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Three Epigenetic Drugs Up-Regulate Homeobox Gene *Rhox5* in Cancer Cells through Overlapping and Distinct Molecular Mechanisms[S]

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ABSTRACT

Epigenetic therapy of cancer using inhibitors of DNA methyltransferases (DNMT) or/and histone deacetylases (HDACs) has shown promising results in preclinical models and is being investigated in clinical trials. Homeodomain proteins play important roles in normal development and carcinogenesis. In this study, we demonstrated for the first time that an epigenetic drug could up-regulate homeobox genes in the *r*eproductive *h*omeobox genes on chromosome *X* (*Rhox*) family, including murine *Rhox5*, *Rhox6*, and *Rhox9* and human *RhoxF1* and *RhoxF2* in breast, colon, and other types of cancer cells. We examined the molecular mechanisms underlining selective induction of *Rhox5* in cancer cells by three epigenetic drugs: 5-aza-2′-deoxycytidine (DAC; decitabine), arsenic trioxide (ATO), and MS-275 [entinostat; *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide]. DAC induced Rhox5 mRNA expression from both distal promoter (Pd) and proximal promoter, whereas MS-275 and ATO induced gene expression from the Pd only. DAC and ATO inhibited both DNMT1 and DNMT3B protein expression, whereas MS-275 significantly reduced DNMT3B protein. In contrast to DAC, neither MS-275 nor ATO induced DNA demethylation on the Pd region. All three drugs led to enhanced acetylation of histones H3 and H4 at the promoter region. The occupancy of the activating histone mark dimethylated lysine 4 of H3 at Pd was enhanced by DAC and MS-275 but not ATO. Because they modulate gene expression with different potencies through shared and distinct epigenetic mechanisms, these epigenetic drugs may possess great potential in different applications for epigenetic therapy of cancer and other diseases.

Increasing evidence supports the hypothesis that cancer development depends not only on genetic alterations but also on aberrant epigenetic changes that convey heritable gene expression patterns critical for neoplastic initiation and progression (Jones and Baylin, 2007). The use of identified epigenetic changes in cancer as targets for cancer therapy has gained significant progress in the past few years (Yoo and Jones, 2006). To better understand epigenetic regulation in cancer cells and to explore the potential use of epigenetic drugs in the therapy of cancer, it is important to determine which genes are regulated and which molecular mechanisms are responsible for gene induction by epigenetic drugs.

Both DNA methylation and histone modifications are major epigenetic events for gene activity (Jenuwein and Allis, 2001; Berger, 2007; Jones and Baylin, 2007). Epigenetic drugs are classified into two major categories: DNMT inhibitors and HDAC inhibitors. DAC has been approved by the Food and Drug Administration (Silver Spring, MD) to treat myelodysplastic syndrome. DAC is a potent inhibitor for DNMTs and can also cause DNA damage, which may induce gene expression (Palii et al., 2008). In addition, DAC has been shown to have effects on histone H3-lysine 9 methylation (Nguyen et al., 2002). HDAC inhibitors mainly affect histone acetylation to regulate gene expression. One commonly used HDAC inhibitor is trichostatin A (TSA). DAC and TSA often have synergistic effects in gene regulation. Given

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ABBREVIATIONS: DNMT, DNA methyltransferase; HDAC, histone deacetylase; DAC, decitabine, 5-aza-2′-deoxycytidine; TSA, trichostatin A; MS-275, entinostat, *N*-(2-aminophenyl)-4-[*N*-(pyridine-3-ylmethoxy-carbonyl)aminomethyl]benzamide; ATO, arsenic trioxide; *Rhox*, reproductive homeobox genes on chromosome X; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; Pd, distal promoter(s); Pp, proximal promoter(s); CpG, cytosine and guanine separated by a phosphate; AcH3, acetylated histone 3; AcH4, acetylated histone 4; H3K4Me2, dimethylated lysine 4 of H3; LBH589, panobinostat.

the relatively high toxicity of both DAC and TSA along with the unstable nature of DAC and the nonspecific demethylation associated with its use, clinical application of these two epigenetic modulators is limited (Yoo et al., 2007). To develop effective epigenetic therapy for cancer and other diseases, many investigators have sought to develop more selective epigenetic modulators to decrease toxicity. MS-275 (entinostat), a class I HDAC-selective inhibitor (Saito et al., 1999; Khan et al., 2008), is a promising epigenetic drug with low toxicity and long half-life, and it has been used in clinical trials for cancer patients (Gojo et al., 2007). Another strategy is to screen for epigenetic modulators from commonly used chemotherapeutic drugs. Arsenic trioxide (ATO) has been approved to treat acute promyelocytic leukemia by the Food and Drug Administration. It has also been identified as an inhibitor for DNMT to reactivate DNA methylation-silenced genes in cancer cells (Cui et al., 2006). In addition, ATO can promote histone H3 acetylation at caspase-10 gene promoter (Li et al., 2002).

Homeodomain transcription factors play important roles in normal development, and emerging evidence suggests that some homeodomain proteins play crucial roles in cancer initiation and metastasis (Christensen et al., 2008). Reproductive homeobox genes on the X chromosome (*Rhox*) is a recently identified homeobox family, and more than 30 functional *Rhox* genes have been identified in the mouse (Maclean et al., 2005; Wang and Zhang, 2006). The founding member of the *Rhox* gene cluster in the mouse, *Rhox5*, is an androgen-dependent gene expressed in Sertoli cells that promotes the survival and differentiation of the adjacent male germ cells (Maclean et al., 2005). It has recently been shown that RHOX5 mediates transcriptional repression of the netrin-1 receptor gene *Unc5c* (Hu et al., 2008), the latter of which functions as a tumor suppressor gene in colon cancer (Bernet et al., 2007). It is interesting that DNA methylation plays important roles in lineage-specific gene silencing of *Rhox* family members in mouse embryogenesis and in embryonic stem cells (Oda et al., 2006). Among *Rhox* genes, *Rhox5* (formerly named *Pem*) has been demonstrated to be expressed in approximately 50 to 65% of cancer cells; it has been identified as a cancer germline antigen (Wilkinson et al., 1990; Ono et al., 2000). In the human genome, three X-linked homeobox genes, *RhoxF1*, *RhoxF2*, and *RhoxF2B*, belong to *Rhox* gene family (Geserick et al., 2002; Wayne et al., 2002; Wang and Zhang, 2006). *RhoxF1* and *RhoxF2* are expressed in fractions of human cancer cell lines, as well as in restricted normal tissues such as testis (Wayne et al., 2002).

We and our collaborators have previously investigated the epigenetic regulation of genes by DNMT and HDAC inhibitors (Yu et al., 2002; Kaneko et al., 2004; Guo et al., 2006). We established the first tumor model in which an epigenetic drug (DAC) was used for de novo induction of a cancer germline antigen (P1A) in addition to demonstrating successful immunotherapy of cancer by targeting the newly induced antigen P1A (Guo et al., 2006). In the current study, we have shown, for the first time to our knowledge, that epigenetic drugs could selectively induce several homeobox genes of the *Rhox* family in both murine and human cancer cells. Using *Rhox5* gene as a model, we have examined DNMT protein reduction, DNA methylation status, and occupancy of histone acetylation/methylation marks at the *Rhox5* gene promoter in cancer cells treated with three separate epigenetic drugs. We found that these three drugs affected multiple overlapping and distinct epigenetic marks to induce *Rhox5* gene expression in cancer cells.

