Reversal of the Hypomethylation Status of Urokinase (uPA) Promoter Blocks Breast Cancer Growth and Metastasis*

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Metastasis is a leading cause of mortality and morbidity in cancer. Urokinase (uPA), only expressed by the highly invasive cancer cells, has been implicated in invasion, metastasis, and angiogenesis of several malignancies including breast cancer. Because uPA expression is strongly correlated with its hypomethylated state, we utilized the uPA gene in the highly invasive MDA-231 human breast cancer cells as a model system to test the hypothesis that pharmacological reversal of the uPA promoter hypomethylation would result in its silencing and inhibition of metastasis. S-Adenosyl-l-methionine (AdoMet) has previously been shown to cause hypermethylation and inhibit demethylation. Treatment of MDA-231 cells with AdoMet, but not its unmethylated analogue S-adenosylhomocysteine, significantly inhibits uPA expression and tumor cell invasion in vitro and tumor growth and metastasis in vivo. The effects of AdoMet on uPA expression were reversed by the demethylating agent 5-azacytidine, supporting the conclusion that AdoMet effects are caused by hypermethylation. Knockdown of the methyl-binding protein 2 also causes a significant inhibition of uPA expression in vitro and tumor growth and metastasis in vivo. These treatments did not have any effects on estrogen receptor expression, suggesting that inhibition of hypomethylation will not affect genes already silenced by hypermethylation. These data are consistent with the hypothesis that hypomethylation of critical genes like uPA plays a causal role in metastasis. Inhibition of hypomethylation can thus be used as a novel therapeutic approach to silence the pro-metastatic gene uPA and block breast cancer progression into the aggressive and metastatic stages of the disease.

Metastasis of tumor cells to different organs is the leading cause of cancer-associated morbidity and mortality in patients with breast cancer (1, 2). One of the key mediators of this process is urokinase (uPA), a member of the serine protease family that catalyzes the conversion of inactive zymogen plasmin to its active form plasmin (1, 3). When activated, plasmin digests most components of the ECM, such as laminin, fibronectin, and collagen (3). Produced by tumor cells and tumor surrounding stroma, uPA is involved in the process of tumor progression and has been implicated in the invasion, metastasis, and angiogenesis of several malignancies including breast cancer (1). Breast cancer is a hormone-dependent malignancy that is often initiated as a less aggressive hormone responsive type and gradually progresses to a highly invasive hormone-insensitive phenotype associated with mutations in estrogen receptor (ER) or interference with ER signaling (4). The molecular mechanism regulating this transition is poorly understood.

Abnormal patterns of DNA methylation are hallmarks of most malignancies, including breast cancer (5). Widespread global DNA hypomethylation accompanied by region-specific hypermethylation and increased levels of expression and activity of DNMT1 are associated with the malignant phenotype (5). It is not clear whether hypomethylation plays a causal role in cancer and which of the processes involved in tumorigenesis are controlled by hypomethylation. We have previously shown that uPA is differentially expressed in the highly invasive hormone-insensitive human breast cancer cell line MDA-231, and that the silencing of uPA expression in the non-invasive hormone responsive MCF-7 cells is because of methylation of the uPA promoter (6). Our data raised the possibility that the enhanced demethylation activity in MDA-231 cells is responsible for activation of genes required for metastasis such as uPA causing the highly invasive and metastatic characteristics of this breast cancer cell line (6). We therefore test the hypothesis that hypomethylation plays a causal role in metastasis by using the expression of the pro-metastatic uPA gene in the highly invasive MDA-231 breast cancer cells as a model system. If hypomethylation is responsible for uPA activation and thus plays a role in metastasis, inhibition of demethylation should result in methylation and inactivation of uPA and reversal of metastasis. If this is true, this study can have novel therapeutic implications for inhibiting invasive breast cancer metastasis.

Pharmacological administration of the methyl donor S-adenosyl-l-methionine (AdoMet), which is a safe and natural compound, has been previously proposed to increase DNA methylation (7–10). AdoMet could act by either stimulating DNA methyltransferase activity or by inhibiting demethylation activity (11). It has recently been shown that AdoMet inhibits active demethylation in human 293 cells and can prevent demethylation of ectopically methylated DNA resulting in hypermethylation (11). We therefore used AdoMet to test our hypothesis that pharmacological inhibition of demethylation can result in inhibition of uPA expression as well as metastasis.

