Alteration of the Methylation Status of Tumor-Promoting Genes Decreases Prostate Cancer Cell Invasiveness and Tumorigenesis In vitro and In vivo

Nicholas Shukeir,1,2 Pouya Pakneshan,1,2 Gaoping Chen,1,2 Moshe Szyf,1,3 and Shafaat A. Rabbani1,2

Departments of Medicine, Oncology, and Pharmacology, McGill University, Montreal, Quebec, Canada

Abstract
We tested the hypothesis that cell invasiveness and tumorigenesis are driven by hypomethylation of genes involved in tumor progression. Highly invasive human prostate cancer cells PC-3 were treated with either the methyl donor S-adenosylmethionine (SAM) or methyl DNA-binding domain protein 2 antisense oligonucleotide (MBD2-AS). Both treatments resulted in a dose- and time-dependent inhibition of key genes, such as urokinase-type plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2), and vascular endothelial growth factor expression to decrease tumor cell invasion in vitro. No change in the levels of expression of genes already known to be methylated in late-stage prostate cancer cells, such as glutathione S-transferase P1 and androgen receptor, was seen. Inoculation of PC-3 cells pretreated with SAM and MBD2-AS into the flank of male BALB/c nu/nu mice resulted in the development of tumors of significantly smaller volume compared with animals inoculated with PC-3 cells treated with vehicle alone or MBD2 scrambled oligonucleotide. Immunohistochemical analysis of tumors showed the ability of SAM and MBD2-AS to significantly decrease tumoral uPA and MMP-2 expression along with levels of angiogenesis and survival pathway signaling molecules. Bisulfite sequencing analysis of tumor genomic DNA showed that inhibition of both uPA and MMP-2 expression was due to methylation of their 5′ regulatory region. These studies support the hypothesis that DNA hypomethylation controls the activation of multiple tumor-promoting genes and provide valuable insight into developing novel therapeutic strategies against this common disease, which target the demethylation machinery. (Cancer Res 2006; 66(18): 9202-10)

Introduction
Prostate cancer remains one of the most commonly diagnosed cancers in men and is leading a cause of cancer deaths (1). It starts as an androgen-dependent, noninvasive disease, which can easily be treated at this early stage with surgery and hormone therapy (2–4). However, if left undetected or untreated, prostate cancer eventually develops into a more aggressive, hormone-independent, highly invasive disease (5). Despite recent advances in the treatment options available for prostate cancer, little progress has been made in either curing or blocking the progression of late-stage, highly invasive prostate cancer resulting in high morbidity and mortality rates. The high rate of morbidity and mortality is due to the ability of prostate cancer cells to metastasize to several distant organs in the body, including the skeleton (5–7). In general, metastasis to distant organs involves four major steps (8): adhesion of tumor cells to the extracellular matrix (ECM), ability of tumoral cells to degrade the ECM and extravasate into surrounding blood vessels, survival against the natural host defenses and settling at the preferred organ site, and extravasation into the organ and formation of new tumors. This is facilitated by the ability of highly advanced prostate cancer cells along with the surrounding stroma to produce several growth factors and proteases that have the ability to inhibit the apoptotic machinery, increase tumor cell proliferation, and cause an increase in new blood vessel formation (neovascularization). Some of the key growth factors and proteases secreted by prostate cancer cells include vascular endothelial growth factor (VEGF; refs. 9, 10), matrix metalloproteinases (MMP; refs. 11–13), and urokinase-type plasminogen activator (uPA; refs. 14–16).

The epigenome, particularly the modifications of cytosines in CpG dinucleotides, has been gaining great attention as an anticancer target (17–21). A main modification associated with the cytosine rings at CpG dinucleotides is methylation (22). One of the hallmarks of cancer is abnormal methylation patterns (23) with malignancies generally governed by widespread DNA hypomethylation of tumor-promoting genes along with site-specific DNA hypermethylation of tumor suppressor genes (23, 24).

