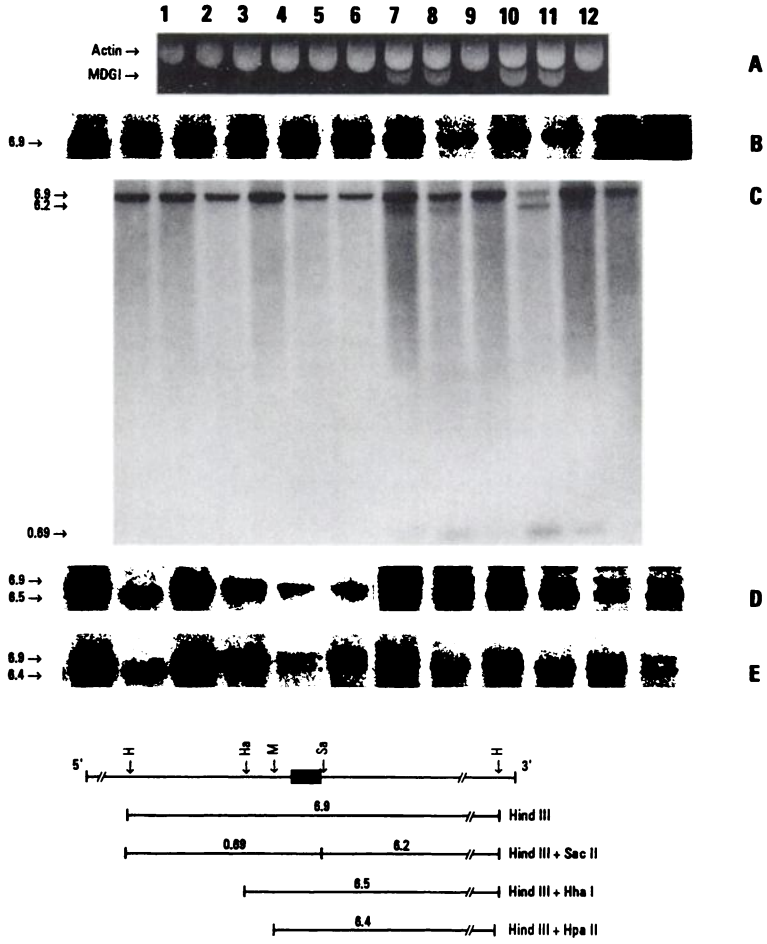

Fig. 3. Effect of 5-aza-deoxycytidine on the methylation state and expression of the *MDGI* gene of human breast cancer cell lines. *A*, RNA derived from MCF-7 (*Lane 1*), Hs578T (*Lane 2*), MDA231 (*Lane 3*), T47D (*Lane 4*), BT-20 (*Lane 5*), and HBL-100 (*Lane 6*) cell lines before (*Lanes 1–6*) and after (*Lanes 7–12*) 5-aza-deoxycytidine treatment were subjected to RT-PCR as described in "Materials and Methods." The MDGI and β-actin products are shown. DNA from the above cell lines was subjected to Southern blot analysis. DNA was cleaved with *Hin*dIII (*B*), *Hin*dIII and *Sac*II (*C*), *Hin*dIII and *Hha*I (*D*), or *Hin*dIII and *Hpa*II (*E*). Restriction sites for *Hin*dIII (*H*), *Msp*I and *Hpa*II (*M*), *Hha*I (*Ha*), and *Sac*II (*Sa*) and a 420-bp probe are shown. Molecular sizes in kb were estimated from a λ-*Hin*dIII digest.

samples following *Bam*HI and *Sma*I digestion (Fig. 2*F*). This observation did not seem to result from the lack of excess restriction endonuclease or from inhibition of enzyme activity, because a similar pattern was obtained when a 10-fold excess enzyme was used and when the internal control ΦX174 DNA was completely cleaved (data not shown). The *Hpa*II and *Hha*I sites from paired normal breast DNA were completely cleaved (Fig. 2, *D* and *E*), whereas the *Sac*II site in these DNA samples was partially cleaved (Fig. 2*C*). Therefore, the 6.9-kb band seen in the *Hin*dIII and *Sac*II digest of DNA from normal breast tissues came from stromal cells, as demonstrated in Fig. 1.

Because RNA for expression analysis was available from six pairs of normal and neoplastic breast tissues, we wished to determine whether hypermethylation of the *Hha*I, *Hpa*II, *Sma*I, and *Sac*II sites was correlated with gene activity. Breast tumors that showed hypermethylation at *Sac*II, *Sma*I, *Hpa*II, and *Hha*I sites did not express the *MDGI* gene, as determined by RT-PCR, whereas normal breast tissue that showed hypomethylation of these sites expressed *MDGI* (Fig. 2*A*). No PCR products were seen when the reverse transcriptase was omitted from the RT reaction (data not shown). In some breast tumor RNA samples, the 281-bp MDGI product was not readily detectable after the first round of PCR. However, when their first-round PCR samples were used as a template for another 40-cycle amplification, the 281-bp MDGI product was observed (data not shown). These results suggest that the faint 6.2-kb band reflecting an unmethylated *Sac*II site in breast tumor DNA samples (Fig. 2*C*) came from non-neoplastic cell contamination. The results indicate that epigenetic changes in the *MDGI* gene may be involved in silencing of this gene.