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§The abbreviations used are: uPA, urokinase; ER, estrogen receptor; AdoMet, S-adenosyl-l-methionine; SAH, S-adenosylhomocysteine; 5-azaC, 5-azacytidine; MSP, methyltransferase-specific PCR; MB2D, methylated DNA-binding protein 2; MB2D-AS, human MB2 antisense oligonucleotide; MB2-D-SS, reverse sequence oligonucleotide; RT, reverse transcriptase; GFP, green fluorescent protein; CTL, control; MOPS, 4-morpholinepropanesulfonic acid.
In addition, recent studies suggest that methylated DNA-binding protein 2 (MBD2) is required for tumorgenesis (12, 13) but not required for normal cell growth (12, 13), suggesting that it targets unique processes in tumorgenesis. The high levels of MBD2 expressed in MDA-231 cells, its requirement for tumorgenesis, and its methylated DNA binding properties prompted us to determine whether MBD2 is involved in regulation of uPA, and whether pharmacological inhibition of MBD2 would result in silencing of uPA expression, its increased methylation, and inhibition of metastasis.

In this study, we demonstrate that expression of uPA in the metastatic breast cancer cell line and its hypomethylated state could be reversed pharmacologically by agents that modulate methylation like AdoMet and an antisense inhibitor of MBD2. This study confirms that uPA plays a role in growth, invasion, and metastasis of breast cancer, and that uPA is activated by hypomethylation in tumor cells. Moreover, our data are consistent with the hypothesis that DNA hypomethylation plays a causal role in metastatic cancer and suggests that pharmacological reversal of hypomethylation can be used as a novel therapeutic approach for blocking breast cancer progression into the aggressive and metastatic stages of the disease.

MATERIALS AND METHODS

Cell Lines and Reagents—We obtained all cell lines from the American Type Culture Collection (ATCC, Manassas, VA). The MDA-231 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin-streptomycin sulfate (Invitrogen). The BT549, and Hs578T human breast cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin-streptomycin sulfate (Invitrogen). We treated the MDA-231 cells with AdoMet (New England Bio-labs, Mississauga, Canada) and SAH (Sigma), added to the regular growth media under sterile conditions, for 3 or 6 days. The media was changed every second day during treatments. A cell growth curve analysis was performed on the control MDA-231 cells and cells treated with AdoMet or SAH. All cells, plated at exactly the same density of the viable cells per plate, were trypsinized and the number of viable cells were counted daily throughout the 6-day treatments. Results represent the mean ± S.E. of three independent experiments and significant difference from control as determined by Student’s t test is indicated by an asterisk (*p < 0.05).
cells plated in triplicates, were trypsinized and the number of viable cells determined by 0.4% trypan blue (Sigma) was counted daily for 6 days.

The 5′-azacytidine (5′-azaC) was obtained from Sigma. The MDA-231 cells were first treated with 100 μM AdoMet for 6 days and then with 30 μM 5′-azaC for 3 days. In another set of experiments, the MDA-231 cells were treated with both 100 μM AdoMet and 30 μM 5′-azaC for 6 days. Then we extracted the genomic DNA and cellular RNA using DNAzol and TRIzol, respectively (Invitrogen), following the manufacturer’s instructions.

**Antisense Oligonucleotides**—The MBD2 antisense (MBD2-AS) and the reverse sequence (MBD2-RS) oligonucleotide sequences are 5′-TCAACAGTATTTCCACAGTA-3′ and 5′-ATGGACCCTTTATGACAACT-3′, respectively (14). Synthesized by Integrated DNA Technologies (Corvalle, IA), they both have a PS backbone, 2′-o-methyl modification of the ribose in the first four and last four bases, and are purified by ion exchange high performance liquid chromatography. We transfected these oligos into the MDA-231 cells using 10 μl/ml Lipofectin (Invitrogen) with different concentrations of the antisense oligos for different time points. At the end of the treatment, we isolated total cellular RNA and analyzed the levels of uPA and MBD2 mRNA expression by Northern blot analysis.

**Northern Blot and RT-PCR Analysis**—We electrophoresed 20 μg of cellular RNA on a 1.1% agarose-formaldehyde gel in MOPS buffer and transferred to a nylon membrane (Amersham Biosciences), and then hybridized the blots with 32P-labeled human uPA, MBD2, and 18S cDNA for 14 h at 65 °C. Autoradiography of the blots was carried out at −80 °C using X-AR film (Easton Kodak Co.). We quantified the levels of mRNA expression by densitometric scanning (Gel Doc, Bio-Rad).