A key feature of late-stage prostate cancer is the down-regulation of tumor suppressor genes and up-regulation of tumor-promoting genes. Whereas hypermethylation of tumor suppressor genes, such as glutathione S-transferase P1 (GSTP1), has attracted great attention, very little attention has been paid to hypomethylation of tumor-promoting genes, which is often observed in late-stage cancers. Two of the main proteases, which have been shown to be up-regulated in late-stage prostate cancer, are uPA and MMP-2 (11, 16). uPA, has been shown previously to be abundantly expressed by late-stage prostate cancer cells (PC-3) with complete absence of uPA expression in normal prostate epithelial cells (PrEC) and early-stage prostate cancer cells (LnCAP). This differential expression is due to demethylation of the uPA promoter and indeed can be up-regulated in LnCAP cells by the use of demethylating agents, such as 5′-azadecoxycytidine (5-azaC; ref. 25). Because metastasis involves the coordinate activation of several genes, inhibition of metastasis should require coordinate inhibition of these genes. We hypothesized that if hypomethylation is a common mechanism for activation of tumor-promoting genes, then demethylation inhibitors would suppress this group of genes leading to reduced growth and invasive capacity of cells.

In the current study, we have investigated two approaches to inhibit active demethylation in human prostate cancer cells. S-adenosylmethionine (SAM), a methyl donor of DNA methylation
reactions, has been shown previously to inhibit replication-independent active demethylation and cause hypermethylation (26, 27). SAM treatment was shown previously to cause hypermethylation of uPA in breast cancer cells (28). We also showed that knockdown of methyl DNA-binding domain protein 2 (MBD2) could reverse the hypomethylated state of uPA and silence it in breast cancer cells (28). Increased MBD2 mRNA expression is observed in late-stage PC-3 cells in comparison with the levels of MBD2 mRNA expression in early-stage LnCAP cells (29). Recently, MBD2 has been implicated in tumorigenesis but was not required for the growth of normal cells (30–33). In this study, we determined whether SAM treatment and MBD2 knockdown would silence several prometastatic genes in prostate cancer. The therapeutic implications of our data will then be discussed.

Materials and Methods

Cell line and reagents. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin sulfate (Invitrogen, Burlington, Ontario, Canada).

For growth curve analyses, PC-3 cells were seeded in triplicates at an initial density of 50 x 10^3 per well in six well plates (Falcon Plastics, Oxnard, CA). The cells were treated either with vehicle alone as control or SAM. At the end of each day, cells were trypsinized, resuspended, and counted using a model Z-1 Coulter counter (Coulter Electronics, Bedfordshire, United Kingdom).

Antisense oligonucleotides. MBD2 antisense oligonucleotide (MBD2-AS) and MBD2 scrambled oligonucleotide (MBD2-SCR) sequences are 5’-TCAACAGTAGTTCGCCAGTG-3’ and 5’-ATGGACCCTGTATGACAACT-3’, respectively, and were characterized previously in vitro and in vivo (28, 33). The oligonucleotides were synthesized and purified by Biognostik (Gottingen, Germany). Transfection was done using 5 μL/mL polyfectamine in vitro (Invitrogen) was used to extract total cellular and tumoral RNA according to the manufacturer’s instructions. For Northern blot analysis, 20 μg RNA was electrophoresed on a 1.1% agarose-formaldehyde gel and then transferred onto a Nylon membrane (Amersham Biosciences, Baie d’Urfe, Quebec, Canada). The membranes were then hybridized with 32P-labeled cDNA encoding uPA, MB2, or 18S overnight at 65°C, washed, exposed at −80°C, and then developed. The levels of mRNA expression were quantified using densitometric analyses (Scion Corp., Frederick, MD). For reverse transcription-PCR (RT-PCR) analyses, 2 μg RNA was used. The primers used to amplify the genes of interest are shown in Table 1. The PCRs were carried out using standard protocols and the final PCR products were electrophoresed on a 1.1% agarose gel.