Materials and Methods

Cancer Cell Lines and Mouse Normal Cells. Cancer cell lines of both human and mouse origins were originally purchased from the American Type Culture Collection (Manassas, VA), unless indicated otherwise. Most murine cancer cell lines have been described in our previous study (Guo et al., 2006). Mouse mammary cancer line EMT6 was a gift from Yale Cancer Center (New Haven, CT). Normal mouse mammary epithelial cell line MM3MG was obtained from American Type Culture Collection. All cells were cultured in recommended culture media supplemented with 5 or 10% fetal bovine serum and antibiotics.

Drug Treatment. DAC, MS-275, and ATO were purchased from Sigma-Aldrich (St. Louis, MO). Cancer or normal cells were plated in 100-mm dishes to obtain ~20% confluence. After overnight incubation, cells were treated daily with one of the three compounds at indicated doses for ~48 to 120 h (DAC for 48 h, MS-275 for 72 h, and ATO for 120 h).

RNA Isolation, RT-PCR, and Real-Time RT-PCR. Total RNA from normal and cancer cells was extracted by using RNeasy mini kit (QIAGEN, Valencia, CA) and used for RT-PCR. Mouse and human testis polyA+ RNA was ordered form Clontech (Mountain View, CA) and used as positive control for *Rhox* gene mRNA expression. First-strand cDNA was synthesized by using ImProm-II reverse transcription system, with an oligo(dT) primer (Promega, Madison, WI). PCR was performed for *Rhox* genes by using primer pairs listed in Table 1. For semiquantitative RT-PCR, ~30 to 35 cycles of amplification in total were performed. β-Actin or GAPDH was amplified as an internal control. Amplified DNA products were subjected to electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining.

Quantitative real-time RT-PCR was performed with an ABI StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). TaqMan MGB probes (5-carboxyfluorescein dye-labeled) and primers for *Rhox5* (Assay ID: Mm00476718_m1) and internal

TABLE 1
Sequences of the primers used in PCR assays

Gene Name	Primer Sequence
mRNA expression	
Rhox5-F	AATGGAAATCCTGGGGGTAG
Rhox5-R	AAATCTCGGTGTCGCAAAAG
Rhox6-F	GAATAGGCTGGCTCAACTGC
Rhox6-R	ATCCGAAACCAATTCTGCAC
Rhox9-F	GGAGGAGAAGAGGAGGGAGA
Rhox9-R	CCAGCCTGTGGATTTCTCAT
β-Actin-F	TGGAATCCTGTGGCATCCATGA
β-Actin-R	TAAAACGCAGCTCAGTAACAGT
RhoxF1-F	GTGCGGGTTTGGTTTAAGAA
RhoxF1-R	CCAGAAAAACCCATCTCCAA
RhoxF2-F	GCAGTGCAGATTTGGTTTGA
RhoxF2-R	GGCTGTGGTCCCAGAAGTAA
GAPDH-F	CCCTTCATTGACCTCAACTACATGG
GAPDH-R	CCTGCTTCACCACCTTCTTGATGTC
Promoter-specific mRNA expression	
Rhox5-Pd-F	TGCACAGTCCTTCAAGCTCACC
Rhox5-Pp-F	AGCTCAGAATCTGCCGAAGC
Rhox5-R	ATCTCACTCCACGACAAGCAGGAC
Bisulfite sequencing	
Rhox5-Pd-F	TTTTAGGTGTGAAGAGGTGAGTTAGA
Rhox5-Pd-R	TCACCAAAACAAAAAAAACCATAA
ChIP assay	
Rhox5-1-F	CACAGCCAATGACATCATCC
Rhox5-1-R	CAGGCAGAGGTGACCAATTT

F, forward; R, reverse.

control *Gapdh* (Assay ID: Mm99999915_m1) were ordered from Applied Biosystems.

Western Blot Analysis. Protein extracts were isolated from ~80% confluent cultured cells in plates using the M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's directions. Protein content was determined by the Bradford (1976) method. Equal amounts of protein were resolved on 6 or 12% SDS polyacrylamide gels and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA). The resulting blots were blocked with 5% nonfat dry milk and probed with antibodies specific for RHOX5, DNMT1, DNMT3A, and DNMT3B (Abcam Inc., Cambridge, MA), and β-actin (Sigma-Aldrich). Blots were then incubated with appropriate peroxidase-conjugated secondary antibodies, and proteins were detected using the SuperSignal chemiluminescence system (Thermo Fisher Scientific).

Bisulfite Sequencing. Genomic DNA from normal cells and mock- and drug-treated cancer cells was extracted using a QIAamp DNA mini kit (QIAGEN). Bisulfite modification of genomic DNA was carried out using an EpiTect bisulfite kit (QIAGEN). PCR primers specific for distal promoter (Pd) region of *Rhox5* gene were designed (Table 1), and PCR experiments were done using GoTaq DNA polymerase (Promega). PCR products were purified using QIAquick PCR purification kit (QIAGEN) following the manufacturer's instructions. Purified DNAs were cloned by using TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. PCR-confirmed insert-positive colonies were picked randomly for DNA sequencing.

Chromatin Immunoprecipitation Assay. ChIP assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's directions. DNA cross-linking was performed by adding 1% formaldehyde into cell cultures at room temperature for 10 min, and glycine was then added (final concentration, 0.125 M) for 5 min to stop the cross-linking reaction. Cells were lysed with a lysis buffer with protease inhibitors and sonicated to shear genomic DNA to lengths ~500 base pairs. One percent of the cell lysate was used for input control, and the rest was used for immunoprecipitation using ChIPAb+ antibodies against acetyl-histone H3 and acetyl-histone H4 (pan-acetylated forms of H3 and H4) (Millipore), as well as dimethyl-histone H3 lysine 4 (Abcam Inc.). Mouse IgG (Millipore) was used as nonspecific control antibody. After collecting immunoprecipitates using protein G-agarose columns, protein-DNA complexes were eluted and heated at 65°C overnight to reverse the cross-linking. After digestion with RNAase A and proteinase K, DNA fragments were purified by spin columns and analyzed by PCR. Specific primer sets were designed to amplify a target sequence within *Rhox5* gene as listed in Table 1.

Statistical Analyses. Student's *t* test was used to compare significance of the difference between groups. Dose-response curves for *Rhox5* gene induction were plotted using Prism version 5 (GraphPad Software Inc., San Diego, CA) as described previously (Kundakovic et al., 2009).

