

Pharmacological Inhibition of DNA Methylation Induces Proinvasive and Prometastatic Genes *In Vitro* and *In Vivo*¹

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Abstract

The mechanism of action of DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR), a potential anticancer agent is believed to be activated by the demethylation of tumor suppressor genes. We tested here the hypothesis that demethylating agents also demethylate and activate genes involved in invasion and metastasis and therefore might increase the risk of developing tumor metastasis. The effect of 5-aza-CdR on noninvasive human breast cancer cells MCF-7 and ZR-75-1 was evaluated by cell proliferation, invasion, and migration assay. The ability of 5-aza-CdR to activate a panel of silenced prometastatic and tumor suppressor genes was evaluated using reverse transcription–polymerase chain reaction and bisulfite DNA sequence analysis *in vitro* and for change in tumor growth and gene expression *in vivo*. Treatment of MCF-7 and ZR-75-1 with 5-aza-CdR diminished cell proliferation, induced tumor suppressor *RASSF1A*, and altered cell cycle kinetics' G₂/M–phase cell cycle arrest. While these effects of 5-aza-CdR slowed the growth of tumors in nude mice, it also induced a battery of prometastatic genes, namely, *uPA*, *CXCR4*, *HEPARANASE*, *SYNUCLEIN* γ , and transforming growth factor-beta (*TGF- β*), by demethylation of their promoters. These results draw attention to the critical role of demethylation as a potential mechanism that can promote the development and progression of tumor metastasis after demethylation therapy as an anticancer treatment.

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Introduction

Hypermethylation of tumor suppressor genes is a common mechanism of gene silencing observed in cancer [1]. Knock down of DNA methyltransferase 1, the enzyme that is involved in the copying of DNA methylation during cell division, by either pharmacological [2,3] or genetic approaches [4], was shown to inhibit cancer in animal models. The DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-CdR), was shown to demethylate and induce the activity of methylated tumor suppressor genes [5]. 5-Aza-CdR is currently being tested in different clinical trials as an anticancer agent [6–8]. Breast cancer is the most common malignant disease affecting women of all age group globally; during this multistep process of tumor progression, the disease was initiated as a less-aggressive nonmetastatic hormone-responsive–type, which gradually develop as a highly invasive and metastatic hormone-insensitive malignancy. Most of the mortalities among these patients are due to delayed diagnosis, poor prognosis, and metastases to the distant organs especially lung and bones, which are more frequent sites of breast cancer metastases [9]. The early steps of the metastatic cascade involving the degradation of the extracellular matrix and subsequent invasion by cancer cells are accomplished by several proteinases, including among others, urokinase-type plasminogen activator (uPA), heparanase and matrix metalloproteinase (MMPs),

and other proteins, by which different lines of data suggest to contribute to metastasis, such as transforming growth factor-beta (*TGF- β*), *S100A4*, *SYNUCLEIN* γ , and *CXCR4* [10–20]. We have previously shown that *uPA* promoter region is hypomethylated, and its expression is upregulated in the invasive breast cancer cell line MDA-MB-231 (hormone insensitive), whereas the same promoter region is methylated in noninvasive breast cancer cells such as MCF-7 (hormone-responsive). Furthermore, 5-aza-CdR treatment induces the expression of *uPA* in noninvasive MCF-7 cells [21]. Similar effects were also observed in a hormone-responsive human prostate cancer cell line, LNCaP, after the treatment with 5-aza-CdR [22]. We have also shown that reverting the hypomethylated status of uPA promoter in MDA-MB-231 cells by

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either S-adenosyl-L-methionine treatment or knock down of methyl DNA binding domain protein 2 causes significant inhibition in uPA expression and invasion *in vitro* and tumor growth *in vivo* [23]. We previously hypothesized that reactivation of expression of prometastatic genes by DNA demethylation is a common contributing factor to promoting tumor metastasis [24]. Therefore, we suggested that although inhibition of DNA methylation results in decreasing tumor growth by the activation of tumor suppressor genes, it might, at the same time, result in the activation of prometastatic genes, suggesting divergence of growth control and invasion in breast cancer cells [24,25].

In the present study, we tested this hypothesis by examining the effects of 5-aza-CdR, a classic DNA methylation inhibitor, which is currently tested in clinical trials, on the expression and DNA methylation of the tumor suppressor gene, *RASSF1A*, and a set of genes involved in metastasis/angiogenesis of breast cancer, such as *uPA*, *HEPARANASE*, *CXCR4 RECEPTOR*, *STROMAL CELL-DERIVED FACTOR 1 α* (*CXCL12*), *BREAST CANCER-SPECIFIC GENE 1* or *SYNUCLEIN γ* (*SNCG*), *VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)*, and *TGF- β* as well as genes involved in cell cycle, such as cyclin-dependent kinase inhibitors (*p21^{WAF1/Cip1}*, *CYCLIN D1*, and *CYCLIN E1*) and *BAX*, a proapoptotic gene of the Bcl-2 family [10,11,14,15,17,26–34]. We then tested whether 5-aza-CdR will convert nonmetastatic breast cancer cell lines MCF-7 and ZR-75-1 to invasive and metastatic cells using *in vitro* and *in vivo* assays. Our data support a mechanism for the potential capacity of 5-aza-CdR to induce the expression of previously quiescent prometastatic genes and for the need to carefully evaluate and monitor patients receiving DNA methylation inhibitors.

Materials and Methods

Cell Culture and Treatment

Human breast cancer cell line, MCF-7 and ZR-75-1 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were maintained in minimum Eagle's medium (Earle's salts; Invitrogen Life Technologies, Ontario, Canada) with 2 mM L-glutamine, 10% FBS, 10 μ g/ml insulin, and 100 U/ml of penicillin-streptomycin sulfate. ZR-75-1 cells were grown in RPMI 1640 medium (Invitrogen Life Technologies) with 10% FBS, 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Cells were incubated at 37°C in 5% CO₂. In the 5-aza-CdR treatment group, MCF-7 and ZR-75-1 cells were treated for 7 days with 5-aza-CdR (Sigma Aldrich, Ontario, Canada) at final concentrations of 0.1, 1, and 5 μ M in the regular medium. The culture medium was changed every second day in both control and treatment groups by maintaining the desired concentration of the drug.

Cell Proliferation Assay

MCF-7 and ZR-75-1 cells were plated in quadruplicate at a density of 50,000 cells/well in 2 ml of respective culture media in six-well plates. Cells were grown in the regular medium with 5% FBS in the control and treatment (5-aza-CdR) groups. Cell growth curve was analyzed at two different doses of 5-aza-CdR (1 and 5 μ M). Cells were trypsinized, and the number of viable cells was determined by 0.4% trypan blue and counted at different time points starting from day 0 (without 5-aza-CdR) using a Coulter counter (Model ZF; Coulter Electronics, Hertfordshire, UK). Cell culture medium

was replenished every second day along with the indicated dose of 5-aza-CdR in the treatment group.

Fluorescence-Activated Cell Sorting Analysis

The effect of 5-aza-CdR on cell cycle distribution was determined by flow cytometry after staining the cells with propidium iodide. Briefly, control and 5-aza-CdR-treated (5 μ M for 7 days) MCF-7 and ZR-75-1 cells were trypsinized and washed with phosphate-buffered saline (PBS), and fixed with chilled 70% ethanol. The fixed cells were then centrifuged and washed with PBS. The cells were then treated with 1 U of DNase-free RNase to the cell suspension (10⁶ cells in 1 ml), and incubated for 30 minutes at 37°C. Fifty microliters of 1 mg/ml propidium iodide was added directly to the cell suspension, and flow cytometry analysis was performed on a flow cytometer (FACS Calibur; BD Biosciences, Ontario, Canada). Results were analyzed further using the WinMDI software (version 2.8; Scripps Research Institute, La Jolla, CA).

Boyden Chamber Matrigel Invasion Assay

Cells' invasive capacity was determined using a two-compartment Boyden chamber Matrigel invasion assay (Transwell; Costar Corning Corporation, Burlington, MA) as described previously [21]. The 8- μ m-pore polycarbonate filters were coated with basement membrane Matrigel (50 μ g/filter). A total of 5 \times 10⁴ cells treated with or without 5-aza-CdR (1 and 5 μ M) in 0.1 ml of medium were added to the upper chamber and placed on top of a lower chamber prefilled with 0.8 ml of serum-free medium supplemented with 25 μ g/ml fibronectin (Sigma Aldrich) and then incubated at 37°C for 24 hours. After incubation, the medium was removed, and the polycarbonate filters with invaded cells were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde (Sigma Aldrich) in 0.1 M phosphate buffer, pH 7.4, at room temperature for 30 minutes. They were then stained with 1.5% toluidine blue, washed, and mounted onto glass slides. The number of cells invaded was examined under a light microscope. Ten fields under a magnification of \times 400 were randomly selected, and the mean number of cells invading was calculated.