Matrigel chamber invasion assay. The invasive capacity of control and experimental PC-3 cells was determined using two compartments: Boyden chambers (Transwell Costar, Cambridge, MA) and basement membrane Matrigel (BD Biosciences, Mississauga, Ontario, Canada). All cells were analyzed for their viability and an equal number of viable cells (50 x 10^3) was added to the upper chamber and allowed to invade through the Matrigel onto the filters for 18 hours. At the end of the incubation period, the filters were washed, fixed, and stained and the number of cells were counted. Ten random fields for each set of experiments were analyzed and the average number of cells invaded was calculated.

uPA ELISA assay. uPA levels secreted into the medium from control and experimental PC-3 cells were determined using IMUBIND uPA ELISA test kit (American Diagnostica, Stamford, CT). Briefly, cells were cultured in the presence of vehicle, SAM, MBD2-AS, or MBD2-SCR. At the end of the incubation period, medium was collected and the ELISA assay was done according to the manufacturer's instructions. Additionally, the number of cells was counted at the end of the incubation period and used for normalization purposes.

Western blot analysis. To examine the levels of MBD2 in control and experimental PC-3 cells, total nuclear extracts were isolated and analyzed on a SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Immunoblotting was done using standard protocols using a rabbit polyclonal MBD2 antibody. The specificity of the antibody was tested by preabsorbing a second membrane with the anti-MBD2 antibody following preadsorption with recombinant MBD2 protein. The lack of signal following preabsorption indicates the specificity of the antibody to MBD2.

Animal protocols. For in vivo studies, PC-3 cells were treated with either vehicle alone or with 500 μmol/L SAM for 6 days or were transfected with MBD2-AS or MBD2-SCR for 3 days. At the end of the treatment period, cells were harvested and the cell pellets were washed with sterile saline solution and then spun down and resuspended in 20% Matrigel-saline solution. Cells (2 x 10^6) were then inoculated s.c. into male BALB/c nu/nu mice (National Cancer Institute-Fredrick, Frederick, MD). Tumors were measured at weekly intervals for up to 10 weeks and tumor volume was

Table 1. Primer sequence and annealing temperatures used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA forward</td>
<td>ACATTCACTGGTGCAACTGC</td>
<td>56</td>
</tr>
<tr>
<td>uPA reverse</td>
<td>CAAGGCTGTCGAGCGTGTAG</td>
<td>56</td>
</tr>
<tr>
<td>MMP-2 forward</td>
<td>TGATGAGAGACGAGCATCA</td>
<td>56</td>
</tr>
<tr>
<td>MMP-2 reverse</td>
<td>ATACCTTCTGTGGCGTCTG</td>
<td>58</td>
</tr>
<tr>
<td>VEGF forward</td>
<td>CCTGGTGACATCTTCGCCAGGTA</td>
<td>58</td>
</tr>
<tr>
<td>VEGF reverse</td>
<td>CTACCGCCTGGCTGTGCACA</td>
<td>54</td>
</tr>
<tr>
<td>AR forward</td>
<td>ATCTGGGACATTTGGAATCTA</td>
<td>54</td>
</tr>
<tr>
<td>AR reverse</td>
<td>TTGTGTTCCTCATCCAGGA</td>
<td>58</td>
</tr>
<tr>
<td>GSTP1 forward</td>
<td>AAGTCTCCAGGACGAGACCT</td>
<td>58</td>
</tr>
<tr>
<td>GSTP1 reverse</td>
<td>AGTCAGCGAAGGAGATCGG</td>
<td>56</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>CCCCATTCTAGCCTAATTAGTCTG</td>
<td>56</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>GAGGGGCACTCCAGCTTCTG</td>
<td>56</td>
</tr>
</tbody>
</table>

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
determined according to the equation: tumor volume = (length × width²) / 2. At the end of the study, animals were sacrificed and their primary tumors were removed for further analysis.