Results

Different Frequency of Rhox mRNA Expression in Cancer Cells. We first detected mRNA expression of five representative *Rhox* genes (murine *Rhox5*, *Rhox6*, and *Rhox9* and human *RhoxF1* and *RhoxF2*) in cancer cell lines by RT-PCR (Fig. 1). Testis cDNA was used as positive control for *Rhox* gene expression (Fig. 1A, lane 1). Normal mouse primary mononucleocytes did not express *Rhox5* and *Rhox6* but did express *Rhox9* (Fig. 1A, lane 2). In the murine cancer cell lines examined, 83% (10/12), 25% (3/12), and 33% (4/12) expressed *Rhox5*, *Rhox6*, or *Rhox9* mRNA, respectively, at easily detectable levels (Fig. 1A). In human cancer cell lines, 77% (10/13) and 8% (1/13) expressed *RhoxF1* and *RhoxF2*, respectively (Fig. 1B). As expected, both *RhoxF1* and *RhoxF2*

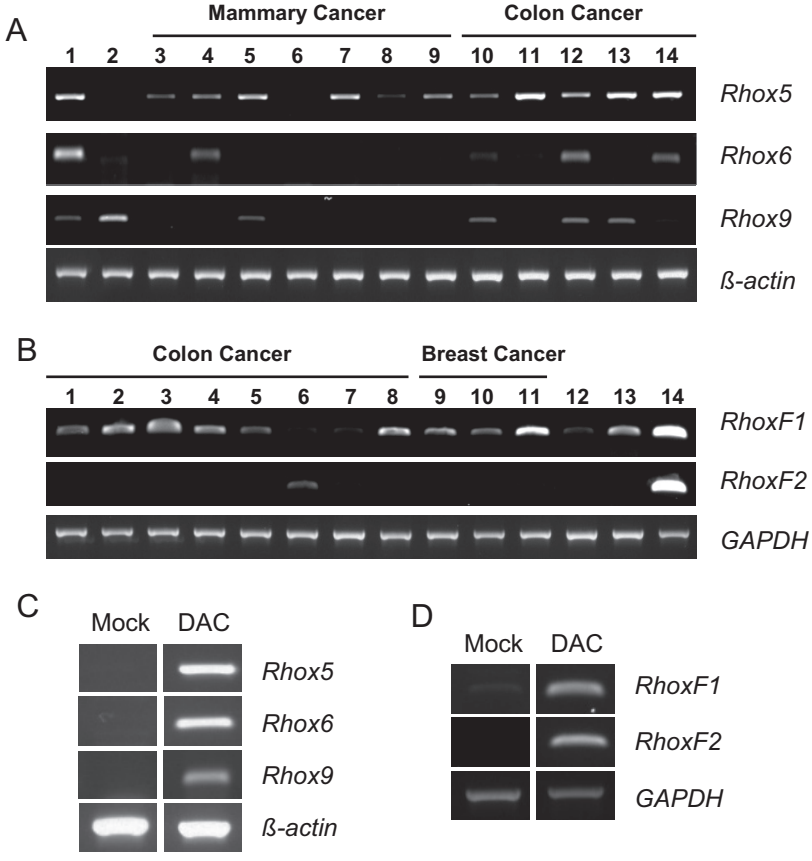

Fig. 1. Expression of representative *Rhox* family genes in murine and human cancer cell lines and their induction by DAC treatment. A, expression of murine *Rhox* genes (*Rhox*5, *Rhox6*, and *Rhox9*) in cancer cell lines as detected by RT-PCR. Lane 1, mouse testis cDNA served as positive control for *Rhox* gene expression. Lane 2, primary spleen mononucleocytes. Lanes 3 to 9, mammary cancer cell lines 4T1, C3-L5, C127I, EMT6, TUBO, MM2MT, and TS/A. Lanes 10 to 14, colon cancer cell lines CA07/A, CA51, CMT93, CT26, and MC38. B, expression of human *Rhox* genes (*RhoxF1* and *RhoxF2*) in colon cancer cells (lanes 1–8: Caco-2, DLD1, HCT116, HT29, LOVO, SW480, SW620, and WiDr), breast cancer cells (lanes 9–11: MCF7, MDA-MB-231, and MDA-MB-468), cervical cancer HeLa cells (lane 12), and pancreatic cancer cells PANC3.27 (lane 13). Lane 14, human testis cDNA was used as positive control. C, induction of mRNA for *Rhox*5, *Rhox6*, and *Rhox9* in murine EMT6 cancer cells treated with DAC (1.0 μM for 48 h). D, up-regulation of RhoxF1 and RhoxF2 mRNA in human breast cancer MDA-MA-231 cells treated with DAC (1.0 μM for 72 h).

were expressed in positive control testis tissue (Fig. 1B, lane 14).

Multiple *Rhox* Genes Are Up-Regulated in Cancer Cells Treated with DAC. To screen potential *Rhox* gene(s) regulated by epigenetic mechanisms, we treated cancer cell lines expressing low levels of *Rhox* genes with the DNA-demethylating agent DAC. As shown in Fig. 1C, EMT6 mammary cancer cells expressed low or trace levels of *Rhox5*, *Rhox6*, or *Rhox9*. When EMT6 cancer cells were treated with DAC, all three genes were induced at the mRNA level as detected by RT-PCR. In MDA-MB-231 and MCF7 human breast cancer cells expressing low levels of *RhoxF1* and *RhoxF2*, DAC treatment further enhanced the levels of gene expression (Fig. 1D; data not shown).

These preliminary results clearly indicated that multiple *Rhox* genes could be regulated by a DNA-demethylating agent, suggesting that epigenetic mechanisms may play important roles in regulation of *Rhox* family members. Because promoters of the *Rhox5* gene have been well defined previously, we chose *Rhox5* as a representative gene in this family for further study.

DAC, MS-275, and ATO Could Induce *Rhox5* in Cancer Cells but Not in Normal Cells. We next sought to investigate whether *Rhox5* induction by DAC was dose-dependent and whether there was cancer cell selectivity. As shown in Fig. 2A, EMT6 cancer cells treated with DAC at a dose as low as 0.13 μM expressed a detectable level of Rhox5 mRNA, whereas DAC did not induce *Rhox5* in normal mammary epithelial cells (MM3MG). The dose-response of *Rhox5* gene induction by DAC was further confirmed by real-time RT-PCR (Fig. 2B). At dose of 2.0 μM, DAC continued to up-regulate Rhox5 mRNA, but the efficiency of gene induc-