Cell Wounding Assay

Confluent MCF-7 monolayer was grown in six-well plates as described above with 2% fetal bovine serum. The cell monolayer was mechanically wounded in the center of each well with a sterile 200- μ l pipette tip and maintained in the presence or absence of 5-aza-CdR (1 and 5 μ M) for 48 hours where the culture medium was changed every 24 hours. Cells were then washed three times to get rid of the detached cells and debris. Wound healing was monitored at different time points with an inverted bright field microscopy under the 4 \times objective. Only cell-free area was selected, measured, and quantified using the Image Pro-Plus software (Media Cybernetics, Inc., Bethesda, MD) and calculated as percentage wound healing using the equation: %wound healing = [1 - (wound area at T_x hours/wound area at T_0)], where the T_x is the respective time point and T_0 is the time immediately after wounding.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total cellular RNA from control and 5-aza-CdR-treated MCF-7 and ZR-75-1 cells were extracted using TRIzol (Invitrogen Life Technologies) according to the manufacturer's protocol. Two micrograms of total RNA was used for reverse transcription and amplification. The primers used for reverse transcription-polymerase chain

reaction (RT-PCR) were designed so that there is an intron between the amplified regions to recognize any DNA contamination. Two sets of different RT-PCR primers were used in the present study; their sequence and annealing temperature are given in Table 1. The reactions were carried out using standard protocols. The DNA was amplified under the following conditions: 95°C for 5 minutes, 30 cycles at 95°C for 30 seconds, annealing temperature of the respective primer for 30 seconds as given in Table 1, and at 72°C for 30 seconds, and the final extension at 72°C for 5 minutes. Different numbers of cycles were used to ensure linearity of amplification. The gels were quantified by using the Scion Software (Scion Corporation, Frederick, MD) for the densitometry.

Sodium Bisulfite Sequencing

Genomic DNA was extracted from control and 5-aza-CdR-treated (5 μM for 7 days) MCF-7 cells using DNazol (Invitrogen Life Technologies) following the manufacturer's instructions. Sodium bisulfite treatment of the genomic DNA was performed as described previously [21,22]. Two sets of primers were designed to amplify the modified DNA fragment within the uPA promoter region with outer primers (5'-GTAAGGGGTTTGAGGTAGT-3' and 5'-ATAACCAAACCTCCCAACTA-3') and inner primers (5'-TTTAGGTAAGTTGGGTTT-3' and 5'-TCTCTCTCCTCTATAAACTC-3') of the uPA promoter sequence. The amplification reaction was performed under the following conditions: 95°C for 5 minutes; 34 cycles at 95°C for 1 minute, at 51°C for 2.5 minutes, and at 72°C for 1 minute; final extension at 72°C for 5 minutes. For CXCR4, the bisulfite DNA was subjected to primary and nested PCR using two sets of primers from the promoter and exon 1 region; outer primers (5'-TTATTTATTAGTAAGGATGGA-3' and 5'-AATACCTCCAATATCCTAAC-

3') and inner primers (5'-AAATGTTTTTTGGGAGGTTTTG-3' and 5'-CACTAATCCCCTCCATAATA-3') at 95°C for 5 minutes; 34 cycles at 95°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 30 seconds; final extension at 72°C for 5 minutes. For SNCG genomic bisulfite sequencing, first-step PCR was carried out using the outer set of primers (5'-GGTTGAGTTAGTAGGAGTTTA-3' and 5'-CCTACCATACCCCACTTACCC-3'), and nested PCR was carried out using the inner set of primers (5'-TATTTTGGAGGAAGGTGAGGTTG-3' and 5'-CCACTTACCCACATACATAACC-3') at 95°C for 5 minutes; 34 cycles at 95°C for 30 seconds, at 58°C for 30 seconds, and at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

Finally, PCR products of the uPA, CXCR4, and SNCG were purified by using QIAquick PCR purification kit (Qiagen Inc., Ontario, Canada) and subcloned into pGEMT cloning vector (Promega, Madison, WI) according to the manufacturer's instruction, and plasmid DNA from different bacterial clones was prepared and analyzed for DNA sequences (Genome Centre, McGill University, Quebec, Canada). At least 10 clones for each gene were sequenced per treatment group.

In Vivo Tumor Growth in Breast Cancer Model

Six-week-old female Balb C *nu/nu* mice were obtained from Charles River, Inc. (St. Constant, Quebec, Canada). Before inoculation, MCF-7 cells were treated with 5 μM of 5-aza-CdR for 7 days in regular growth media. Growth medium and drug was changed every second day during treatment. On day 8, treated and untreated MCF-7 cells were trypsinized and washed with Hank's balanced buffer; 2.5×10^6 cells were resuspended in 100 μl of saline with 20% Matrigel (Becton Dickinson Labware, Ontario, Canada). An anaesthetic cocktail of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg) was injected intramuscularly, and 2.5×10^6 cells were inoculated using 30-gauge needles into the mammary fat pad of anaesthetized mice. All experimental animals (eight animals in each group) were implanted with 0.25 mg of estradiol pellet through subcutaneous route using a trocar. All the experimental animals were taken care of in accord with the McGill University Animal Care Committee.

Both control and experimental animals were monitored at weekly intervals for the development of tumors for 6 weeks. Tumor volume was determined according to the formula: tumor volume = shorter diameter² × longer diameter / 2. Results were presented as the mean of tumor volumes recorded from both groups. At the end of the study, control and experimental animals were sacrificed; primary tumors were removed and fixed in 10% buffered formalin and Bouin's solution, respectively. Before fixation, tumors were divided into two halves, one half was fixed in formalin and embedded in paraffin for histologic analyses by light microscopy. The other half was snap-frozen in liquid nitrogen to analyze the expression of various genes by RT-PCR.

Immunohistochemical Analyses

Bouin's solution-fixed tumor samples were processed for immunohistochemistry. The fixed tissues were embedded in paraffin. Paraffin-embedded tumor samples were cut into 5-μm-thick sections; for immunohistochemical analysis for uPA, CXCR4, and heparanase, a prometastatic marker was carried out as using the avidin-biotin-peroxidase complex technique. Briefly, the sections were deparaffinized in xylene followed by rehydration through a series of ethanol to water gradients. The sections were treated with 1% normal goat

Table 1. Different Sets of Primer Sequences and Annealing Temperatures Used for RT-PCR Analysis.

Genes	Primer Sequence for RT-PCR	T _m Value (°C)
Tumor suppressor gene		
<i>RASSF1A</i>	5'-ACCTCTGTGGCGACTTCATCT-3' 5'-AGGTGAACCTTGCAATGCGC-3'	52
Involved in metastasis		
<i>uPA</i>	5'-ACATTCACTGGTGCAACTGC-3' 5'-CAAGCGTGTGAGCGCTGTAG-3'	56
<i>SNCG</i>	5'-CAAGAAGGGCTTCTCCATCGCCAAGG-3' 5'-CCTCTTTCTCTTTGGATGCCACACC-3'	60
<i>HEPARANASE</i>	5'-TTGATGCCAAGAAGCAATCAAC-3' 5'-GTAGTGATGCCATGTAACCTGAATC-3'	55
<i>CXCR4</i>	5'-GGAGGGGATCAGTATATACA-3' 5'-GAAGATGATGGAGTAGATGG-3'	55
<i>CXCL12</i>	5'-AGAGCCAACGTCAGCATCT-3' 5'-CGTCTTTGCCCTTTCATCTC-3'	55
Involved in angiogenesis		
<i>VEGF</i>	5'-CCTGGTGGACATCTCCAGGAGTA-3' 5'-CTCACCCGCTCGGCTTGTACACA-3'	58
Involved in cell cycle		
<i>TGF-β</i>	5'-GTACCTGAACCCGTTGTGCT-3' 5'-TACAGCTGCCGACGCAGCA-3'	54
<i>p21^{WAF1/Cip1}</i>	5'-CTCAGAGGAGGCCCATG-3' 5'-GGGCGATTAGGGCTTCC-3'	55
<i>CYCLIN D1</i>	5'-TGGATGCTGGAGTCTGCGAGGAA-3' 5'-GGCTTCGATCTGCTCCTGGCAGGC-3'	60
<i>CYCLIN E1</i>	5'-AGTTCTCGGCTCGCTCCAGGAAGA-3' 5'-TCTTGTGTCGCCATAATCCGGTCA-3'	56
Involved in apoptosis		
<i>BAX</i>	5'-ATGGACGGCTCCGGGGAG-3' 5'-TGGAAAGAAGATGGGCTGA-3'	51
<i>GAPDH</i>	5'-CCCTTCATTGACCTCAACTACATGTT-3' 3'-GAGGGGCCATCCACAGTCTTCTG-5'	56