***MDGI* Gene Expression and Methylation of Human Breast Cancer Cell Lines.** The methylation of the *Hpa*II, *Hha*I, and *Sac*II sites in the *MDGI* gene was examined in six human breast cancer cell lines. DNA from six cell lines was methylated at the *Sac*II site, as indicated by the presence of a 6.9-kb band after *Hin*dIII and *Sac*II digestion (Fig. 3*C*, *Lanes 1–6*). *Hin*dIII- and *Hha*I-cleaved DNA from the cell lines revealed that partial hypermethylation of the *Hha*I site was observed in five of six cell lines (Fig. 3*D*, *Lanes 1–6*). Similar results were obtained at the *Hpa*II (Fig. 3*E*, *Lanes 1–6*) and *Sma*I (data not shown) recognition sites. *Hin*dIII- and *Msp*I-cleaved DNA from all samples resulted in an identical banding pattern (data not shown). As shown in Fig. 3*A*, *Lanes 1–6*, none of the breast cancer cell lines used in this study expressed the *MDGI* gene, as determined by RT-PCR.

5-aza-Deoxycytidine Studies. To further examine the hypothesis that epigenetic changes of the *MDGI* gene are responsible for its inactivation, six breast cancer cell lines were treated with the methylation inhibitor 5-aza-deoxycytidine. This led to partial demethylation of the *Sac*II (Fig. 3*C*, *Lanes 7–12*), *Hha*I (Fig. 3*D*, *Lanes 7–12*), and *Hpa*II (Fig. 3*E*, *Lanes 7–12*) sites. Four cell lines that showed demethylation at the *Sac*II, *Hpa*II, and *Hha*I sites reexpressed the message of MDGI, as assessed by PCR (Fig. 3, *Lanes 7, 8, 10,* and *11*).

Discussion

Our results demonstrate directly that potentially reversible epigenetic mechanisms are responsible for silencing MDGI expression in human breast cancer cell lines. Furthermore, they suggest that *MDGI*

gene silencing occurs in many primary breast tumors, and that this involves aberrant DNA methylation. Although mutation is the classic mechanism for the silencing of tumor suppressor genes in neoplasms (36), there are precedents for silencing of well-characterized suppressors by epimutation (30–32, 37–39).

Although hypomethylation of specific sites in the 5′ region has been shown to be critical for gene expression (20, 21, 40–43), hypomethylation in the intron sequences can be equally important in regulation of gene expression (44). Our data suggest that hypomethylation of the *Sac*II site located in the first intron was also involved in *MDGI* gene expression. This site was methylated in 6 of 6 breast cancer cell lines and in 19 of 35 breast tumors (54%). The methylation pattern of the *Sac*II site in the first intron of human *MDGI* appeared to be cell type and tissue specific. This site was hypomethylated in normal epithelial cells but not in tumor cells.

The importance of hypermethylation as a repressor of MDGI transcription was reinforced by experiments involving the demethylating agent 5-aza-deoxycytidine. Treatment of breast cancer cells with this cytosine analogue led to the demethylation of the *Hha*I and *Hpa*II sites and the *Sac*II site in the first intron. As a result of demethylation of these sites, the MDGI transcripts were detected in four cell lines.

The specific mechanisms by which DNA methylation inhibits gene expression are not known. However, methylation has been shown to interfere with the binding of transcription factors in several systems (40, 41, 45–47). Furthermore, many of the intron-binding proteins have been shown to represent enhancer-binding proteins (48–50). Therefore, it is possible that methylation at the *Sac*II site in the first intron of the *MDGI* gene interferes with the binding of the mammary-specific nuclear factor(s) essential for transcription, thus keeping the *MDGI* gene silent, although both normal glands and tumors were exposed to the same hormonal milieu. It is possible that hypermethylation of the *Hha*I, *Hpa*II, and *Sac*II sites might involve an alteration in the DNA-protein interaction over gene-sized domains, leading to inability to set up the conformational elements characteristic of active chromatin. Several methylation-dependent binding proteins have been described (51–53). The binding of these proteins might direct the methylated *MDGI* gene into an inactive chromatin conformation, thereby inhibiting *MDGI* gene expression. This interpretation is supported by earlier transfection experiments in which the methylated herpes simplex thymidine kinase constructs appeared to inhibit transcription only after chromatin structure was formed in the vector DNA (54, 55).

Our data indicate that epigenetic modification of the *MDGI* gene correlates with its silencing in human breast cancer. This result is consistent with our recent report, which demonstrated that mutation in the coding region of the *MDGI* gene is not a common explanation for loss of function of this suppressor gene in human breast cancer (14). These data, coupled with previous findings that tumors and immortalized cells that do not have mutations in coding region sequences of tumor suppressor genes often contain hypermethylated CpG islands (30, 31, 37–39) suggest that aberrant methylation may prove to be a relatively common mechanism for silencing tumor suppressor genes in human neoplasia.

Acknowledgments

We thank Dr. M. Szyf for critical review of the manuscript, Xiaoying Wan for laboratory assistance, and Peter Wilkinson for manuscript assistance.

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