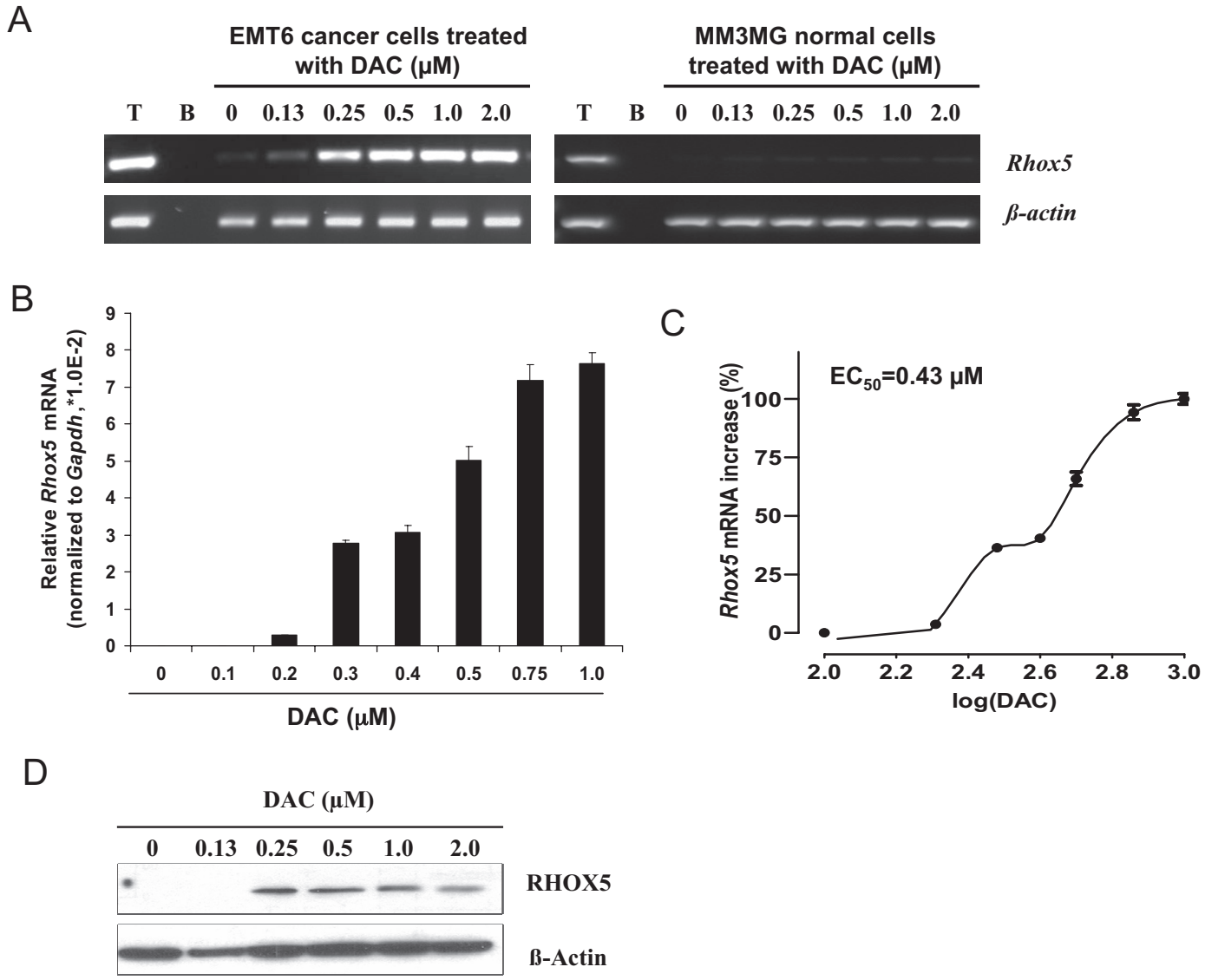

Fig. 2. DAC selectively induced *Rhox5* expression in cancer cells. A, left, dose-dependent induction of Rhox5 mRNA in EMT6 cancer cells treated with DAC for 48 h, as detected by RT-PCR. Right, absence of Rhox5 mRNA induction in DAC-treated normal mammary epithelial cells (MM3MG). β-Actin was amplified as internal control. Lane T, testis cDNA. Lane B, no input cDNA. B, quantitative real-time PCR analysis of relative Rhox5 mRNA level as normalized by *Gapdh* in EMT6 cells treated with different concentrations of DAC for 48 h. C, dose-response curve for Rhox5 mRNA induction after 48-h DAC treatment was plotted using methods described previously (Kundakovic et al., 2007). The *x*-axis represents the log of drug concentration, and the *y*-axis indicates a percentage of the maximal Rhox5 mRNA increase. Basal mRNA value was defined as 0% responses, whereas the highest value (mRNA level of maximal gene induction) was defined as 100% responses. The EC_{50} value is the effective concentration of drug that leads to 50% of maximal *Rhox5* gene induction. D, up-regulation of RHOX5 protein by DAC treatment in EMT6 cells. β-Actin was served as protein loading control.

tion was decreased compared with 1.0 μM DAC (data not shown). The EC_{50} value of DAC for *Rhox5* gene induction was 0.43 μM (Fig. 2C). Consistent with the RT-PCR data, RHOX5 protein was induced by DAC treatment as detected by Western blot (Fig. 2D).

We then examined whether *Rhox5* could be induced by a second epigenetic drug, MS-275, in EMT6 cancer cells. RT-PCR showed that *Rhox5* was induced by increasing concentrations of MS-275 (Fig. 3A, left). MS-275 did not induce *Rhox5* in normal cells at the same doses used in cancer cells (Fig. 3A, right). Quantitative RT-PCR further supported that induction of *Rhox5* by MS-275 was dose-dependent (Fig. 3B). The EC_{50} value of MS-275 on *Rhox5* gene induction was 2.58 μM (Fig. 3C). The induction of RHOX5 protein by MS-275 in EMT6 cells was also confirmed by Western blot analysis (Fig. 3D).

For the third epigenetic drug, ATO, we wanted to investigate whether *Rhox5* could be up-regulated by this chemotherapeutic drug at clinically achievable doses ($<$2.0 μM) (Shen et al., 1997). EMT6 cancer cells treated with higher doses ($\geq$1.5 μM) for 120 h revealed that 80% of cells were dead upon observation under microscope (data not shown). RT-PCR indicated that there was selective induction of Rhox5 mRNA in cancer cells treated with lower doses of ATO (Fig. 4A). Real-time RT-PCR demonstrated that there was dose response of Rhox5 mRNA induction by ATO, and a plateau of gene induction was achieved by ATO at doses from 1.5 to 2.0 μM (Fig. 4B). As shown in Fig. 4C, the EC_{50} value of ATO on *Rhox5* gene induction was 1.02 μM. The induction of *Rhox5* by ATO at the protein level could also be detected by Western blot (Fig. 4D).