We used total RNA (2 μg) isolated from the primary tumors for reverse transcription and amplification. The primers used for RT-PCR were designed so that there is an intron between the amplified regions to recognize any DNA contamination. We used three sets of primers to amplify uPA (5′-ACATTCACCTGGCTGCAACTGC-3′, 5′-CAAGCGCTGTA-CAGCGCTGTA-3′, MBD2 (5′-AGGGATGTCCTACTATCTCAG-3′, 5′-AGATGGTCTGCATCGTCTG-3′), and GAPDH (5′-CCCTCCATTGACCTCAAATCATGATGTCTGCCATCAGT-3′) 5′-GAGGGGCCCATCCATCGTCTG-3′ for each mRNA sample. The PCR were carried out using standard protocols and the DNA was amplified under the following conditions: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and the final extension of 72 °C for 5 min. We then analyzed the PCR products on a 1% agarose gel.

**Boydren Chamber Invasion Assay**—Using two-compartment Boydren chambers (Transwell, Costar, Cambridge, MA) and basement membrane Matrigel (BD Biosciences), we examined the invasive capacities of the cells before and after treatment with AdoMet, MBD2-AS, or MBD2-RS oligos as previously described (6, 15). We coated the 8-μm pore polycarbonate filters with basement membrane Matrigel (50 μg/filter) and analyzed 5 × 10^5 cells in each chamber as described (6). We then fixed the filters for 30 min in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature, we washed them with phosphate-buffered saline, and finally stained them with 1.5% toluidine blue and mounted them onto glass slides. Cells were examined under a light microscope. Under ×400 magnification, we examined 10 randomly selected fields and the average number of cells invaded was calculated.

**uPA Enzyme Activity Assay**—We examined the enzymatic activity of uPA in cell-conditioned medium of the cells alone or after treatment with AdoMet, MBD2-AS, or MBD2-RS using Spectrozyme UK (American Diagnostica, Greenwich, CT), a synthetic chromogenic substrate of uPA (16). We used high molecular weight recombinant uPA (American Diagnostica) to obtain a standard curve and the direct uPA activity assay was carried out as the manufacturer recommended. We used a V_max plate reader ( Molecular Devices, Sunnyvale, CA) to monitor the photometric absorbance of the reaction mixtures at 405 nm after 30 min incubation at room temperature.

**Western Blot Analysis**—To examine changes in MBD2 levels in MDA-
231 cells after treatment with AdoMet, MBD2-AS, and MBD2-RS, total nuclear extract was isolated from the untreated and treated MDA-231 cells as previously described (12). Equal amounts of proteins were analyzed on SDS-polyacrylamide gels and were then transferred to nitrocellulose membranes using standard protocols. Immunoblotting was performed using the sheep polyclonal MBD2 antibody purchased from Upstate Cell Signaling (Lake Placid, NY) and the /H9252-actin antibody was purchased from Sigma. The secondary antibodies were purchased from Bio-Rad, and chemiluminescence was used for protein detection (PerkinElmer Life Sciences).

Methylation Specific PCR (MSP)—We performed the sodium bisulfite treatment of the genomic DNA as previously described (16, 17). We designed two sets of MSP primers to amplify the methylated (5'-AGCGTTGCGGAAGTACGCGG-3', 3'-AAACCCGCCCCGACGCCGCC-5'), or unmethylated (5'-AGTTGTTGGAATGATGAG-3', 3'-AAACCCACCCCAACACCACC-5') promoter sequence. We used these primers to amplify the uPA promoter under the following conditions: 95 °C for 3 min, 10 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s, 20 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s, and final extension of 72 °C for 5 min.

Animal Protocols—For the animal study, we used MDA-231 cells transfected with the cDNA for green fluorescent protein (GFP). These cells exhibit no significant difference in their invasive capacity in the Matrigel invasion assay (15). We obtained 4–6-week-old female BALB/c (nu/nu) mice from Charles River, Inc. (St. Constant, Quebec, Canada). Before inoculation, MDA-231-GFP cells were treated with 100 μM AdoMet for 6 days, and transfected with 200 nM MBD2-AS and MBD2-RS for 72 h. At the end of the treatment, we inoculated 5 × 10⁶ untreated (CTL) and treated MDA-231-GFP cells with 20% Matrigel (BD Biosciences) into the mammary fat pad of these female BALB/c nude mice. We determined the tumor volumes at timed intervals for a 10-week period after inoculation. At the end of the study, we sacrificed the animals by terminal bleeding and removed their lung, liver, spleen, kidney, and the primary tumors for further analysis. We examined 20 μl of the blood of each mouse, smeared on a glass slide, under the fluorescent microscope for the presence of GFP-labeled tumor cells in circulation. We sliced the lung, liver, spleen, and kidney to 1-mm thick slices of fresh tissue for direct examination under the fluorescent microscope for the presence of GFP-expressing tumor foci. We counted the number of GFP-expressing tumor foci per field of examination from 10 random sites of five different slides for each organ and calculated and graphed the average for each group. Using RNAqueous™-4PCR (Ambion), we extracted total RNA from the primary tumors for RT-PCR analysis to examine levels of uPA and MBD2 mRNA expression in these tumors.