serum (Vector Laboratories Inc., Burlingame, CA), to block the non-specific binding, for 30 minutes at room temperature before incubation with the primary antibodies: monoclonal antibody against uPA (American Diagnostics Inc., Stamford, CT) and monoclonal antibodies against CXCR4 and heparanase (Santa Cruz Biotechnologies, Santa Cruz, CA); primary anti-uPA, CXCR4, and heparanase antibodies were used at 1:50 dilution overnight at 4°C under the conditions described by manufacturer. Throughout the experiment, after each step, all sections were washed three times, 10 minutes each, with Tris buffer (pH 7.6). Biotinylated goat anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) and HRP donkey anti-goat or goat anti-mouse IgG (Bio-Rad Laboratories Ltd., Ontario, Canada) were used as the secondary antibodies at a dilution of 1:200 for 1 hour at room temperature. The sections for uPA staining were treated with vectastain ABC-AP kit (Vector Laboratories Inc., Burlingame, CA). The immunoreaction was visualized with Fast Red TR/Naphthol AS-MX phosphate (Sigma Aldrich) containing 1 mM levamisole (uPA) and DAB HRP substrate kit (Vector Laboratories Inc., Ontario, Canada) for CXCR4 and heparanase for 5 to 10 minutes. The sections were counterstained with hematoxylin (Fisher Scientific, Ltd., Ontario, Canada) and mounted with Kaiser's glycerol jelly or paramount. For all negative controls included in the study, the primary antibody was substituted with PBS. Ten random fields were selected from the previously coded slides and manually scored as arbitrary units for the expression level of these genes [35].

Statistical Analysis

Results were analyzed as the mean \pm SEM, and comparisons of the experimental data were analyzed by a two-tailed independent-sample Student's *t* test at 0.05 level of significance.

Results

Effect of 5-Aza-CdR on Cell Proliferation, Cell Cycle, and Migration

To verify that 5-aza-CdR exhibits the previously described effects on cell growth *in vitro* of the noninvasive breast cancer cell lines MCF-7 and ZR-75-1, we measured the effects of two doses of 5-aza-CdR on cell viability, cell doubling time, and cell cycle kinetics [36]. The results presented in Figure 1, *A* and *B*, show that 5-aza-CdR is increasing cell doubling time significantly at 1 and 5 μ M doses. A FACS analysis of cell cycle distribution on control and 5-aza-CdR-treated cells (5 μ M for 7 days) reveals a significant increase in the proportion of cells in the G₂ phase, with a concomitant decline in S phase (Figure 1 *C*) in the treatment groups of both cell lines. Our results showed that 5-aza-CdR decreases cell proliferation under the conditions used in our study.

To determine whether 5-aza-CdR treatment affects the expression of uPA, a key protease involved in tumor cell invasion, MCF-7 and ZR-75-1 cells were treated with different doses of the 5-aza-CdR. Results from these studies showed a significant ($P < .05$) dose-dependent increase in the uPA expression at 1.0 and 5.0 μ M concentrations of 5-aza-CdR (Figure 1 *D*). At these doses, more than 95% of tumor cells were viable. This change in the expression of uPA had a dose-dependent increase in tumor cells' invasive capacity as determined by the Boyden chamber Matrigel invasion assay (Figure 1 *D*). The effect of 5-aza-CdR on cell migration was analyzed using MCF-7 cells, rather than ZR-75-1 cells that grow in patches. A significant ($P < .05$)

increase in wound healing (%) was observed in 5-aza-CdR-treated (5 μ M) MCF-7 cells compared with control cells at 32 and 48 hours after wounding (Figure 1, *E* and *F*). Healing at the lower dose of 5-aza-CdR (1 μ M) was also found to be significant ($P < .05$) compared with control MCF-7 cells. For both, control and 5-aza-CdR-treated groups, the rate of wound closure was highest at 24 and 32 hours after wounding. Migration assay on ZR-75-1 cells was not feasible, because these cells grow in patches and are unable to form a uniform monolayer.