It has been demonstrated previously that inhibitors of both DNMTs and HDACs at higher doses induced cell growth arrest and cell death in cancer cells in several studies (Saito et al., 1999; Weiser et al., 2001; Yu et al., 2002; Shao et al., 2004; Palii et al., 2008). As a confirmation, we showed here

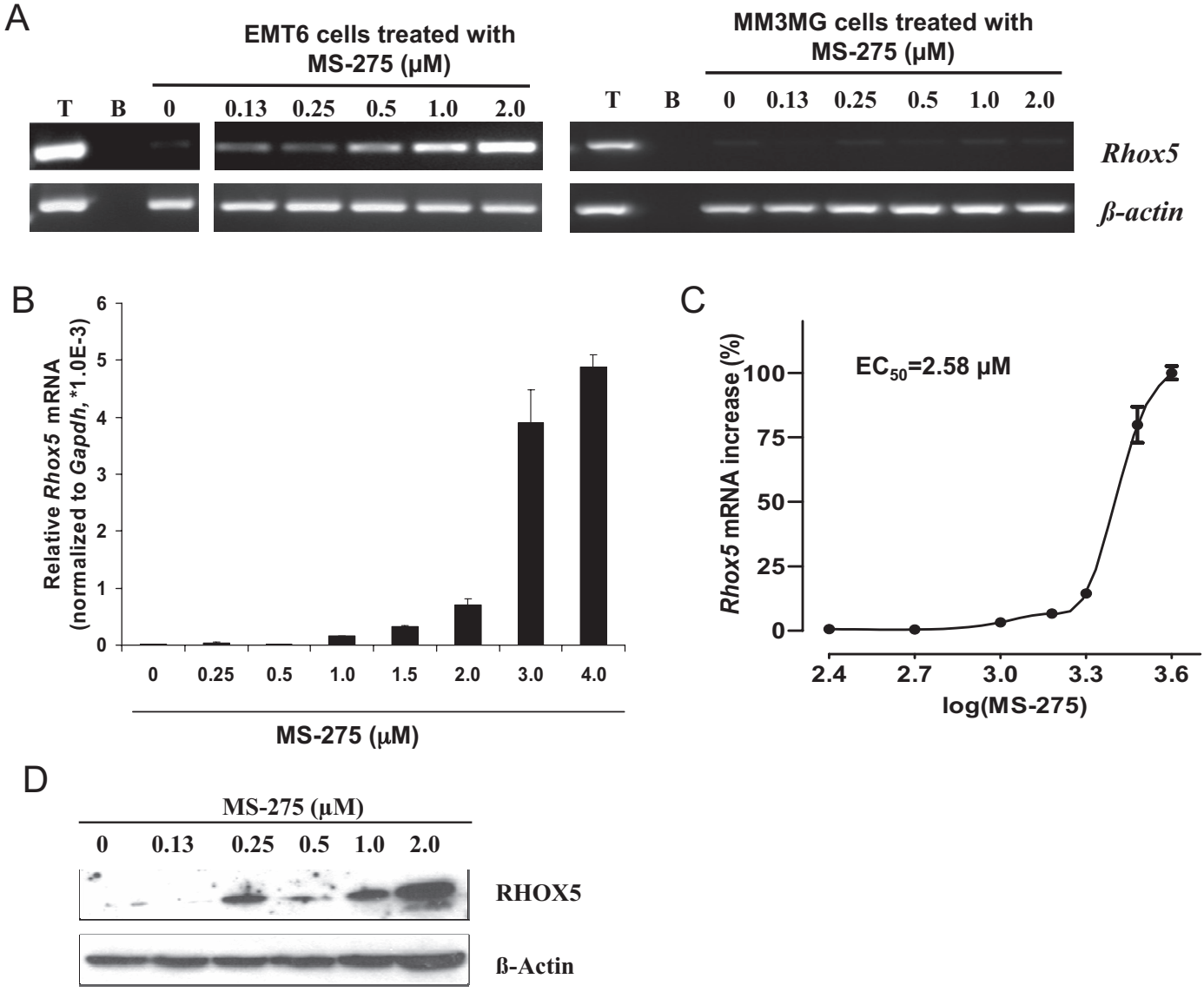

Fig. 3. Selective induction of *Rhox5* expression in cancer cells by MS-275 treatment. A, induction of Rhox5 mRNA in EMT6 cancer cells and normal cells MM3MG treated with different concentrations of MS-275 for 72 h. Lane T, testis cDNA positive control. Lane B, no input cDNA. B, quantitative analysis of relative Rhox5 mRNA level in EMT6 cells treated with different concentrations of MS-275 for 72 h. C, dose-response curve for Rhox5 mRNA induction after 72-h MS-275 treatment. D, up-regulation of RHOX5 protein in EMT6 cells treated with MS-275.

that EMT6 cancer cells treated with DAC, MS-275, or ATO at higher doses resulted in significant reduction in total numbers of viable cells and increased apoptosis (Supplemental Fig. 1).

In summary, all three drugs, DAC, MS-275, and ATO, could selectively induce *Rhox5* gene expression in EMT6 cancer cells but not in normal mammary epithelial cells or primary hepatocytes. These drugs could also induce *Rhox5* expression in CA07/A colon cancer cells and P815 mastocytoma cells (data not shown). In EMT6 cancer cells, the basal level of Rhox5 mRNA was five copies when normalized to 10^6 copies of Gapdh mRNA. In terms of maximal induction, it was ~75,000 copies of Rhox5 mRNA (15,000-fold) for DAC, 5000 copies (1000-fold) for MS-275, and 50 copies (10-fold) for ATO, respectively, when normalized to 10^6 copies of Gapdh mRNA (Figs. 2B, 3B, and 4B).

All Three Drugs Decreased DNMT Protein Levels in Treated Cancer Cells. To dissect the epigenetic mechanisms underlining drug-induced *Rhox5* gene expression, we examined changes of protein levels in three important DNMT enzymes, DNMT1, DNMT3A, and DNMT3B, in mock- or drug-treated cancer cells. As expected, DAC decreased both DNMT1 and DNMT3B protein levels in a drug dose-dependent manner in EMT6 cells (Fig. 5A). It potently inhibited both DNMT1 and DNMT3B even at a low dose of 0.25 μM. ATO demonstrated potent inhibition of DNMT1 and DNMT3B at doses at or above 1.0 μM (Fig. 5B). In contrast, MS-275 did not inhibit DNMT1, but we observed significant reductions of DNMT3B at all doses examined (Fig. 5C). For DNMT3A, we did not detect expression of this protein in EMT6 cells, and these drugs did not induce DNMT3A in these cells. However, because of the lack of expression, we