Immunohistochemistry—Paraffin-embedded tumor samples were cut into 5-μm thick sections for immunohistochemical analysis. Immunohistochemical staining for uPA was performed using the avidin-biotin-peroxidase complex method. Briefly, the sections were dewaxed in xylene, and rehydrated through a series of ethanol to water gradients. The sections were then incubated in 1% normal goat sera (Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature before

**Fig. 3. Effects of AdoMet (SAM) on uPA expression in the invasive BT549 and HS578T human breast cancer cells.** The cells were treated with the vehicle alone (CTL) and 100 μM AdoMet and SAH for 6 days. A, total cellular RNA was isolated at the end of the treatments and the level of uPA mRNA was determined by Northern blot analysis as described under “Materials and Methods.” The signals obtained were quantified by densitometry of the autoradiograms and normalized to the signal obtained for 18 S. B, the enzymatic activity of uPA in the conditioned medium of the treated or untreated cells was determined using the enzyme-linked immunosorbent-based assay Spectrozyme UK. C, the invasive capacity of the cells following AdoMet treatment was measured using the Boyden chamber Matrigel invasion assay. Results represent the mean ± S.E. of two independent experiments and significant difference from the control as determined by Student’s t test is indicated by an asterisk (p < 0.05).
treatment with the primary antibody (monoclonal antibody against human uPA from American Diagnostica) at 1:25 dilution overnight at 4 °C. Biotinylated goat anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) was used as the secondary antibody at 1:200 for 30 min at room temperature. The slides were treated with Vectastain ABC-AP kit (Vector Laboratories Inc.) with dilution of 1:200 for 30 min at room temperature, and subsequently developed with Fast Red TR/Naphthol AS-MX phosphate (Sigma) containing 1 mM levamisole for 10–15 min. The slides were then counterstained with hematoxylin (Fisher) and mounted with Kaiser’s glycerol jelly. All sections were washed three times for 10 min with Tris buffer (pH 7.6) after each step. For negative control sections, the primary antibody was omitted.

A computer-assisted image analysis system was used to quantify the immunostaining. Images of stained sections were photographed with a Leica digital camera and processed using BioQuant image analysis software, version 6.50.10 (BioQuant Image Analysis Corp., Nashville, TN). The threshold was set by determining the positive staining of control sections and was used to automatically analyze all recorded images of all samples that were stained in the same session under identical conditions. The software calculated the area of immunohistochemical stained regions automatically in each microscopic field. Pixel counts of the immunoreaction product were calculated automatically and were given as total density of the integrated immunostaining over a given area, which reflected the relative amount of uPA detected by the antibodies.

**Statistical Analysis**—Results are expressed as the mean ± S.E. of at least triplicate determinations and statistical comparisons are based on Student’s t test and analysis of variance. A probability value of <0.05 was considered to be significant.

**RESULTS**

**Effect of AdoMet on uPA Expression and Activity in MDA-231 Cells**—We have previously shown that the levels of uPA mRNA expression in breast cancer cell lines correlate with their state of methylation and that 5’-azacytidine, an inhibitor of DNA methylation, induces uPA in the non-metastatic breast cancer cell line MCF-7 (6). Because uPA is unmethylated and highly expressed in the metastatic breast cancer cell line MDA-231 (6), we used these cells as a model system to test the hypothesis that agents that inhibit demethylation would silence the expression of pro-metastatic genes such as uPA. The ubiquitous methyl donor of DNA methyltransferase reaction S-adenosylmethionine or AdoMet was previously shown to reverse hypomethylation in cell lines and tumors in animals (9) and was also recently shown to inhibit the active demethylation of ec-topically methylated genes in HEK 293 cells (11). We therefore treated MDA-231 cells with increasing concentrations of AdoMet from 25 to 100 μM for 3 and 6 days and determined the levels of expression of uPA and MBD2 mRNA. MBD2 was previously shown to be required for tumorigenesis but not normal cell growth in a number of human cancer cell lines and in mice (12, 13). These AdoMet treatments did not have any effect on ER expression (data not shown). This is not surprising because ER is silenced in MDA-231 cells by either mutations or...
DNA methylation, and inhibition of demethylation or hypermethylation should not change the state of methylation of a gene that is already methylated prior to the treatment.