DNA extraction and bisulfite modification and sequencing. Tumoral genomic DNA was extracted using the DNeasy tissue kit (Qiagen) according to the manufacturer’s instructions and subjected to sodium bisulfite modification as described previously (34). A nested PCR was done to amplify the indicated region of the uPA and MMP-2 promoters. The following uPA primers were outer, forward 5'-GTAGGGGTTGAGGTA-3' and reverse 5'-ATAACCAACTCCCAACTA-3' and nested, forward 5'-TTAGTAAAGTTGAGGTTAG-3' and reverse 5'-TCTCTCTCTTC-TATAAACTC-3'. The following MMP-2 primers were outer, forward 5'-TTGGTTGATTTGTTTTTG-3' and reverse 5'-TCTTAA-CACCCCTTTATATT-3' and nested, forward 5'-GATTGTTAGGAGTTAGG-3' and reverse 5'-CAACCTCCACACCC-3'. The PCR conditions used were as follows: initial denaturation at 95°C for 10 minutes, 95°C for 1 minute, 51°C for 2.5 minutes, and 72°C for 1 minute with a final extension at 72°C for 5 minutes. The final PCR products were electrophoresed on a 2% agarose gel. The expected bands were excised and purified using the QIAquick Gel Extraction kit (Qiagen) and then TA cloned using the pGEM-Vector Easy System (Promega, Nepean, Ontario, Canada) and sequenced.

Histologic analysis. Tumors removed from sacrificed animals were paraffin embedded and subjected to immunohistochemical analysis. Staining for uPA (American Diagnostica), MMP-2 (Cell Signaling Technology, Inc., Beverly, MA), CD31 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and phosphorylated Akt (pAkt; Cell Signaling Technology) was carried out by using the avidin-biotin peroxidase complex method as described previously (35). Sections were treated with the primary antibodies at 1:50 dilution overnight at 4°C. Biotinylated secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was used for 30 minutes at room temperature. The slides were developed and intensity of immunostaining quantified using BioQuant image analysis software version 6.50.10 (BioQuant Image Analysis Corp., Nashville, TN).

Statistical analysis. All results are represented as the mean ± SE of at least triplicate experimentation. Statistical analysis was carried out using either Student’s t test, ANOVA, or regression analysis. P < 0.05 was considered to be significant.

Results

SAM down-regulates uPA and MBD2 expression in PC-3 cells. PC-3 cells were treated with different doses of SAM for various times. Total cellular RNA was collected and analyzed for the expression of uPA and MBD2 mRNA levels. Our data indicate that increasing concentrations of SAM significantly reduced the expression of both uPA and MBD2 mRNA levels with maximal reduction observed with 500 μmol/L SAM (Fig. 1A). This reduction in both uPA and MBD2 mRNA levels was time dependent with maximal effect achieved after 6 days of treatment (data not shown). Similar effects were not observed with S'-adenosylhomocysteine (SAH), the inactive analogue of SAM (Fig. 1A). The reduction of uPA mRNA was accompanied by a dose-dependent reduction in the levels of uPA protein secreted into the culture medium by treated cells as analyzed by ELISA (Fig. 1B). Furthermore, Western blot analysis revealed that SAM treatment caused a significant reduction in the levels of MBD2 protein (Fig. 2B).

To determine whether SAM treatment might have an effect on PC-3 cell growth, cellular growth patterns were examined by carrying out a growth curve analysis. The highest concentration of SAM (500 μmol/L) significantly reduced the doubling time of treated PC-3 cells compared with control PC-3 cells treated with vehicle alone (data not shown).

Determination of the effect of uPA mRNA and protein reduction on the invasive capacity of PC-3 cells was carried out using a Matrigel Boyden chamber invasion assay as described in Materials and Methods. SAM treatment caused a dose- and time-dependent reduction in the number of viable cells that were able to invade through Matrigel (Fig. 1C). Our data do not reflect differences in viability of SAM-treated and untreated cells because an equal

Figure 1. Effect of SAM on uPA levels and invasive capacity of PC-3 cells. Human PC-3 cells were treated with different doses of SAM or SAH for up to 6 days (A). At the end of incubation, total cellular RNA was collected and analyzed for the levels of uPA and MBD2 mRNA levels by Northern blot analysis as described in Materials and Methods. Further evaluation of the effect of SAM on uPA protein levels was carried out by carrying out an ELISA assay on conditioned medium collected for PC-3 cells treated with 500 μmol/L SAM for 6 days (B). The invasive capacity of PC-3 cells treated with different doses of SAM for different durations (C) was determined by determining the ability of control and treated cells to invade through Matrigel using a Matrigel Boyden chamber invasion assay as described in Materials and Methods. To establish that SAM treatment is effective in other cells lines that express uPA, two other human prostate cancer cell lines (DU-145 and PC-3M; D) were treated with 500 μmol/L SAM for 6 days. At the end of treatment, total cellular RNA was extracted and analyzed for the expression of uPA by RT-PCR. Columns, mean of three different experiments; bars, SE. * P < 0.05, significant difference from control.
number of viable cells for control and SAM-treated samples were used for these experiments.