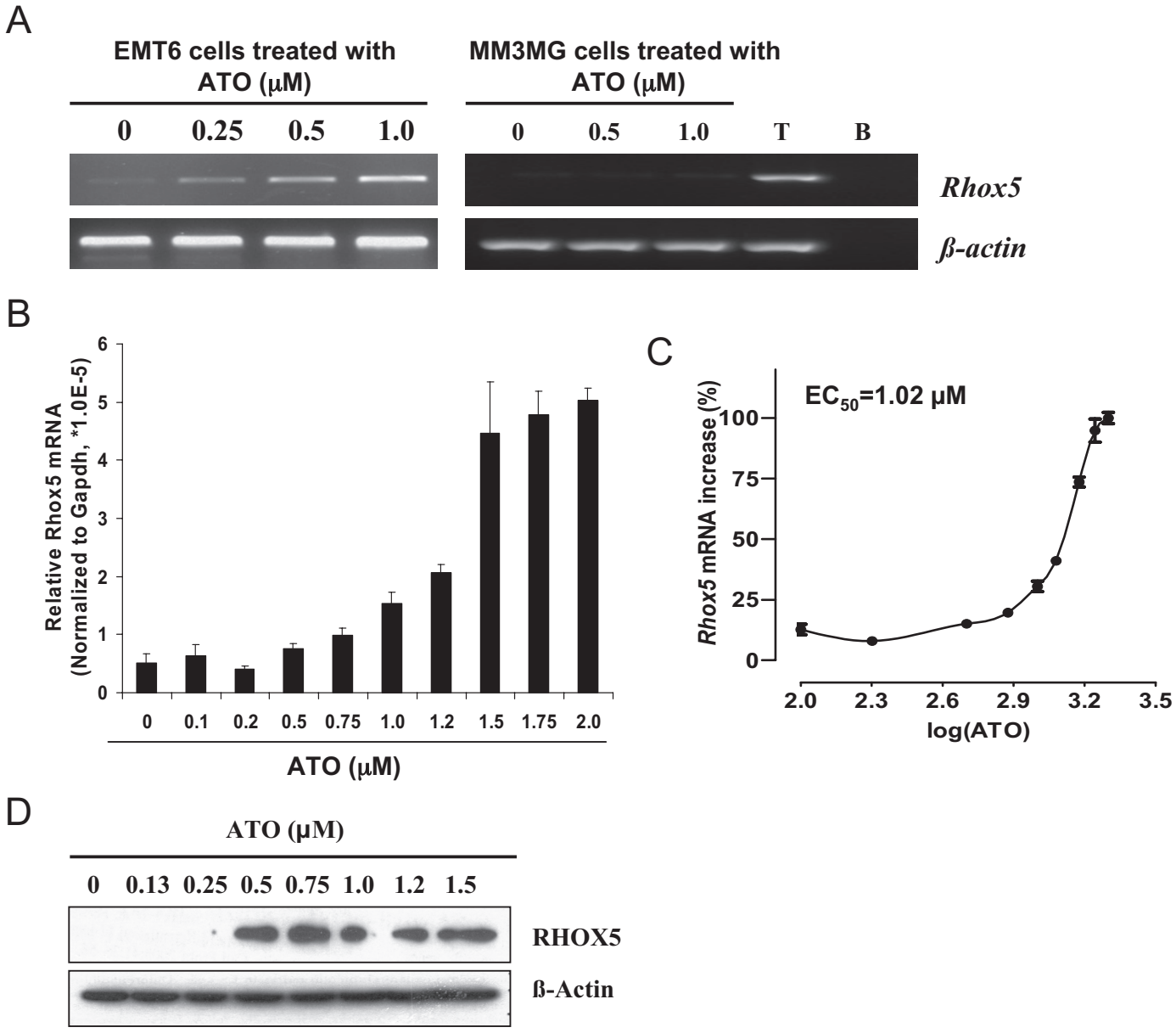

Fig. 4. Selective induction of *Rhox5* expression in ATO-treated cancer cells. A, induction of Rhox5 mRNA in EMT6 cancer cells and normal cells MM3MG treated with different doses of ATO for 120 h. Lane T, testis cDNA positive control. Lane B, no input cDNA. B, quantitative analysis of relative Rhox5 mRNA level in EMT6 cells treated with different concentrations of ATO for 120 h. C, dose-response curve for Rhox5 mRNA induction after 120-h ATO treatment. D, dose-dependent induction of RHOX5 protein in ATO-treated EMT6 cells.

could not examine the potential inhibitory effects of these drugs on DNMT3A.

ATO and MS-275 Activated the Distal Promoter of *Rhox5* Exclusively, whereas DAC Activated Both Pd and Pp of the Gene. Previous studies (Maiti et al., 1996; Barbulescu et al., 2001; Rao et al., 2002, 2003) have demonstrated that *Rhox5* gene could be transcribed from Pd and/or proximal promoters (Pp) (Fig. 6A). We have studied mRNA transcription from both promoters in EMT6 cancer cells either mock-treated or treated with a single epigenetic drug. Based on the existence of an additional 30 nucleotides in exon 3 from the mRNA transcribed from Pp, we designed promoter-specific PCR primers used in RT-PCR to detect specific transcripts from either the Pd or Pp. CT26 colon cancer cells were used as a positive control because they used both Pd and Pp for *Rhox5* gene expression. This is similar to the situation in mouse testis as confirmed by RT-PCR (data not shown). In mock-treated EMT6 cancer cells, a trace level of Rhox5 mRNA transcribed from Pd was detected by RT-PCR (Fig. 6B). DAC could induce transcripts from both Pd and Pp in EMT6 cells. However, both ATO and MS-275 treatment induced transcription from Pd but not Pp in EMT6 cancer cells (Fig. 6B).

DAC, but Not ATO and MS-275, Induces DNA Demethylation around the Distal Promoter. All three drugs used in this study could inhibit one or more DNMTs in EMT6 cancer cells (Fig. 5). Therefore, we examined the DNA methylation status in the Pd region in mock- and drug-treated EMT6 cancer cells, as well as in a *Rhox5*-expressing CT26 cancer cells and *Rhox5*-silenced normal mammary epithelial cells. The *Rhox5* gene promoters are CpG-poor. We designed two sets of PCR primers to cover the two segments within Pd and Pp regions for PCR amplification of bisulfite-treated genomic DNA. These two regions (one region from −928 to −766 and one region from −259 to −53) contain binding sites for important transcription factors (Barbulescu et al., 2001).

As shown in Fig. 6C, it was clear that the Pd region of the gene (with four CpG dinucleotides between −928 and −766) was hypermethylated in normal epithelial cells and hypomethylated in CT26 cancer cells. In EMT6 cancer cells, the promoter DNA was extremely hypermethylated. Therefore, DNA hypermethylation status of the gene in EMT6 cells was consistent with a low level of *Rhox5* gene expression. We next examined any change in DNA methylation after a course of epigenetic drug treatment that was previously shown to result in *Rhox5* induction in EMT6 cells. As expected, DAC induced a shift from hypermethylation to hypomethylation in the Pd region ($p < 0.05$, between mock-treated versus DAC-treated cells). It was surprising that there was little, if any, change in DNA methylation status within the promoter in EMT6 cells after MS-275 or ATO treatment. Similar patterns of changes in DNA methylation were found in a second region (BS-2) covering nucleotides between −259 and −53 in EMT6 cancer cells before and after treatment with the drugs (Supplemental Fig. 2). In summary, DAC, but not ATO or MS-275, induced DNA demethylation in *Rhox5* promoter regions in EMT6 cancer cells.

***Rhox5* Gene Induction Correlated Strongly with Increased Activating Histone Marks Associated with the Promoter Region.** We sought to examine the changes of the histone code in the Pd region in EMT6 cancer cells treated with these three drugs. This analysis was performed by means of ChIP assays. We selected the three most important activating histone epigenetic marks (AcH3, AcH4, and H3K4Me2) as indicators of an open and active chromatin structure around the promoter. This type of chromatin structure is a hallmark of an actively transcribed gene. As shown in Fig. 6D, treatment with any of the three drugs caused accumulation of acetylated histones H3 and H4 at Pd region. This is the first time that increased acetylated H4 within a gene promoter was observed in cancer cells treated with ATO. Upon examination of the activating histone methylation mark H3K4Me2, we observed a significant enhancement in EMT6 cells treated with DAC and MS-275 but not ATO.