The results shown in Fig. 1A illustrate that while 3 days of treatment with AdoMet had no effect on uPA expression, 6 days treatment with AdoMet resulted in strong inhibition of both uPA and MBD2 mRNA expression in MDA-231 cells. A similar effect was not observed with the inactive analogue of AdoMet, SAH (Fig. 1B). To determine whether AdoMet and SAH treatments have any effects on cell viability and growth, the cell doubling time of the control MDA-231 cells and the cells treated with AdoMet and SAH was examined by a cell growth curve analysis. All cells, plated at a similar density of the viable cells per plate in triplicates, were trypsinized and the number of viable cells was counted daily throughout the 6-day treatment. The results of the analysis showed that AdoMet and SAH did not cause a statistically significant change in the MDA-231 cell doubling time throughout the treatments (Fig. 1C).

This reduction in uPA mRNA expression by AdoMet was accompanied as expected with a reduction in uPA enzymatic activity in treated cells as determined using the Spectrozyme UK assay (Fig. 2A). We then determined whether reduction of uPA expression by AdoMet was also accompanied by reduction in the invasive capacity of the cells using the Boyden chamber Matrigel invasion assay (Fig. 2B). This experiment demonstrates that AdoMet treatment inhibits the invasive capacity of the metastatic MDA-231 cells in parallel to its silencing of uPA. The reduction in uPA expression was confirmed by Western blot analysis as shown in Fig. 2C.

We then tested the hypothesis that AdoMet silencing of uPA involves reversing its hypomethylated state by using MSP assay and measuring the state of methylation of CG sequences in the uPA promoter (Fig. 2D). AdoMet treatments resulted in hypermethylation of the uPA promoter because in AdoMet-treated cells an amplification product is observed only with primers that detect methylated CGs, whereas in control cells an amplification product is observed with primers that detect unmethylated CGs. We then examined whether the effects of AdoMet are mediated by DNA methylation or by other mechanisms through AdoMet. If the silencing of uPA expression by AdoMet is a consequence of increased DNA methylation, the effect should be reversed by the DNA demethylating agent 5-azaC, which is in fact what was observed as shown in Fig. 2E.

In summary, these experiments show that AdoMet treatment reverses the hypomethylated state of the uPA promoter in the MDA-231 cells resulting in silencing of this gene, and that this reversal of hypomethylation is associated with inhibition of the invasive capacity of these cells. These data are also consistent with the hypothesis that the hypomethylation of uPA in metastatic MDA-231 cells is responsible for its activation.

To determine whether AdoMet exerts similar effects on uPA expression in other invasive human breast cancer cells or whether this effect is a peculiarity of the MDA-231 cells, we examined the effects of AdoMet treatments on BT549 and HS578T human breast cancer cells, which are among the ER-negative highly invasive human breast cancer cell lines, and are characterized by high levels of uPA expression and demethylation of uPA promoter (6). We treated these cells with 100 \(\mu M\) AdoMet and SAH for 6 days and determined uPA mRNA levels by Northern blot analysis (Fig. 3A). Treatment of these two cell lines with AdoMet resulted in significant reduction of uPA mRNA expression and its enzymatic activity (Fig. 3B). This reduction of uPA expression and activity was accompanied by significant reduction of the invasive capacity of the cells as determined by Boyden chamber Matrigel invasion assay (Fig. 3C). These experiments further confirmed our hypothesis that reversal of the hypomethylation state by AdoMet results in silencing of uPA gene expression and activity and thus inhibits the invasive capacity of the cancer cells.

**Effects of Antisense Knockdown of MBD2 on uPA Expression and Activity in MDA-231 Cells**—Because MBD2 was previously shown to be required for tumorgenesis (12, 13), and because MBD2 mRNA expression is also inhibited by AdoMet treatment (Fig. 1), we determined whether knockdown of MBD2...
would reverse the hypomethylated state and inhibit the expression of uPA and metastasis of MDA-231 cells similar to AdoMet. We used sequence-specific second generation gapmer 20-mer antisense oligonucleotides directed against human MBD2 mRNA (MBD2-AS) and its reverse control oligonucleotide (MBD2-RS) to block MBD2 expression. To directly examine the role of MBD2 in controlling uPA expression in MDA-231 cells, we treated MDA-231 cells with increasing concentrations of either MBD2-AS or MBD2-RS for 72 h. The results show that MBD2-AS treatment results in a dose-dependent inhibition of MBD2 mRNA in comparison with cells treated with MBD2-RS oligonucleotides. Knock down of MBD2 mRNA also results in a dose-dependent inhibition of uPA mRNA expression (Fig. 4A). Seventy-two hours treatment of MDA-231 cells with 200 nM MBD2-AS oligos results in a 60% knockdown of MBD2 mRNA, which is paralleled with a 70% reduction in uPA expression in comparison with cells treated with the control MBD2-RS oligonucleotide (Fig. 4B). This inhibition of uPA mRNA expression is also accompanied by a parallel reduction in uPA enzymatic activity as determined by the Spectrozyme UK assay (Fig. 5A). We then determined whether MBD2-AS knockdown would also result in inhibition of the invasiveness of MDA-231 cells using the Boyden chamber Matrigel invasion assay. The results shown in Fig. 5B illustrate that MBD2 knockdown results in inhibition of the invasive capacity of the cells, which corresponds to the level of MBD2 mRNA knockdown and uPA silencing similar to the results obtained following AdoMet treatment. A Western blot analysis of the MDA-231 cells transfected with 200 nM MBD2-AS and MBD2-RS oligonucleotides for 72 h confirmed that the MBD2-AS knocks down the MBD2 protein levels (Fig. 5C). MBD2 antisense knockdown results in partial hypermethyl-
vation of the uPA promoter as demonstrated by MSP analysis (Fig. 5D). Whereas control oligonucleotide treatment does not alter the methylation status of the uPA promoter, which is only amplified with primers recognizing the unmethylated promoter, MBD2-AS treatment results in the increased presence of a product amplified with the methylation specific primers as well as products amplified with primers recognizing the unmethylated CGs. The partial reversal of hypomethylation corresponds to the partial silencing of uPA compared with the results obtained with AdoMet, which causes almost complete reversal of the methylation pattern and silencing of uPA (Fig. 2).