Effect of 5-Aza-CdR on the Genes Involved in Invasion and Metastasis

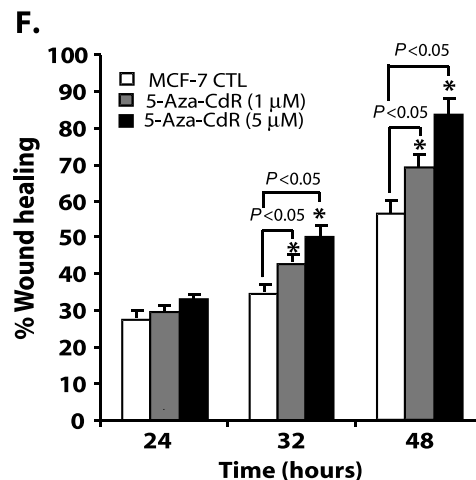
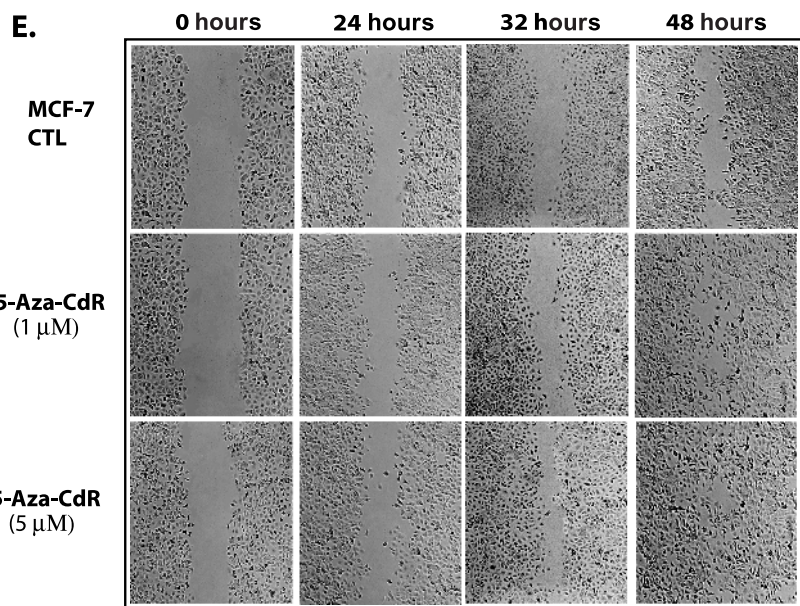
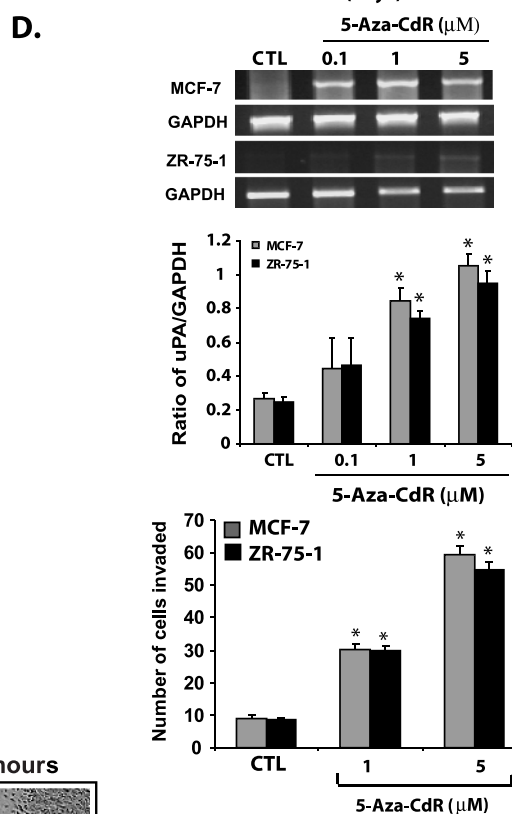
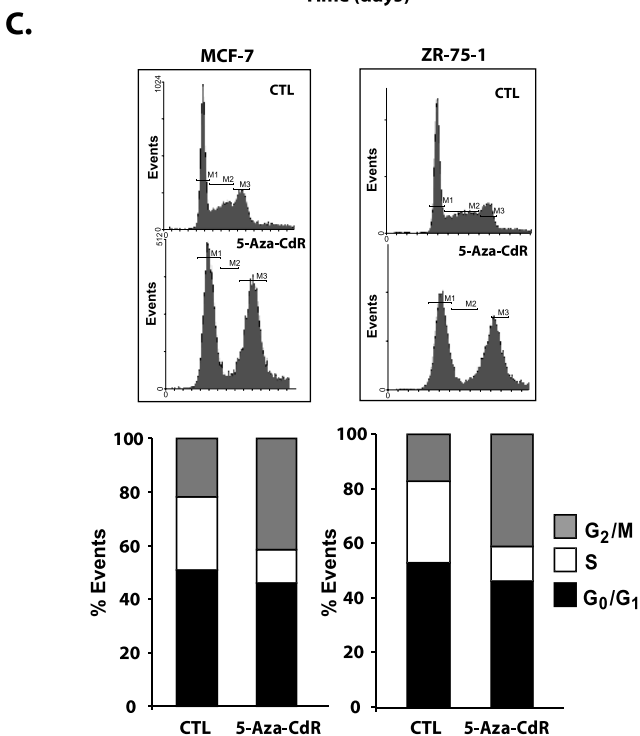
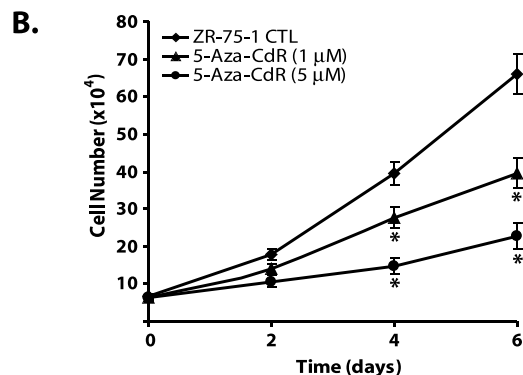
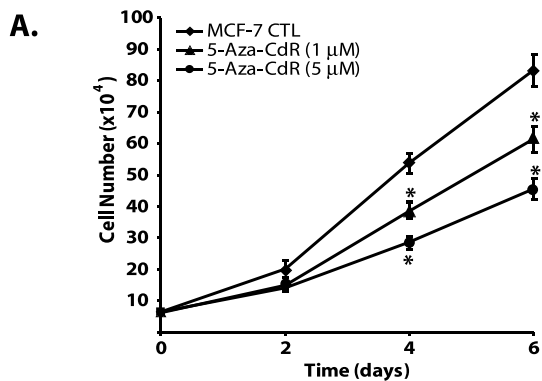
What is the mechanism by which 5-aza-CdR stimulates cell invasion and migration? We tested the hypothesis that 5-aza-CdR induces a battery of genes, which were previously reported to be involved in cell invasion and metastasis, including *uPA*, *SNCG*, *HEPARANASE*, *CXCR4*, and *CXCL12*. MCF-7 and ZR-75-1 cells were treated with 5 μ M 5-aza-CdR for a duration of 7 days. At the end of the experiment, total cellular RNA was isolated and analyzed for the expression of mRNA levels of these genes as well as the tumor suppressor gene, *RASSF1A*, which is hypermethylated in 42% to 65% of primary breast tumors, including these cell lines [26]. We found that, as reported, 5-aza-CdR treatment upregulated the expression of the tumor suppressor gene, *RASSF1A*, in both cell lines. However, in addition to the tumor suppressor gene, a number of genes demonstrated to play a causal role in metastasis were induced as well, namely *uPA*, *SNCG*, *HEPARANASE*, the chemokine receptor *CXCR4*, the cytokine *TGF- β* , and the angiogenesis factor *VEGF* (Figure 2). There is no significant difference in *CXCL12* or *CYCLIN D1* and *CYCLIN E1* mRNA expression in control and 5-aza-CdR-treated groups of either cell lines. Thus, our RT-PCR data confirm that 5-aza-CdR is responsible for the activation of quiescent genes or upregulation of a panel of prometastatic genes that are normally suppressed in these noninvasive cell lines concurrently with its activation of tumor suppressor genes.

Effect of 5-Aza-CdR on the Methylation Status of the uPA, CXCR4, and SNCG Gene Promoters

To test the hypothesis that the increase in mRNA expression of these genes is due to concurrent demethylation in the promoter and exon 1 region of these genes, we subjected genomic DNA from treated and untreated cells to sodium bisulfite mapping of the indicated regulatory regions of uPA, SNCG, and CXCR4 promoters (Figure 3 *A*). Demethylation of specific CpG sites after 5-aza-CdR treatment was noted for these three genes. The uPA promoter was highly methylated (81.8%) in the MCF-7 control; significant demethylation occurs at an SP1-binding site and four CpG sites located between TATA box and exon 1 as well as extensive demethylation of exon 1. CXCR4 regulatory region (-260 to +150) was almost totally demethylated by 5-aza-CdR (Figure 3 *B*). The CpG site numbers 2 to 14 and 16 on the SNCG promoter region are more prone to demethylation after 5-aza-CdR treatment, consistent with the possibility that demethylation of these sites may play an important role in the induction of this gene (Figure 3 *C*).

Effect of 5-Aza-CdR on In Vivo Tumor Growth

Following the *in vitro* data indicating that 5-aza-CdR induces a battery of genes involved in metastasis as well as tumor suppressor genes, we tested whether 5-aza-CdR-treated cells will exhibit this dual response; that is, reduced tumor growth and increased prometastasis



gene expression *in vivo*. 5-Aza-CdR-treated (5 μ M for 7 days *in vitro*) MCF-7 cells and control cells were inoculated in the mammary fat pad of female Balb C *nu/nu* mice in the presence of an estrogen pellet. As depicted in Figure 4 A, 5-aza-CdR-treated MCF-7 cells showed a reduced rate of increase in tumor volume compared with the control group of animals inoculated with MCF-7 cells treated with vehicle alone. Because 5-aza-CdR treatment was transient and the animals were not further treated with 5-aza-CdR after inoculation, we confirmed by RT-PCR that the genes (*uPA*, *HEPARANASE*, *CXCR4*, *SNCG*, and *VEGF*) induced by 5-aza-CdR *in vitro* were still induced in these tumors (Figure 4 B).

We then measured the levels of expression of uPA, CXCR4, and HEPARANASE, which are key players in tumor invasion and metastases, by immunohistochemistry in control tumors and tumors from animals inoculated with cells treated with 5-aza-CdR. We found that all tumors from the treatment group showed overexpression of uPA, CXCR4, and HEPARANASE both in terms of intensity and density compared with tumors from the control animals (Figure 5). Our data showed that genes induced by 5-aza-CdR *in vitro* are not changed after inoculating these cells in the *in vivo* model, both at the mRNA and protein levels.