To establish whether the effect of SAM is restricted to PC-3 cells or whether it has similar effects on other invasive human prostate cancer cell lines, DU-145 and PC-3M cells were treated with 500 μmol/L SAM for up to 6 days and analyzed for the expression of uPA mRNA by RT-PCR analysis. Treatment of these two cell lines resulted in a significant reduction in the levels of uPA mRNA (Fig. 1D), indicating that SAM treatment silences uPA in several prostate cancer cells.

**MBD2 knockdown in PC-3 cells silences uPA.** PC-3 cells were treated with different doses of MBD2-AS or MBD2-SCR for different times as described in Materials and Methods. We first showed that this treatment resulted in MBD2 knockdown. Northern blot analysis indicated that whereas transfection of MBD2-AS resulted in a significant dose- and time-dependent reduction in the levels of MBD2 mRNA levels (Fig. 2A), no such reduction was seen with the MBD2-SCR (data not shown). The maximal reduction in MBD2 mRNA levels was seen at 72 hours using 200 nmol/L MBD2-AS (Fig. 2A). This reduction in MBD2 mRNA levels also translated to a reduction in MBD2 protein levels as determined by Western blot analysis (Fig. 2B).

Northern blot analysis was carried out to determine the resulting effect of inhibiting MBD2 mRNA and protein levels on the levels of uPA mRNA levels. A time- and dose-dependent inhibition in uPA mRNA levels was observed, which was correlated with the time- and dose-dependent knockdown in MBD2 levels (Fig. 2A). The dose-dependent inhibition in uPA mRNA following transfection of 200 nmol/L MBD2-AS resulted in a similar dose-dependent inhibition in secreted uPA protein levels as analyzed by ELISA (Fig. 2C). As expected, the dose- and time-dependent reduction in uPA levels caused a dose- and time-dependent decrease in the invasive capacity of PC-3 cells to invade through Matrigel as analyzed by Boyden chamber invasion assay (Fig. 2D). MBD2-SCR transfection had no significant effect at reducing the invasive capacity of PC-3 cells (data not shown). These studies show that MBD2 plays an important role both in the expression of uPA and in maintaining the invasive capacity of PC-3 cells.

**Effect of SAM treatment and MBD2 knockdown on gene expression.** To determine whether SAM treatment and MBD2 knockdown is restricted to uPA or whether it would affect other tumor-promoting genes, we measured the effect of these treatments on two other genes shown to be involved in prostate cancer progression (MMP-2 and VEGF) using RT-PCR. Whereas control PC-3 cells as well as MBD2-SCR-treated cells expressed abundant levels of both MMP-2 and VEGF mRNA, SAM treatment and MBD2 knockdown resulted in significant reduction in the levels of mRNA expression of these genes (Fig. 3A).

Because methylation is a mechanism of gene inactivation, a possible concern with hypermethylation therapy is that it would result in further silencing of tumor suppressor genes, such as GSTP1, or activation of tumor-promoting genes, such as androgen receptor (AR). MBD2, on the other hand, was shown previously to bind and silence methylated tumor suppressor genes, such as GSTP1, or activation of tumor-promoting genes, we measured the effect of these treatments on the levels of GSTP1 and MMP-2 and VEGF mRNA, SAM treatment and MBD2 knockdown resulted in significant reduction in the levels of mRNA expression of these genes (Fig. 3A).

Because methylation is a mechanism of gene inactivation, a possible concern with hypermethylation therapy is that it would result in further silencing of tumor suppressor genes, such as GSTP1, or activation of tumor-promoting genes, such as androgen receptor (AR). MBD2, on the other hand, was shown previously to bind and silence methylated tumor suppressor genes, such as GSTP1, or activation of tumor-promoting genes, we measured the effect of these treatments on the levels of GSTP1 and MMP-2 and VEGF mRNA, SAM treatment and MBD2 knockdown resulted in significant reduction in the levels of mRNA expression of these genes (Fig. 3A).