In summary, our data are consistent with the hypothesis that MBD2 is required for maintaining uPA hypomethylation and expression as well as for maintaining the invasiveness of the metastatic MDA-231 breast cancer cell line. Inhibition of MBD2 results in methylation and silencing of the pro-metastatic and hypomethylated uPA gene and thus subsequent reduction in invasiveness of metastatic breast cancer cells.

Effect of AdoMet and MBD2-AS Oligonucleotides on MDA-231 Tumor Growth in Vivo—We then tested whether transient treatment with AdoMet and MBD2 knockdown in vitro would inhibit the ability of MDA-231 tumor cells to grow in vivo. We had previously generated MDA-231 cells stably transfected with the CDNA for green fluorescent protein (MDA-231-GFP) and used these stably labeled cells to follow tumor progression in mice (15). These cells exhibit no significant difference in their invasive capacity in Matrigel invasion assay from non-transfected controls (data not shown). We injected the MDA-231-GFP cells treated with phosphate-buffered saline (CTL), AdoMet (100 μM), MBD2-AS (200 nM), or MBD2-RS (200 nM) oligonucleotides into the mammary fat pad of female BALB/c nu/nu mice. Neither treatment had an effect on GFP expression. Tumor volumes were measured at timed intervals for a 10-week period after tumor cell inoculation. Experimental animals injected with cells treated with AdoMet and MBD2-AS oligonucleotides developed tumors of significantly smaller volume compared with the animals inoculated with cells treated with vehicle alone (CTL) and MBD2-RS oligonucleotides (Fig. 6A).

At the end of the study we sacrificed the animals and smeared 20 μl of the blood from each animal on a slide to be examined immediately. The ability of tumor cells to move from the primary site and infiltrate into the circulation is a first and critical step in metastasis. We therefore examined the slides under the fluorescent microscope for the presence of GFP-labeled tumor cells in the circulation. Essentially no GFP cells were detected in the blood of the animals inoculated with cells treated with AdoMet (Fig. 6B). The number of GFP cells detected in the blood of the animals injected with cells treated with MBD2-AS was significantly less compared with the phosphate-buffered saline and MBD2-RS controls (Fig. 6B). We inspected different organs of mice injected with the different treated MDA-231-GFP cells. We noticed following visual examination of the dissected mice that both AdoMet and MBD2-AS had a statistically significant effect on the size of the spleen, probably reflecting the difference in rudimentary immune response toward circulating tumor cells in the circulation (Fig. 6C).

Effect of AdoMet and MBD2 Knockdown on Tumor Metastasis—To evaluate the effect of AdoMet and MBD2-AS treatment of MDA-231-GFP cells on their ability to metastasize in vivo, we examined different organs collected from these mice for the presence of metastasizing tumor cells after sacrificing the an-

![Fig. 7. AdoMet (SAM) and MBD2-AS oligonucleotides inhibit tumor metastasis.](http://www.jbc.org/)