Discussion

Disruption in the DNA methylation machinery resulting in global hypomethylation [37] and regional hypermethylation [1] is well documented in most cancers. However, most of the focus of attention in the field has been on hypermethylation and silencing of tumor suppressor genes, leading to a number of clinical trials with the DNA methylation inhibitor 5-aza-CdR [7,38]. This approach is effective in inhibiting tumor growth, and it has shown promising results in the treatment of myelodysplastic syndromes [38,39]. However, this approach overlooks the other side of the methylation tale in cancer: global hypomethylation. If global hypomethylation plays a causal role in the cancer, then agents which globally inhibit DNA methylation, such as 5-aza-CdR, might have undesirable effects after their use, which can potentially affect cancer progression [24]. An important requirement in developing effective cancer therapeutics is to block not only primary tumor growth but also tumor metastases to distant organs, which is the major cause of cancer-associated morbidity and mortality [40]. We have previously shown that DNA

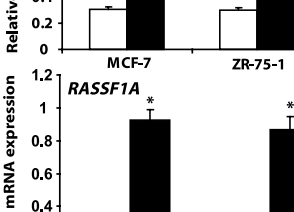
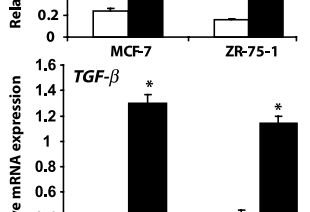
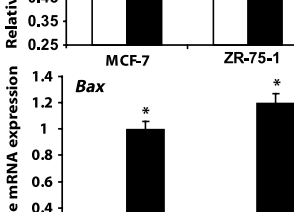
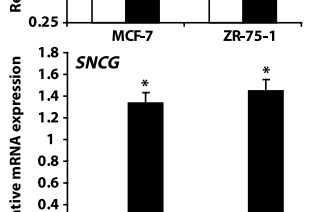
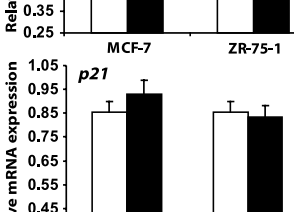
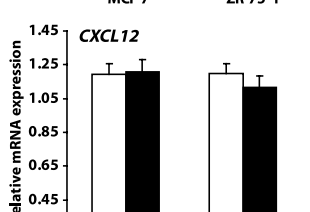
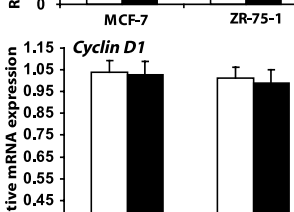
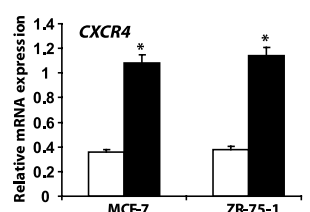
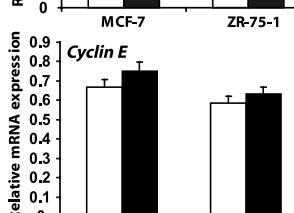
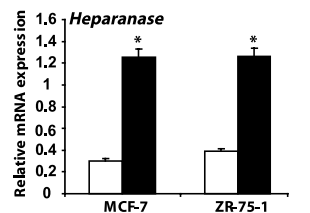
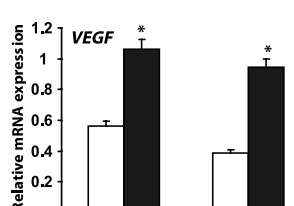
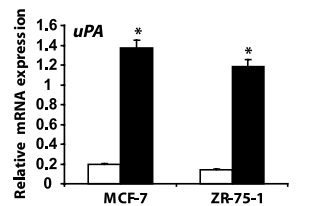
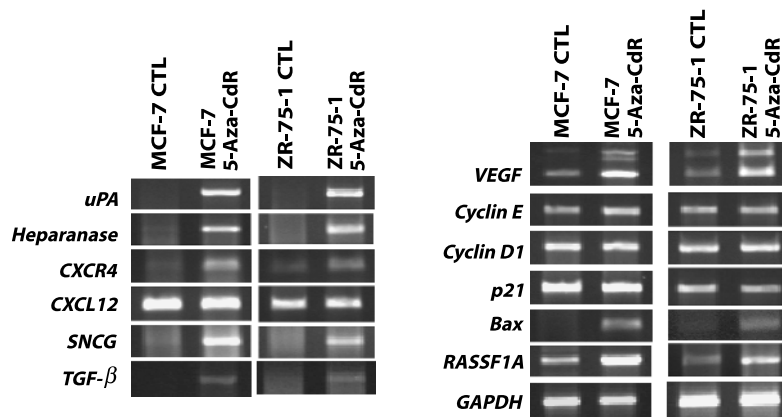
methylation plays divergent roles in growth and metastasis in breast cancer cells [25]. Recent data implicating hypomethylation in the activation of certain prometastatic genes, such as *uPA* [22,23,25], in invasive breast cancer prompted us to test the hypothesis that inhibitors of DNA methylation would promote the transition of non-metastatic breast cancer cells into metastatic cancer by inducing a panel of genes required for metastasis.

Our study tested the hypothesis that hypomethylation coregulates a panel of genes involved in metastasis by a common activation mechanism rather than a specific gene. If this hypothesis is true, it has obvious important therapeutic implications. First, it draws attention to the potential danger in using demethylating agents in anticancer therapy because of their potential effects on activating a large number of quiescent genes including genes involved in promoting tumor metastases. Nevertheless, it puts forward the opportunity of an opposite approach, namely, inhibition of hypomethylation by hypermethylation therapy [23]. Such therapy might provide a unique opportunity for knocking out a whole panel of genes involved in metastasis, thus increasing the chances for successfully blocking metastasis in contrast to therapies, which target one protein at a time. Redundancy in activity of these proteins might require a therapeutic approach that coordinately targets a panel of genes involved in tumor progression.

Our data support this hypothesis. First, we show that 5-aza-CdR concurrently activates the tumor suppressor gene, *RASSF1A*, and a panel of prometastatic genes (Figure 2). We also show that the mechanism of action of 5-aza-CdR on three selected genes, namely, *uPA*, *CXCR4*, and *SNCG*, involves demethylation of their regulatory regions. These genes, *uPA*, *CXCR4*, and *SNCG*, were previously shown to be regulated by hypomethylation in other systems; however, here we show their simultaneous activation in the same noninvasive breast cancer cells with 5-aza-CdR [22,23,29,30,41,42].

Recently, a study has shown that overexpression of the metastatic gene, *HEPARANASE*, in MDA-MB-435 human breast carcinoma and rat C6 glioma cells resulted in a three- to six-fold increase in *VEGF* mRNA levels, which is mediated by the activation of Src family members [43]. In the present study, we also found the upregulation of *HEPARANASE* accompanied by the overexpression of *VEGF* mRNA level in 5-aza-CdR-treated MCF-7 cells. However, it is unclear whether this increase in VEGF expression is due to epigenetic regulation or is an outcome of *HEPARANASE* expression. It has been

Figure 1. Effect of 5-aza-CdR treatment on the proliferation, invasion, and migration of noninvasive cell lines. Noninvasive breast cancer cells, MCF-7 and ZR-75-1, were seeded at the same density and subjected to two different doses of 5-aza-CdR or with vehicle control alone (CTL) in a six-well plate, and growth curve analysis was carried out to determine the effect of 5-aza-CdR on the proliferation rate. At each time point, cells were trypsinized and counted as described in the Materials and Methods section (A and B). All data presented are expressed as mean \pm SEM of quadruplicate wells for each time point. Similar results were obtained in two independent experiments. Cell cycle distribution analysis was carried out by FACS for control and 5-aza-CdR-treated MCF-7 and ZR-75-1 cells (5 μ M for 7 days) after staining the cells with propidium iodide as described in the Materials and Methods section. Data were analyzed by WinMDI version 2.8 and plotted (C). Data are presented as mean of % events from three independent experiments by 100% stacked column graph for control and experimental groups. Expression of uPA, a protease involved in invasion, was analyzed by RT-PCR in MCF-7 and ZR-75-1 cells treated with different doses of 5-aza-CdR (D). Data were presented for control and experimental groups as mean \pm SEM values from two independent experiments. The invasive capacity of MCF-7 and ZR-75-1 cells treated with different doses of 5-aza-CdR was determined using a Boyden chamber Matrigel invasion assay. The bar diagram represents the mean \pm SEM as described in the Materials and Methods section. Wound healing migration assay was carried out on MCF-7 cells in the absence and presence of 5-aza-CdR (1 and 5 μ M) in the culture medium with 2% fetal bovine serum as depicted in panels E and F. Cells were seeded at the same density and grown as monolayer and wounded as discussed in the Materials and Methods section. % Wound healing was recorded at different time points, and percentage of wound healing with respect to T_0 was calculated using the described equation. Similar results were obtained from two different experiments. All data were presented as mean \pm SEM of duplicate values from control and experimental groups from two independent experiments. Significant difference from the control is represented by an asterisk (* $P < .05$).