**SAM and MBD2-AS inhibit PC-3 tumor growth in vivo.** To evaluate whether the in vitro effects of SAM treatment and MBD2-AS transfection on PC-3 invasive capacity would result in the inhibition of tumor growth in vivo, male BALB/c nu/nu mice were...
inoculated s.c. into the right flank with PC-3 cells treated with vehicle alone as control, PC-3 cells treated with 500 μmol/L SAM for 6 days, and PC-3 cells transfected with 200 nmol/L MBD2-AS or MBD2-SCR. Before implantation, we validated that MBD2 knockdown and SAM resulted in silencing of MBD2 and uPA. Tumors were monitored on a weekly basis and tumor volumes were determined as described in Materials and Methods. Control vehicle-treated PC-3 cells and PC-3 cells transfected with MBD2-SCR developed tumors of increasing volume during the study. In contrast, experimental animals inoculated with SAM-treated or MBD2-AS-transfected PC-3 cells developed tumors of significantly smaller volumes compared with animals receiving control PC-3 cells and MBD2-SCR-transfected cells (Fig. 4).

**SAM and MBD2 knockdown inhibit tumoral angiogenesis and proteases.** Tumors were evaluated for the degree of angiogenesis by carrying out immunohistochemical analysis for the levels of CD31 as described in Materials and Methods. Whereas control and MBD2-SCR tumors exhibited a high degree of angiogenesis as seen by the intensity and density of positive CD31 staining, SAM-treated and MBD2-AS tumors exhibited a significantly lower degree of angiogenesis as seen by a marked decrease in both the intensity and the density of positive CD31 staining (Fig. 4B). Furthermore, tumors were also analyzed for the levels of pAkt, the active form of Akt, which is a key intracellular signaling molecule known to be modulated by several key tumorigenic pathways. Intense positive staining was observed in tumors extracted from animals inoculated with control vehicle-treated and MBD2-SCR-transfected PC-3 cells (Fig. 4B). In contrast, a significant reduction in the intensity of positive pAkt staining was observed in both tumors developing from SAM-treated and MBD2-AS-transfected PC-3 cells (Fig. 4B).

Further analysis was carried out to evaluate whether the silencing of MMP-2 and uPA seen in vitro by SAM treatment and MBD2-AS transfection would be stably maintained in tumors developing from experimental cells. Total cellular RNA was extracted from tumors and analyzed for the levels of MMP-2 and uPA mRNA expression by RT-PCR analysis (Fig. 5A). Evaluation of the levels of MMP-2 and uPA protein expression was also carried out by immunohistochemical analysis of control and experimental tumors. Tumors developing from control vehicle-treated cells and MBD2-AS-transfected cells expressed abundant levels of MMP-2 and uPA mRNA and protein (Fig. 5A and B). In contrast, tumors extracted from animals inoculated with either SAM-treated cells or MBD2-AS-transfected cells expressed significantly lower levels of these genes (Fig. 5A and B). The results obtained suggest that the silencing of key tumor-promoting genes achieved in vitro by SAM and MBD2-AS is maintained in vivo. This stable silencing, which follows a transient exposure to either SAM or MBD2 AS, is consistent with the hypothesis that these treatments might result in stable methylation-specific alterations that stably reprogrammed these genes.

**Effect of SAM or MBD2-AS knockdown on uPA and MMP-2 gene methylation.** We then determined the state of methylation of uPA and MMP-2 in tumoral DNA derived from either MBD2-AS, SAM-treated cells, or the control cells. The genomic DNA was subjected to sodium bisulfite modification and the 5′ regulatory regions of the uPA and MMP-2 genes were amplified and analyzed for their methylation patterns. Whereas these genes in tumors excised from animals inoculated with either control or MBD2-SCR-transfected cells were completely unmethylated in the region of interest, tumors excised from animals inoculated with SAM-treated or MBD2-AS-transfected PC-3 cells displayed greater degree of methylation of both uPA and MMP-2 5′ regulatory regions (Fig. 6). This suggests that transient treatment of PC-3 cells with either SAM or MBD2-AS resulted in long-term effects on the methylation state of these genes in tumors derived from these cells in vivo. This observation has interesting implications on the potential utilization of these hypermethylating agents in cancer therapy.