To evaluate the effect of AdoMet and MBD2-AS on metastasis, we sacrificed the mice at week 10 and collected different organs for examining development of tumor metastasis. We collected 1-mm thick slices of lung, liver, spleen, and kidney on a glass slide and examined them directly under the fluorescent microscope for the presence of GFP-expressing tumor foci. We counted the number of tumor foci in 10 random fields per slide, five slides per organ, to determine an average number of tumor foci in these organs. Data shown are representative of seven different animals per group. The graphs represent the mean ± S.E. of seven animals per group and significant difference from the control is determined by Student’s t test and is indicated by an asterisk (*p < 0.05).
imals at week 10. Using fluorescent microscopy, we examined very thin slices of fresh tissue from lung, liver, spleen, and kidney for the presence of GFP-expressing tumor foci recognized by their green fluorescence. Results of these examinations showed that the control animals and animals inoculated with cells treated with MBD2-RS oligonucleotides developed a large number of microscopic tumor metastasis in these organs. In contrast, animals inoculated with cells treated with AdoMet and MBD2-AS oligonucleotides developed significantly fewer metastatic foci in lung, liver, spleen, and kidney. Treatment of MDA-231-GFP cells with AdoMet dramatically reduced tumor metastasis in these mice (Fig. 7).

Levels of Tumoral uPA and MBD2 Production—We then determined whether the in vitro AdoMet and MBD2-AS treatments resulted in stable silencing of either uPA or MBD2 in tumors formed from the treated cells. We extracted total cellular RNA from tumors generated from the different treatment groups and examined the levels of uPA and MBD2 production by RT-PCR. All tumors isolated from the control animals and the animals inoculated with MBD2-RS-treated cells expressed detectable levels of uPA and MBD2 mRNA (Fig. 8A). Several tumors isolated from the animals inoculated with MBD2-AS-treated cells expressed detectable levels of uPA and MBD2 mRNA, whereas none of the tumors isolated from the animals in the AdoMet group expressed detectable uPA and MBD2 mRNA by RT-PCR (Fig. 8A).

The immunohistochemistry analysis of the tumors showed the intensive expression of uPA in the tumors isolated from the animals in the control and MBD2-RS groups (Fig. 8B). In contrast, almost no positive staining for uPA was seen in the tumors isolated from the animals in the AdoMet group. The expression of uPA in the tumors isolated from the animals in the MBD2-AS group was significantly lower than the control and the MBD2-RS (Fig. 8B). These results show that the long term effect of AdoMet and MBD2-AS on expression of uPA is consistent with the stable epigenomic change in DNA methylation caused by these treatments.

**DISCUSSION**

The silencing of tumor suppressor genes by hypermethylation in cancer has been the focus of attention (5). It has been demonstrated that inhibition of DNA methyltransferase suppresses tumorigenesis (18–22) and the demethylation agents have reached the stage of clinical trials as anticancer agents (23). However, it has long been established that global DNA hypomethylation is a common feature of tumors (24, 25), and it was recently demonstrated that loss of genomic methylation could promote tumor development in certain instances (26). One of the mechanisms through which hypomethylation might play a causal role in tumorigenesis is activation of either tumor promoting or metastasis promoting genes (21, 22). If hypomethylation plays a causal role in metastasis, then inhibition of either the proteins responsible for hypomethylation or enhancing DNA methylation might result in inhibition of metastasis through silencing of pro-metastatic genes (6, 27). One excellent example of a gene regulated by methylation is uPA, which is hypomethylated and active in metastatic breast cancer cell lines such as MDA-231 and hypermethylated in non-metastatic breast cancer cells such as MCF-7 (6). The enzymes responsible for hypomethylation in tumors are still unclear and the mere existence of a demethylating activity in tumors is controversial. However, it has been known for some time that the methyl donor AdoMet can inhibit hypomethylation either by enhancing DNA methyltransferase activity, which is the generally accepted model, or by inhibiting active demethylation, which was recently proposed (11). We took advantage of this natural product to test our hypothesis. Our data shows that AdoMet treatment leads to hypermethylation and silencing of uPA (Fig. 2). AdoMet treatment also results in inhibition of tumor cell invasion in vitro (Fig. 2) and metastasis of the treated cells in vivo (Fig. 7). This data is consistent with the hypothesis that hypomethylation plays a causal role in the activation of uPA in metastatic breast cancer cells and perhaps other pro-metastatic genes in other cancers. It stands to reason that AdoMet treatment results in hypermethylation of a number of genes required for metastasis in addition to uPA. Further experiments are required to determine which other genes silenced by AdoMet treatment are responsible for inhibition of metastasis. Nevertheless, our data show that inhibition of hypomethylation results in inhibition of metastasis and that it results in methylation and inactivation of uPA, an important gene implicated in the process of metastasis. Whereas previous experiments showed that uPA could be pharmacologically induced in non-expressing breast cancer cells MCF-7 by the DNA demethylating agent 5′-azadeoxycytidine (6), it was unclear whether hypomethylation was responsible for the activity of uPA in expressing cells such as MDA-231. The fact that pharmacological hypomethylation results in silencing of uPA supports the hypothesis that the hypomethylated state of this gene in MDA-231 cells is responsible for its expression. It remains to be determined whether global hypomethylation is a general mechanism responsible for activation of pro-metastatic genes in a broad range of cancers, or whether hypomethylation of specific genes such as uPA is a stochastic event that confers selective advantage during tumorigenesis and is therefore selected. If global hypomethylation is a general mechanism in tumorigenesis, global hypomethylation inhibitors such as AdoMet could be used in cancer treatment to block progression of cancer into the later more aggressive and metastatic stages of the disease.
Reversal of uPA Promoter Hypomethylation Inhibits Metastasis