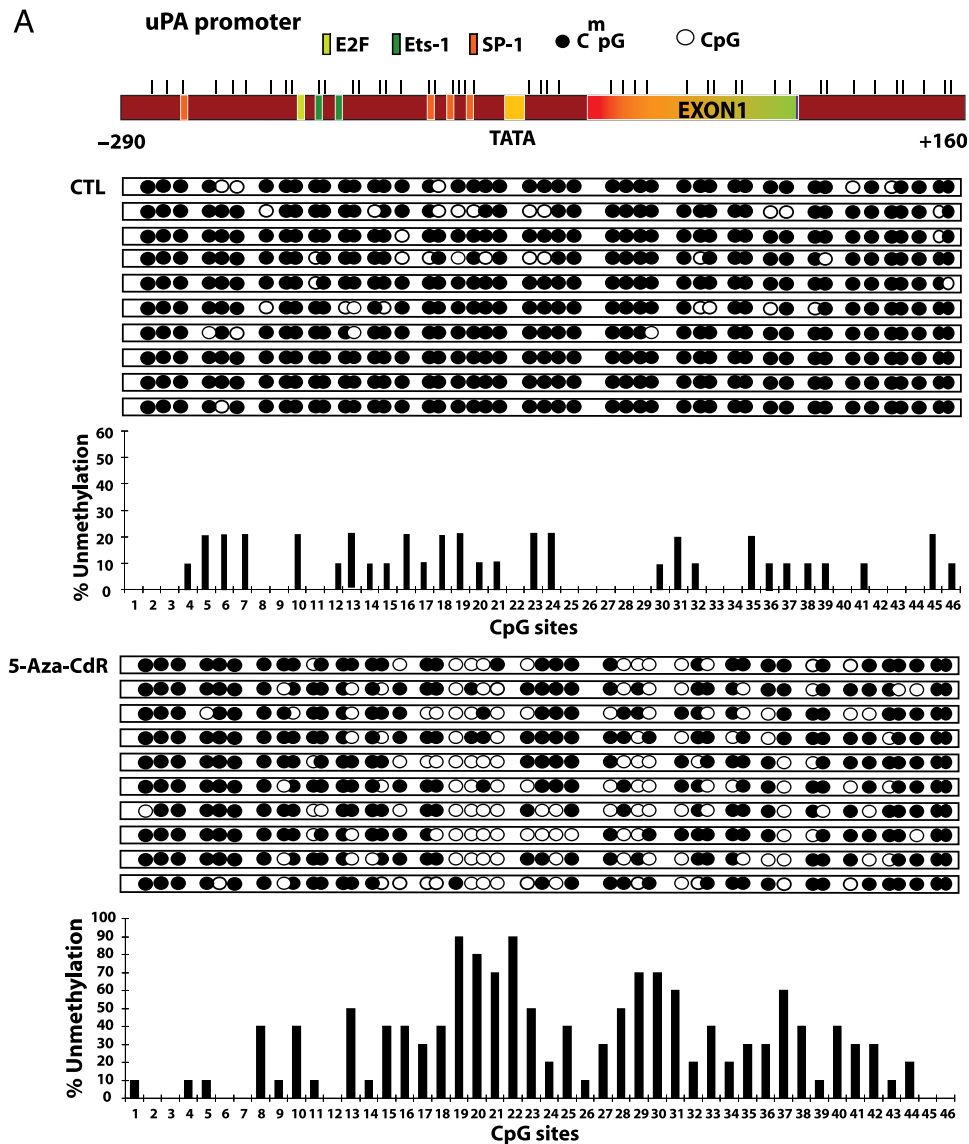


Figure 3. Treatment with 5-aza-CdR causes demethylation of the promoter region of the *uPA*, *CXCR4*, and *SNCG* genes. MCF-7 cells were subjected to 5-aza-CdR treatment (5 μ M) for 7 days, at which point genomic DNA was isolated with DNazol and subjected to bisulfite treatment as described in the Materials and Methods section. Genomic bisulfite-treated DNA was used for first PCR and nested PCR reactions to amplify *uPA*, *CXCR4*, and *SNCG* genes' 5' regulatory regions and the first exon, followed by cloning in pGEMT vector. Ten clones for each gene were picked from control and treatment groups. A physical map of the *uPA* (A), *CXCR4* (B), and *SNCG* (C) promoters is presented. Demethylated CpG site (empty circle) and methylated CpG site (filled circle). Beneath the clones is the bar diagram showing percent unmethylation for each CpG site present within the amplified region.

demonstrated that exogenous expression of *SNCG* in breast cancer cells (MDA-MB-435) led to a significant increase in cell motility and invasiveness in cell culture and to a profound augmentation of metastasis in nude mice [17]. The other genes of interest were *CXCR4* and *CXCL12*; *CXCR4* is a G_i protein-coupled receptor for the ligand

CXCL12 and plays an important role in breast cancer metastasis. Chemokine receptors, such as *CXCR4* and *CXCR7*, play an important role in cell migration and metastasis [42]. Recently, it has been shown that elevated secretion of *CCL2* by bone marrow endothelial cells recruits prostate cancer epithelial cells to the bone microenvironment

Figure 2. Treatment of MCF-7 and ZR-75-1 cells with 5-aza-CdR activates tumor suppressor and prometastatic genes. MCF-7 and ZR-75-1 cells were treated with 5 μ M 5-aza-CdR for 7 days, and at the end of the experiment, total cellular RNA was isolated with TRIzol. RNA from control and treatment groups were analyzed for the expression of genes involved in invasion, metastasis, cell cycle regulation, angiogenesis, and apoptosis by RT-PCR. The expression of tumor suppressor gene, *RASSF1A*, was also analyzed. Changes in the mRNA expression of the respective genes were determined by plotting the relative ratio against GAPDH of at least two different experiments, where white bars represent controls and solid black bars represent 5-aza-CdR-treated groups. Significant difference from the control is represented by an asterisk (* $P < .05$).

[44]. In parallel, Kollmer et al. [45] has also shown that the chemokine *CXCL12* promotes solid metastatic tumor growth by pro-proliferative and antiapoptotic actions in an angiogenesis-dependent manner.

Another ubiquitous cytokine *TGF-β* that is found to be upregulated in our findings is involved in bone metastasis and inhibits growth of normal epithelia and early-stage tumors but stimulates invasion and metastasis of aggressive tumors [46]. Although in some cases, the loss of growth inhibition by *TGF-β* can arise because of mutational inactivation of the *TGF-β* receptors and SMAD signaling molecule [47] or might be due to hypermethylation. The effect of *TGF-β* on tumor growth is biphasic: carcinogenesis and early tumor growth are suppressed by *TGF-β*, whereas this growth factor apparently accelerates tumor progression in more advanced aggressive tumors. Thus, it presents another example of divergent effects on growth and metastasis in breast cancer progression. Most importantly, the high level of *uPA* in serum and extracts from the primary tumors are associated with poor prognosis and overall survival. In fact, *uPA* has been proven to be an important marker of the meta-

static potential in human cancers [23,27,48,49]. *uPA* is required to generate the active zymogen plasmin necessary for the degradation of extracellular matrix and to promote metastasis, and there are reports where tumor metastasis was selectively reduced up to more than seven-fold in *uPA*-deficient mice [50].

The *uPA* promoter region we have evaluated has many transcription factor-binding sites, namely, ETS-1, AP-2, and SP-1, which may play important roles in the regulation of *uPA* expression. In previous studies, using mobility shift assay, we have shown that the transcription factor Ets-1 is involved in inducing *uPA* gene expression. This transcription factor-binding site coincides with methylation sites and is a candidate site that can be affected by DNA methylation [21]. In addition, *uPA* gene activation by 5-aza-CdR can also occur indirectly by additional transcription factors or by *TGF-β* which is known to regulate the production of *uPA* through the stabilization of *uPA* mRNA and through the SMAD-4 signaling pathway [51]. Furthermore, integrin-linked kinase, which is critical for *TGF-β1*-stimulated tumor cell-invasiveness, might also serve as