**Discussion**

Abnormal DNA methylation patterns normally observed in cancer cells are the global hypomethylation of the genome accompanied by regional hypermethylation (23, 24). Aberrant regional hypermethylation tend to localize to CpG islands of tumor suppressor genes leading to their transcriptional inactivation. It has been proposed that the transcriptional silencing of tumor suppressor genes through DNA methylation plays a key role in the transformation of a normal cell to a malignant phenotype (36). Therefore, great attention has been focused on the silencing of tumor suppressor genes in cancer with particular attention paid to reactivation and transcription induction of these tumor suppressor genes through reversal of the hypermethylation modification. Although studies have shown that demethylating agents, through induction of tumor suppressor gene expression, can decrease tumorigenesis (37–39), they have also been shown to induce or further increase the expression of tumor-promoting genes, particularly genes associated with metastasis (40).

Global genomic hypomethylation is also a common feature often observed in cancerous cells. Mechanisms proposed by which
hypomethylation can bring about tumorigenesis include predisposition to genetic instability, especially during early stages of tumor progression, which could result in further genetic changes (41). Recent data suggested that several genes, which are expressed in highly metastatic cells but not in nonmetastatic cells and are involved in metastasis, are regulated by DNA methylation and activated by the demethylating drug 5-azaC. Because the transition of a cell from a nonmetastatic to a metastatic state involves the activation of several genes, such as proteases and growth factors, we proposed that the global hypomethylation activity present in cancer cells is required for coordinate activation of several genes. We tested here this hypothesis by treating highly invasive PC-3 prostate cancer cells with the universal methyl donor SAM and by knockdown of MBD2. We have shown previously that both these treatments could lead to hypermethylation of one prometastatic gene uPA (28). We therefore hypothesized that this treatment would result in silencing other genes involved in prostate cancer progression.

The results obtained show that both SAM and MBD2 knockdown treatment can indeed result in inhibition of several tumor-promoting genes, such as uPA, MMP-2, and VEGF, which resulted in inhibition of tumor cell invasion in vitro and tumor growth in vivo. These results are consistent with the hypothesis that hypermethylation therapy might be a possible approach to late-stage prostate cancer. One interesting observation that might have therapeutic implications is that although the treatment with SAM and MBD2 knockdown was transient, the silencing of metastatic genes and methylation of MMP-2 and uPA was maintained in the tumoral DNA for several weeks (Fig. 5B). This suggests that the hypermethylation therapy results in stable epigenetic reprogramming of these genes, which is then maintained in the absence of any hypermethylating agent.

An important concern with using hypermethylation therapy is that it might result in methylation and silencing of tumor suppressor genes and thus leads to tumor promotion. One possible consideration, which might tone down the potential unwanted effects of hypermethylation therapy is that in advanced prostate cancer, several genes, including AR, and the majority of tumor suppressor genes, such as GSTP1, are already either methylated or partially methylated. We addressed the question of whether either SAM or MBD2 knockdown treatment would result in a change in the levels of expression of these genes. Our results show that no change has occurred in the level of expression of these genes following our treatment, which support the hypothesis that SAM and MBD2 do not target all genes equally and that even a tumor suppressor gene, such as GSTP1, with a propensity to become methylated in prostate cancer cells escapes their effect.
Although SAM and MBD2 knockdown have obviously different mechanisms of action and act on the DNA methylation machinery at different sites, the consequences on gene expression of prometastatic genes, invasibility, tumorigenicity, and DNA methylation seem to be similar, suggesting a common mechanism. The decreased expression of uPA and MMP-2 by SAM and MBD2 knockdown treatment could be indirect, by SAM and MBD2 modulating the activity of proteins regulating MMP-2 and uPA. However, analysis of the uPA gene following SAM treatment showed increased methylation at CpG islands, indicating that establishing the methylation pattern of the uPA gene after SAM treatment is at least partially involved in causing transcriptional repression of uPA. Consistent with a methylation-dependent mechanism is the time course of silencing. The full effect of SAM in methylating and decreasing uPA expression was observed only after a prolonged treatment period (6 days). If the predicted mechanism of SAM action is inhibition of demethylation, silencing would require de novo methylation by de novo methyltransferases. Because de novo methylation in somatic cells could be a slow and inefficient process, the time course might reflect the inefficient de novo methylation process. Further evidence implicating the de novo methylation machinery as a potential mechanism of SAM treatment is the persistence of the inhibitory effect of SAM on uPA, MMP-2, and CD31 (angiogenesis marker) seen in tumoral expression after 10 weeks without any further in vivo treatment. These effects were shown to be in part due to the maintained increased methylation patterns observed in the uPA and MMP-2 promoter in tumoral genomic DNA.