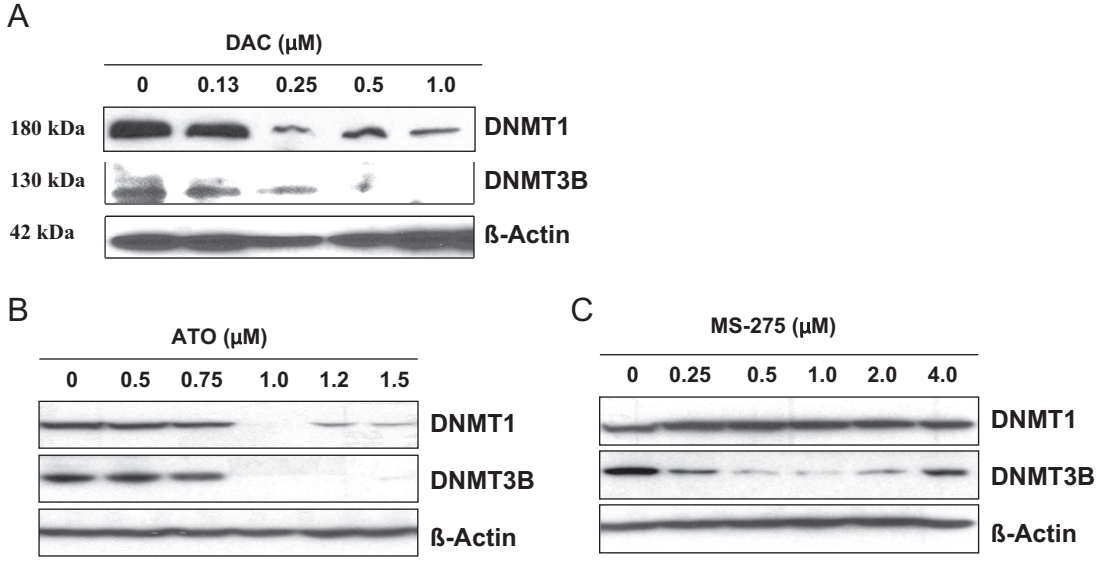

Fig. 5. DNMT1 and DNMT3B protein expression in EMT6 cancer cells after treatment with epigenetic drugs. EMT6 cancer cells were treated with increasing doses of DAC (A), ATO (B), or MS-275 (C). The detailed procedures are described under *Materials and Methods*. DNMT1 and DNMT3B proteins, but not DNMT3A, were detected by Western blot analysis. Representative results from three independent experiments are presented.

Discussion

In this study, we have found for the first time that epigenetic drugs could induce multiple members of the *Rhox* gene family in both murine and human cancer cells. We investigated the epigenetic mechanisms of *Rhox5* gene induction in cancer cells after treatment with three separate epigenetic drugs. We revealed that the Pd region of *Rhox5* gene is hypomethylated in cancer cells (CT26) that express this gene at a high level. In contrast, the same promoter is hypermethylated in normal mammary epithelial cells and in EMT6 cancer cells in which the gene is either silenced or expressed at extremely low levels. It was demonstrated that DNA methylation plays important roles in lineage-specific silencing of *Rhox* family members, including *Rhox5* in embryogenesis (Oda et al., 2006). Our results suggest that DNA hypermethylation plays an important role in repressing *Rhox5* gene in EMT6 mammary cancer cells as well as in normal somatic cells.

Through the use of three separate epigenetic drugs, we were able to induce *Rhox5* expression in cancer cells that express the gene at extremely low levels. The same induction was not observed in normal cells. The results obtained regarding *Rhox5* gene induction with each of the three drugs has led us to a better understanding of the molecular mechanisms regulating *Rhox5* expression. MS-275 was selected as a prototypic inhibitor for class I HDACs, whereas both DAC and ATO are potent DNMT inhibitors. All three drugs induced *Rhox5* gene expression in cancer cells to varying de-