What is the mechanism of action of AdoMet on silencing of uPA gene expression? AdoMet might directly inhibit factors regulating the expression of uPA, it might modulate the general transcription factor machinery, or it might act indirectly by stimulating DNA methylation of the uPA promoter. An interesting aspect of the effects of AdoMet treatment on uPA expression is the delay in onset of uPA silencing; uPA remains highly active up to 3 days following initiation of AdoMet treatment but is almost fully silenced after 6 days (Fig. 1). Such a time course is consistent with an indirect action of AdoMet on uPA, which is brought about through methylation of the uPA promoter. De novo methylation is a slow and inefficient process (28, 29) and the delay might be required for a build up of de novo methylation events in the uPA promoter. AdoMet might act by increasing the rate of de novo methylation or by inhibiting demethylation activity, enabling spurious de novo methylation events to progressively methylate the gene. Another interesting feature of AdoMet treatment is the long term effects on uPA expression and metastasis of a transient in vitro treatment with AdoMet (Figs. 6–8). The persistence of the AdoMet effects after the cells were injected into animals and in the tumors for almost two months in the absence of any further treatment in vivo is consistent with the predicted mechanism of AdoMet increasing DNA methylation. DNA methylation is a stable mark on the genome, which is known to mediate long term effects on gene expression and is normally faithfully maintained in dividing cells (30).

Which proteins are required for maintaining the hypomethylated state of uPA in MDA-231 cells? MB2D is a methylated DNA-binding protein that was previously shown to be required for tumorgenesis (12–14), although its role has been unclear. Our data shows that knockdown of MB2D results in silencing of uPA, increased methylation of the uPA promoter, and inhibition of invasion in vitro and metastasis in vivo (Figs. 4, 5, and 7). This data is consistent with the hypothesis that MB2D is required for maintaining certain genes hypomethylated in tumorgenesis. Our data also support the hypothesis that inhibition of MB2D can be of therapeutic value for inhibition of metastasis (21, 22). The persistence of uPA silencing following a transient treatment with AdoMet suggests that AdoMet can possibly inactivate the proteins responsible for hypomethylation and activation of the uPA gene in MDA-231 cells. If these proteins are still active, they should restore the hypomethylated state of uPA in MDA-231 cells after termination of the treatment. Interestingly, AdoMet treatment results in down-regulation of MB2D transcription (Fig. 1). If MB2D is required for maintaining the hypomethylated state of uPA as suggested by the experiments presented in Fig. 5, down-regulation of MB2D by AdoMet might be responsible for the long term effect of transient AdoMet treatment.

Although further experiments are required to fully understand the mechanisms through which AdoMet and MB2D affect the state of uPA methylation, its expression and breast cancer invasion and metastasis, our studies suggest that inhibition of the hypomethylation machinery might provide a novel therapeutic approach to combating metastasis. The fact that agents that act on the DNA demethylation machinery by different mechanisms produce similar results further strengthens the conclusion that hypomethylation is critical in the process of metastasis. It is then worthwhile to develop inhibitors of DNA demethylation with more potency and higher specificity than AdoMet. Whereas DNA methyltransferase inhibitors target silenced tumor suppressor genes in early stages of tumorgenesis, hypomethylation inhibitors might target pro-metastatic genes thus acting on different stages of tumorgenesis. One concern with inhibition of hypomethylation as a cancer therapy is that it might result in hypermethylation of tumor suppressor genes and promote cancer growth. Hypomethylation inhibitors of hypomethylation should therefore not be used at early stages of cancer when the probability of causing hypermethylation of tumor suppressor genes is high. However, in the later hormone-insensitive stages of breast cancer, when tumor suppressor genes are already silenced by either mutations or aberrant methylation, treatment with inhibitors of hypomethylation would not alter the expression of the silenced tumor suppressor genes, although this needs to be determined by further in depth studies. Our study, therefore, not only highlights the significance of hypomethylation in metastatic cancer progression, it calls for caution in the use of inhibitors of DNA methylation in the late stages of cancer therapy.

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