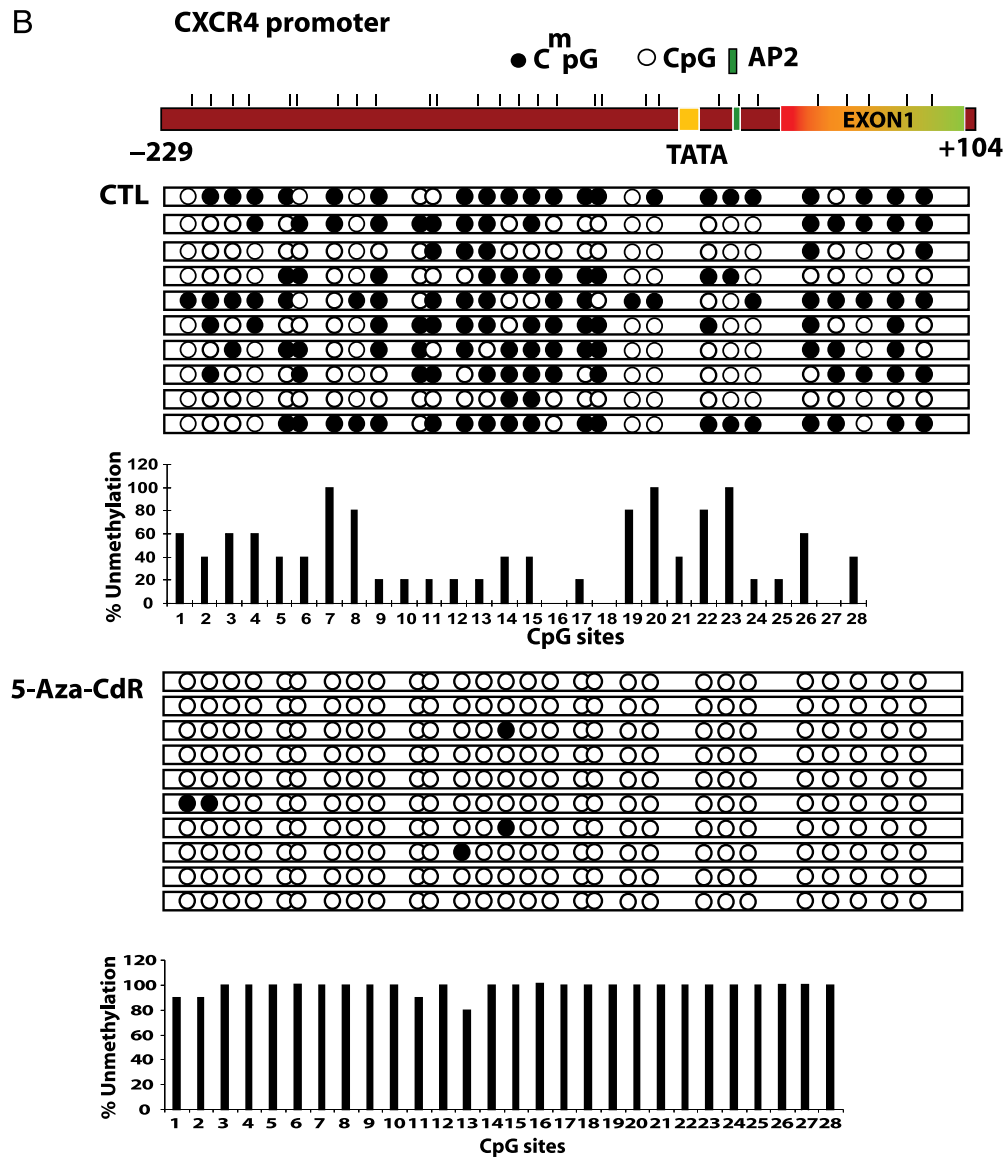


Figure 3. (continued)

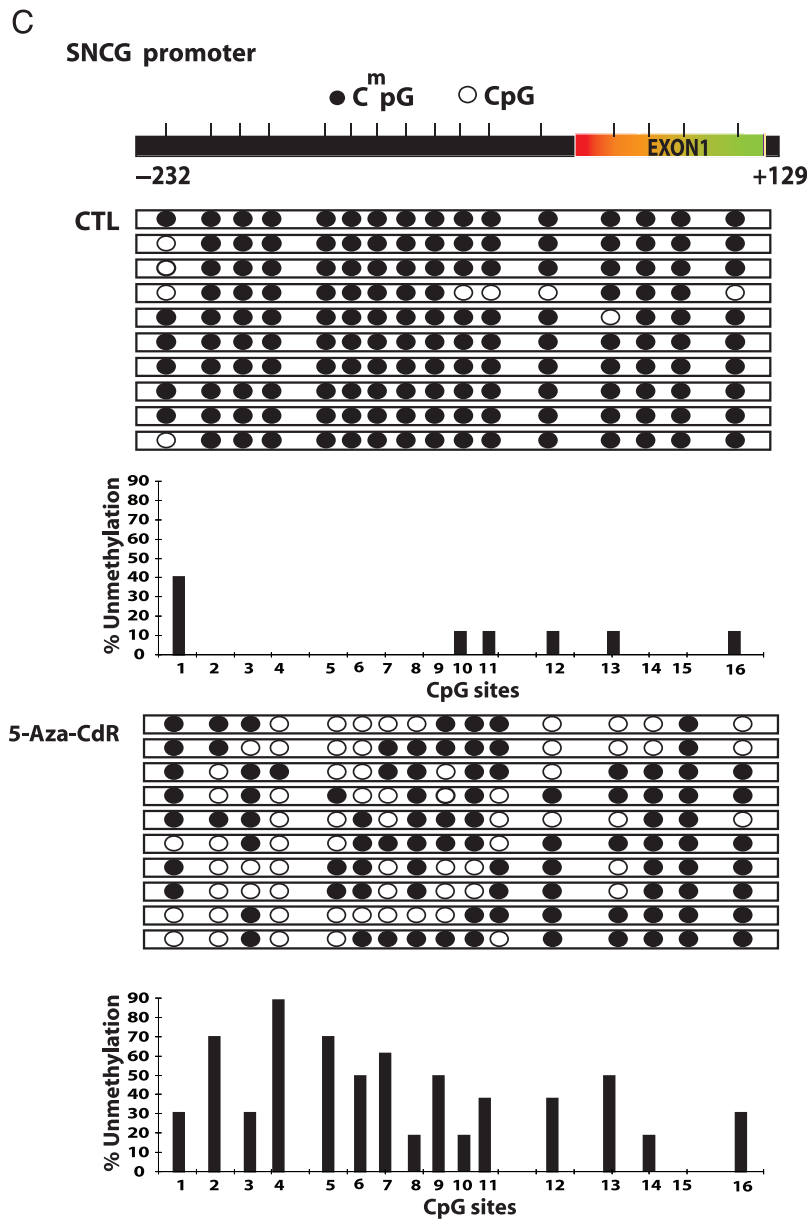


Figure 3. (continued)

a key signaling mediator of *TGF-β* regulation of the uPA/plasminogen activator inhibitor-1 system.

The main question with relevance to the clinical situation was whether 5-aza-CdR-treated breast cancer cells would metastasize *in vivo*. We opted to treat the cells *in vitro* and then inoculate them *in vivo* rather than treat systemically with 5-aza-CdR to measure the direct effect of the DNA-demethylating agent on the cancer cell itself without the confounding factors of the systemic effects of 5-aza-CdR and its potential effects on the stroma. In addition, this paradigm allows us to evaluate the long-term effects of transient 5-aza-CdR treatment. Because the action of 5-aza-CdR results in a stable change in the state of modification of the DNA molecule *per se*, we predicted that the early effects of 5-aza-CdR treatment would linger for the entire period of time of tumor growth *in vivo* well after the 5-aza-CdR was washed away. Our data show that while 5-aza-CdR decreases tumor growth *in vivo*, it also upregulates the expression of many prometastatic genes in the noninvasive breast cancer cells

in vitro and in the primary tumors *in vivo*, which may result in metastases at the distant organ sites of these animals over time. The effects of 5-aza-CdR last long after the 5-aza-CdR is withdrawn, supporting the hypothesis that the effects of 5-aza-CdR are mediated through reprogramming of gene expression, which is consistent with a mechanism involving the change in DNA methylation rather than in an immediate nonspecific effect of the drug which is independent of DNA methylation. Because of the nonmetastatic behavior of MCF-7 cells, we were unable to conclusively demonstrate the ability of 5-aza-CdR to promote tumor metastases in this short-term *in vivo* model of breast cancer.