Whereas studies have implicated MBD2 as a transcriptional repressor, which acts by binding to methylated DNA and recruiting transcriptional repressors (42), other studies have implicated MBD2 as a transcriptional activator that induces gene expression (43, 44). Furthermore, studies have shown that MBD2 is required for tumorigenesis and that a deficiency in MBD2 suppresses tumorigenesis. Thus, our hypothesis was that inhibition of MBD2 expression, by the use of antisense oligonucleotides, would result in increased methylation and transcriptional inhibition of key tumor-promoting genes. Indeed, the results obtained in this study showed that knockdown of MBD2 resulted in decreased expression of tumor-promoting genes, including uPA, MMP-2, and VEGF. Similar to the effects of SAM, MBD2 knockdown had no effect on reactivating AR or further inhibiting GSTP1 expression in PC-3 cells as would be predicted if indeed MBD2 silenced these methylated tumor suppressor genes. The lack of change in expression of GSTP1 following MBD2 knockdown suggests that MBD2 might not be involved in silencing of this gene in PC-3 cells.

The results obtained from our studies suggest that inhibiting the hypomethylation machinery could prove to be a novel therapeutic target for combating late-stage prostate cancer. Whereas inhibitors of DNA methylation target tumor suppressor genes are inactivated during early tumorigenesis, inhibitors of the hypomethylation machinery target metastasis-promoting genes that are activated and expressed during late-stage carcinogenesis. Our results indicate that whereas inhibition of the hypomethylation machinery causes decreased expression of metastasis-promoting genes, no changes in the levels of expression of tumor suppressor genes were observed. Thus, the use of inhibitors of the hypomethylation machinery in late-stage prostate cancer should not lead to further tumor growth through inhibition of tumor suppressor genes but rather to decreased tumorigenesis and decreased metastatic potential through...
inhibition of genes involved in the metastatic process. Further studies are warranted to determine the effect of SAM and MBD2 knockdown on the development of skeletal metastases, a common complication seen in patients with late-stage prostate cancer, which will have a marked effect on prostate cancer morbidity and mortality.

Figure 6. SAM and MBD2-AS treatment result in methylation of the 5′ regulatory region of uPA and MMP-2. Male BALB/c nu/nu mice were inoculated with either control PC-3 cells, PC-3 cells treated with 500 nmol/L SAM for 6 days, or PC-3 cells transfected with 200 nmol/L MBD2-AS or MBD2-SCR for 3 days. At the end of the study, tumoral genomic DNA was extracted and subjected to sodium bisulfite modification to determine the methylation status of the uPA (A) and MMP-2 (B) 5′ regulatory region as described in Materials and Methods. Representative region of each subset of tumors. Ten different clones were analyzed per tumoral subset.

Acknowledgments

Received 5/31/2006; revised 7/19/2006; accepted 7/24/2006. Grant support: Canadian Institutes for Health Research grant MOP 12609 and Prostate Cancer Research Foundation of Canada; Fonds de recherche en santé du Québec training grant (N. Shukeir).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

Alteration of the Methylation Status of Tumor-Promoting Genes Decreases Prostate Cancer Cell Invasiveness and Tumorigenesis In vitro and In vivo

Nicholas Shukeir, Pouya Pakneshan, Gaoping Chen, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/18/9202

Cited articles
This article cites 44 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/18/9202.full#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/18/9202.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.