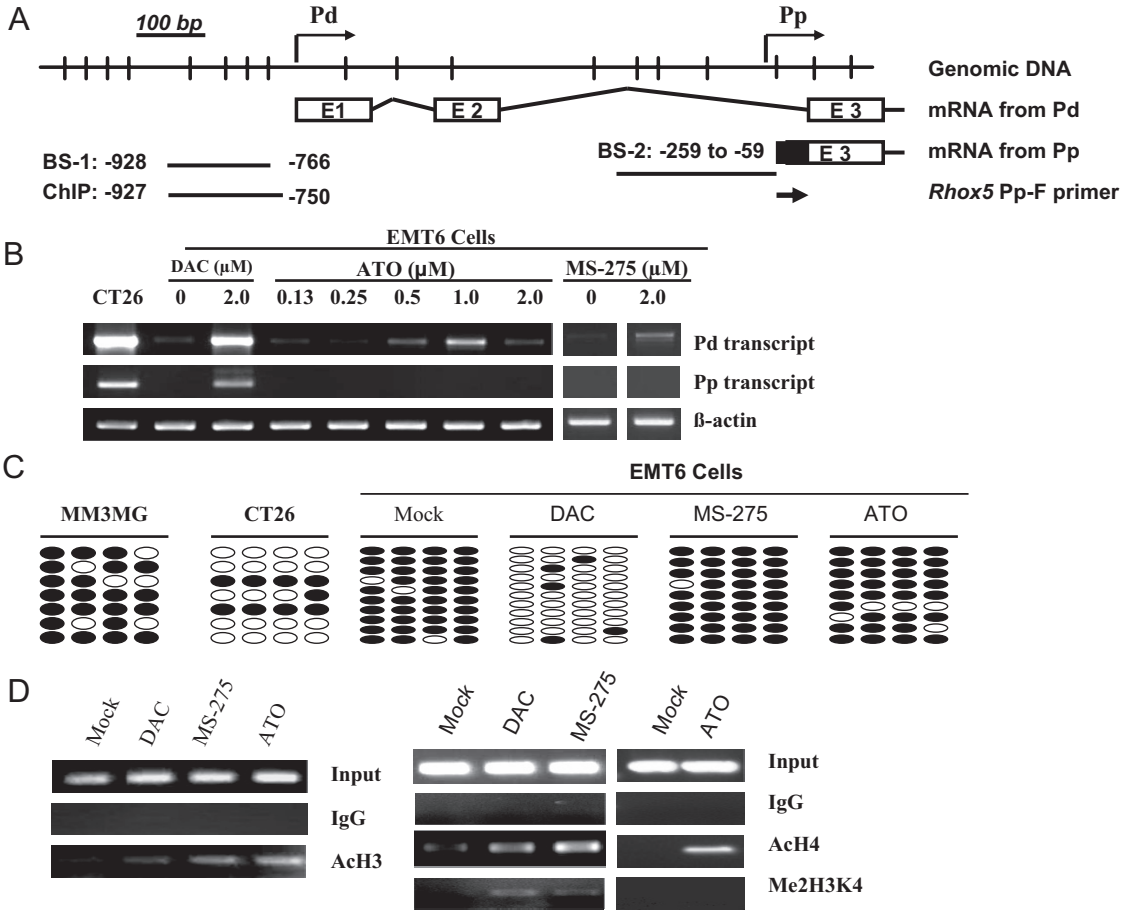

Fig. 6. Induction of *Rhox5* and epigenetic changes in the local chromatin around the Pd in EMT6 cancer cells after epigenetic drug treatment. A, schematic of the mouse *Rhox5* gene promoters, with each vertical line representing one CpG dinucleotide (GenBank accession no. AF410462) (Barbulescu et al., 2001). Numbering starts at the translation initiation codon (ATG) in the exon 3, and it covers a promoter region of 1.2 kilobases (nucleotides from −1139 to +60). The first three exons, with the first two as noncoding exons, are presented below the genomic sequence. There may be multiple transcription initiation sites for both the Pd and the Pp. The main transcription start site from Pd is located at −692, and the main sites from Pp are at approximately −60. The regions amplified for bisulfite sequencing and ChIP assay are presented. Because the extra 30-base pair segment of mRNA exists in the transcript from Pp; thus, a Pp-specific forward primer, *Rhox5*-Pp-F (as shown), was designed to amplify the cDNA derived from Pp-specific mRNA. B, examination of *Rhox5* Pd- and Pp-derived mRNA transcripts in CT26 colon cancer cells and EMT6 mammary cancer cells mock-treated or treated with DAC (2.0 μM for 48 h), ATO (0.13–2.0 μM for 120 h), or MS-275 (2.0 μM for 72 h). CT26 cells, which expressed *Rhox5* from both Pd and Pp, were used as a control. C, DNA methylation status of the *Rhox5* Pd region in normal cells, mock-treated cancer cells, or drug-treated cancer cells. Normal mammary epithelial (MM3MG) cells and CT26 cancer cells were used as negative and positive controls. EMT6 cancer cells were mock-treated or treated with DAC (2.0 μM for 48 h), MS-275 (2.0 μM for 72 h), or ATO (1.0 μM for 120 h). The methylation status of the *Rhox5* Pd was determined by bisulfite sequencing. Independent clones (7–11) from each treatment condition were sequenced and presented as separate rows. Each column represents the four CpG sites located in the Pd region BS-1 (covering nucleotides from −928 to −766). ●, one methylated CpG dinucleotide; ○, one unmethylated CpG dinucleotide. D, occupancy of histone epigenetic marks at the Pd of *Rhox5* gene in mock- or drug-treated EMT6 cancer cells as determined by ChIP assays. Treatment conditions were the same as described in C. The immunoprecipitated chromatin DNA was subjected to PCR with primers covering the promoter region from −927 to −750 (as shown in A). The acetylated histones H3 and H4 (AcH3 and AcH4) and one H3 methylation mark (H3K4Me2) was examined. Input, 1% of input sonicated chromatin DNA for ChIP assay. IgG, nonspecific anti-mouse IgG control.

grees, with DAC being the most potent of the three drugs. We looked for the effects of each drug on DNMTs in cancer cells. Among the three key enzymes, DNMT3A is not expressed in EMT6 cancer cells. Upon analysis of the protein levels of DNMT1 and DNMT3B, we observed a reduction of both enzymes in both DAC- and ATO-treated cancer cells and reduced levels of DNMT3B in MS-275-treated cancer cells. Recent studies have shown that four other HDAC inhibitors [apicidin, depsipeptide, panobinostat (LBH589), and trichostatin A] could down-regulate one or more DNMTs (Xiong et al., 2005; Wu et al., 2008; You et al., 2008; Zhou et al., 2008). This down-regulation may be mediated specifically by promoting ubiquitin-dependent proteasomal degradation of DNMT1 or by decreasing DNMT3B mRNA stability (Xiong et al., 2005; You et al., 2008; Zhou et al., 2008). MS-275 has been shown to inhibit DNMT1 protein and enzymatic activity (Kundakovic et al., 2009). Therefore, we believe that MS-275 may reduce DNMT enzymes through similar post-transcriptional mechanisms as shown by other HDAC inhibitors.

One interesting finding in this study indicated a lack of DNA demethylation on the distal promoter of *Rhox5* gene in cancer cells treated with either ATO or MS-275. This suggests that DNA hypomethylation is not essential for *Rhox5* gene induction. Other investigators have made similar observations for other genes in the absence or presence of an epigenetic drug. Carbonic anhydrase II and ICSBP/IRF8 genes associated with DNA hypermethylation in combination with active histone marks are actively transcribed (Brinkman et al., 2007; Tshuikina et al., 2008). Suberoyl anilide hydroxamic acid, a pan-inhibitor for HDACs, activates the 15-lipoxygenase-1 gene without affecting the status of promoter DNA methylation (Zuo et al., 2008). CDKN2B gene is activated by DNMT inhibitors in leukemia cells without demethylation of its promoter (Flotho et al., 2009). Thus, our results, together with those of others, demonstrated that a silenced gene or one expressed at extremely low levels can be transcriptionally activated without changing the DNA methylation status of its promoter.

The activating histone marks acetylated H3 and H4, as well as H3K4Me2 are clearly indicators for the active promoter. All three drugs induced acetylated H3 and H4, whereas DAC and MS-275 also induced H3K4Me2 mark around the promoter of the *Rhox5* gene in EMT6 cancer cells. Because H3K4Me2 is one of the key activating histone marks, the lack of its induction by ATO may explain the low potency of ATO to induce Rhox5 mRNA expression compared with DAC and MS-275 (10-fold versus 15,000-fold and 1000-fold, respectively).

The pattern of histone epigenetic marks in the Pd in CT26 colon cancer cells where the gene is actively transcribed from this promoter was also analyzed. The pattern in CT26 cells included all three activating histone epigenetic marks—AcH3, AcH4, and H3K4Me2 (data not shown). Therefore, the active gene in naturally active CT26 cancer cells and in potent drug-induced EMT6 cancer cells shares similar pattern of histone epigenetic marks. Although this histone epigenetic pattern by itself may be a good indicator of an active promoter, for maximal transcriptional activity of the promoter, accumulation of activating histone marks along with DNA demethylation is needed. Our results support this notion that DNA methylation and histone modifications often function together to regulate gene expression (Berger, 2007).

The elucidation of the molecular mechanisms for *Rhox5* gene activation by three epigenetic drugs may lead to rational translational applications. The first of these may be the combination of epigenetic drugs and cancer immunotherapy. Cancer germline antigens (such as NY-ESO-1 and P1A) and immunomodulatory molecules that can be up-regulated by DAC have been studied in cancer immunotherapy (Gollob et al., 2006; Guo et al., 2006; Coral et al., 2007; Kato et al., 2007; Campoli and Ferrone, 2008). The second potential application is to use these drugs to study cancer biology. As discussed here, transcription factors encoded by genes of *Rhox* and other homeobox families may play important roles in cancer initiation, progression, and metastasis (Christensen et al., 2008). Some of these genes are regulated by epigenetic mechanisms. Epigenetic drugs may be used to probe mechanisms of gene regulation and as a pharmacological means to regulate these genes. A greater understanding of cancer epigenetics will serve to assist further development of epigenetic therapy for the treatment of cancer.

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