Altering the DNA methylation machinery is a potentially powerful approach to cancer therapy [52]. However, it is crucial to gain full understanding of the mechanisms involved in these two processes, namely, hypermethylation and demethylation, to properly use these drugs targeting the DNA methylation machinery in anticancer therapy. Because metastases is one of the most serious facets of cancer,

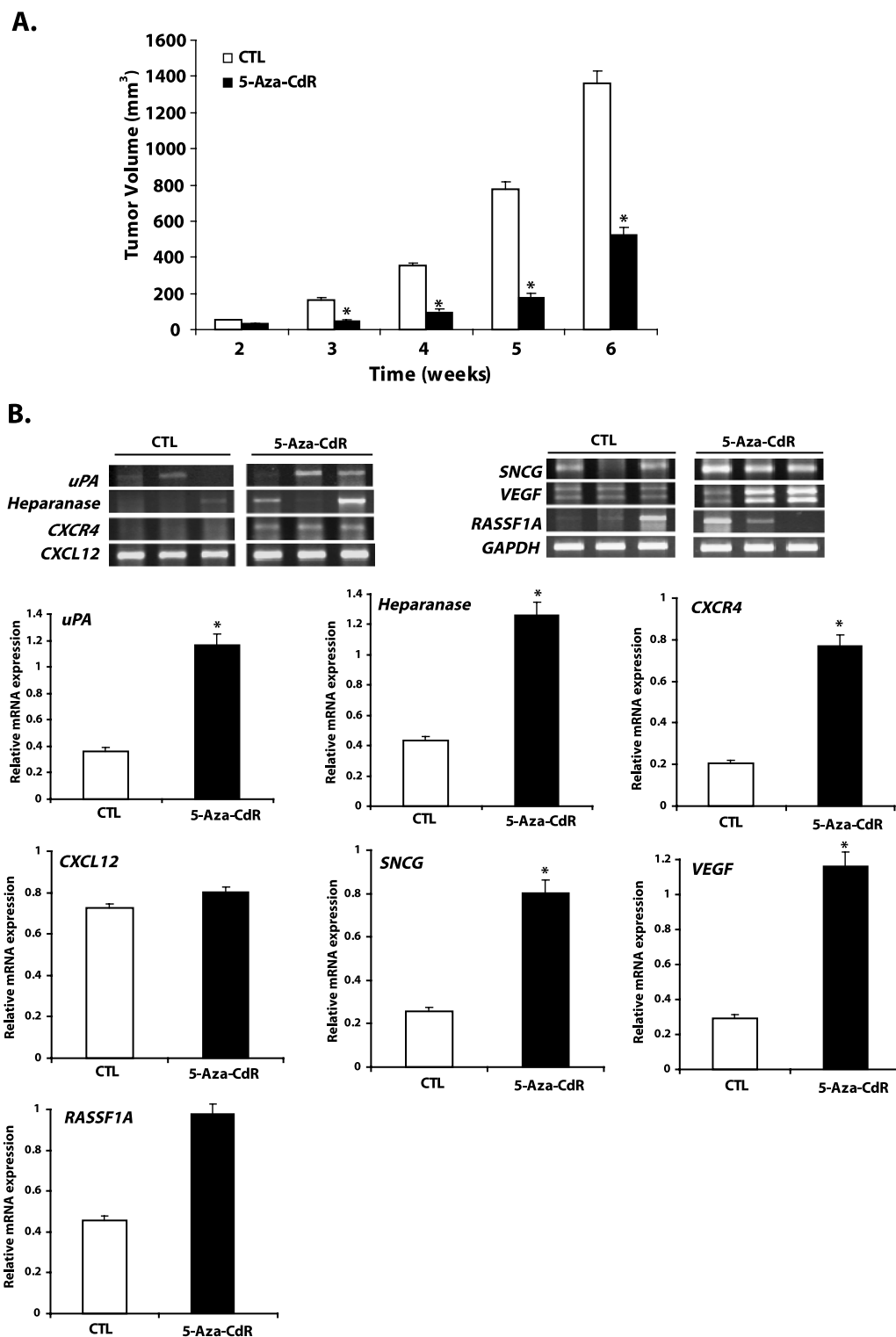


Figure 4. 5-Aza-CdR suppresses tumor growth and upregulates the expression of prometastatic, angiogenetic, and tumor suppressor genes in primary tumor. Female Balb C *nu/nu* mice were inoculated with either control MCF-7 cells or MCF-7 cells treated with 5 μ M 5-aza-CdR for 7 days in the mammary fat pad region in the presence of 0.25 mg of estradiol pellet for a 60-day release. Tumors were measured weekly, and tumor volume was determined as described in the Materials and Methods section. Result represents the mean \pm SEM of eight animals in each group. Significant difference from the control is represented by an asterisk (* $P < .05$) (A). At the termination of the *in vivo* experiment (week 6), primary tumors were excised and snap-frozen. Total cellular RNA was isolated from these tumors using TRIzol and was analyzed by RT-PCR for the expression of genes responsible for invasion and metastases (*uPA*, *HEPARANASE*, *CXCR4*, *CXCL12*, and *SNCG*) and angiogenesis (*VEGF*) and for the tumor suppressor gene (*RASSF1A*) as shown (representative three tumor samples from each group) in panel B. The bar diagram represents the mean \pm SEM of tumors from three animals in each group (white bars represent mean \pm SEM of controls and solid black bars represent mean \pm SEM of the 5-aza-CdR-treated group). Significant difference from the control is represented by an asterisk (* $P < .05$).

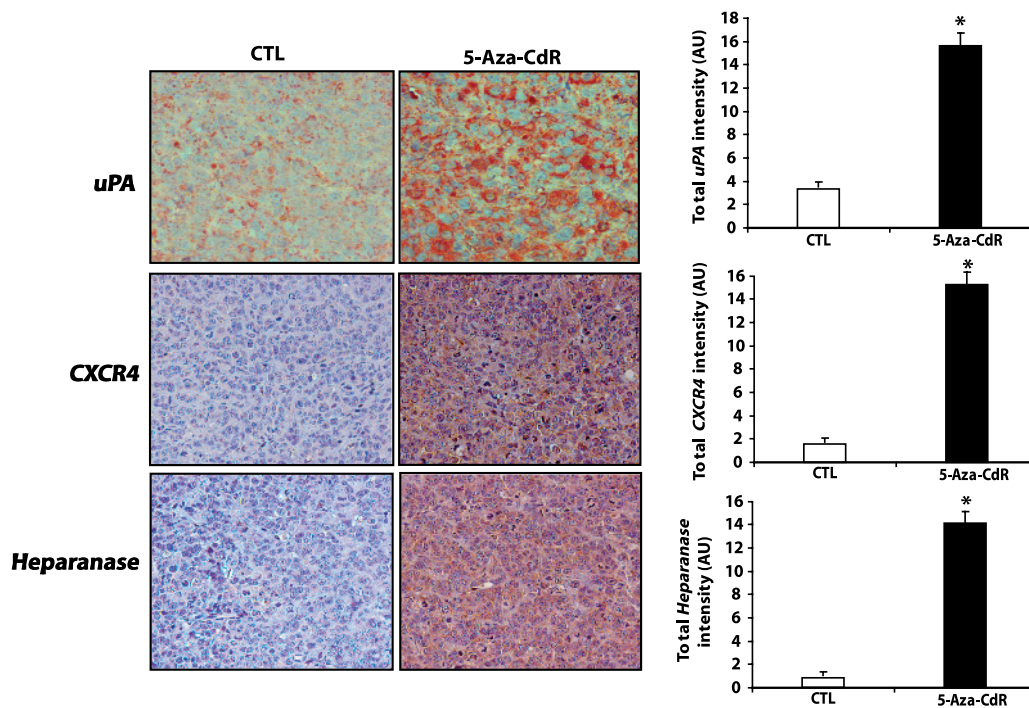


Figure 5. 5-Aza-CdR alters the expression of invasive and prometastatic markers in the primary tumor tissues. At the termination of the *in vivo* experiment, primary tumors were excised and processed for immunohistochemical analyses as described in the Materials and Methods section to determine the levels of production of *uPA*, *CXCR4*, and *HEPARANASE*. A representative microphotograph for each group is shown; original magnification, $\times 200$. The intensity and density of positive staining was quantified as described in the Materials and Methods section. Ten random fields of observation were recorded in each group and were represented graphically as arbitrary units. Results represent the mean \pm SEM of three animals from each group. Significant difference from the control is represented by an asterisk ($*P < .05$).

and it plays a critical role in mortality from cancer, high vigilance is warranted when using demethylating agents in cancer therapy. Our challenge is to design agents that target the growth-promoting effect of DNA methyltransferases but are devoid of the hypomethylation action of critical prometastatic genes.

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