Histone Deacetylase Inhibitors Reverse CpG Methylation by Regulating DNMT1 through ERK Signaling

SIBAJI SARKAR, ANA L. ABUJAMRA, JENNY E. LOEW, LORA W. FORMAN, SUSAN P. PERRINE and DOUGLAS V. FALLER

Cancer Center, Boston University School of Medicine, Boston, MA, 02118, U.S.A.

Abstract. *Methylation of CpG repeats in the upstream/promoter regions of genes is an established mechanism of gene silencing in many cell types. DNA methylation results in the recruitment of histone deacetylases (HDACs) to promoter regions, thereby repressing expression of genes. General inhibitors of class I and II HDACs (HDACi), such as sodium butyrate and suberoylanilide hydroxamic acid, suppress the growth of prostate cancer cells in vitro and in vivo. In this study, we investigated the mechanism of re-expression of silenced cell cycle inhibitors and retinoic acid receptor B2 (RARB2). HDACi inhibited cell cycle progression, and reversed promoter methylation and silencing of three tumor suppressor genes: RARB2 and the cell cycle regulating cyclin-dependent kinase inhibitors p16 and p21. HDACi repressed MAP kinase I (ERK) activation and down-regulated DNA (cytosine-5-)-methyltransferase 1 (DNMT1) levels. Direct inhibition of ERK activity similarly decreased DNMT1 protein levels and reversed the basal hypermethylation of the promoters and silencing of the RARB2, p21 and p16 tumor suppressor genes. Suppression of DNMT1 level by siRNA also reversed methylation of these tumor suppressor genes with similar kinetics. Collectively, these data demonstrate that HDACi, by inhibiting ERK activity, regulate DNMT1 and ultimately DNA methylation. These results demonstrate that HDACs regulate gene methylation, in addition to the established and reciprocal ability of CpG methylation to recruit HDACs to repress transcription.*

Abbreviations: CpG: Cytosine phosphate guanosine; AZA: 5-aza-2'-deoxycytidine; HDAC: histone deacetylase; HDACi: histone deacatylase inhibitors; SB: sodium butyrate; SAHA: suberoylanilide hydroxamic acid; RARB2: retinoic acid receptor beta 2; MAPK1, mitogen-activated protein kinase 1; ERK1: MAP kinase I; AKT1: v-akt murine thymoma viral oncogene homolog 1; DNMTs: DNA (cytosine-5-)- methyl transferases; DNMT1: DNA (cytosine-5-)-methyltransferase 1; CDK1: cyclin-dependent kinase 1; MEK1: MAP2K1, mitogen-activated protein kinase kinase 1; MEK2: MAP2K2, mitogen-activated protein kinase kinase 2; p21: CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1); p16: CDKN2A, cyclin-dependent kinase inhibitor 2A; siRNA: small interfering RNA; MS-PCR: methylation-specific polymerase chain reaction; RT-PCR: reverse transcriptase-polymerase chain reaction; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Correspondence to: Douglas V. Faller, MD, Ph.D., Cancer Center, Boston University School of Medicine, 72 East Concord Street, K-701, Boston, MA 02118, U.S.A. Tel: +1 6176384173, Fax: +1 6176384176, e-mail: dfaller@bu.edu

Key Words: Tumor suppressors, cell cycle arrest, DNA *N*-methyl transferase, ERK.

Methylation of CpG repeats in the upstream/promoter regions of genes is an established mechanism of gene silencing and is correlated with the suppression of critical genes, including tumor suppressors, in many types of tumors. The methyl-CpG-binding protein 2 (MeCP2) facilitates histone deacetylation and DNA methylation-dependent transcriptional silencing. For example, MeCP2 plays an important role in neurite extension of PC12 cells by regulating the expression of key genes involved in differentiation (1). Memory formation and synaptic plasticity are regulated by DNA methylation and histone acetylation (2). Gene silencing by methylation is also prevalent in stem cells, which undergo differentiation while proliferating (3).

DNA methylation results in the local recruitment of histone deacetylases (HDACs) to promoter regions with co-localization of MeCP2 proteins, eventually inhibiting the binding of RNA polymerase II (Pol II) and thereby repressing the expression of genes and their products. Such observations led to the paradigm that DNA methylation lies 'upstream' of histone acetylation in this pathway of transcriptional repression.

While it is known that DNA methyl transferases (DNMTs) produce methylation of CpG repeats in the upstream promoter regions of genes, physiological upstream regulators of the activity and levels of DNMTs have not been identified. Recent studies suggest that DNA methylation in colon cancer cells and in NIH 3T3 cells may be regulated in some circumstances by *ERK* (MAP kinase1) activity (4). DNA

methylation, and the enzymes which cause methylation, might be modulated directly or indirectly by the extracellular milieu through signal transduction pathways, which may include ERK or other cellular kinases.

General inhibitors of class I and II HDACs, alone or in combination with the methylation inhibitor 5-azacitydine, or its congener 5-aza-deoxycytidine (AZA), or other agents are being studied in clinical trials for treatment of diverse types of tumors (5). General (class non-specific) HDAC inhibitors (HDACi), including sodium butyrate (SB) and suberoylanilide hydroxamic (SAHA), suppress the growth of prostate cancer cells *in vitro* and *in vivo*. While the exact mechanisms underlying this growth inhibition have not been fully elucidated, it appears that HDACi induce the expression of several tumor suppressor genes, such as the cyclin-dependent kinase inhibitors (CDKI) *p21* and *p16* (6-9), as well as genes whose products can induce differentiation, such as retinoic acid receptor β2 (*RARB2*) (10). These types of tumor suppressor genes are frequently silenced in many classes of tumors, including prostate cancer (11). This suggests that exposure to HDACi may influence genes silenced by DNA methylation, either independently of their DNA methylation or by altering their methylation, and thereby modulate the growth of tumor cells.

We initiated this work to investigate: (i) whether HDACi reverse the promoter methylation of silenced genes; (ii) whether such promoter demethylation reverses the expression of silenced genes and, if so, (iii) whether HDACi regulate DNMT function by a signaling mechanism. We report that exposure to HDACi inhibits the activating phosphorylation of ERK, which then causes down-regulation of *DNMT1*. Suppression of *DNMT1* in turn results in demethylation of key tumor suppressor genes, such as *RARB2*, *p21* and *p16*. Specific repression of *DNMT1* by small interfering RNA (siRNA) is sufficient to demethylate and depress expression of these tumor suppressor genes, and suppression of ERK activity by various means is sufficient to inhibit DNMT1 levels. These studies thus elucidate a pathway in which ERK activity, regulated by HDACi, lies upstream of *DNMT1*, and *DNMT1* suppression, by repression of ERK activity, results in the demethylation of silenced genes.

Materials and Methods

Material. RPMI-1640 media was purchased from Invitrogen (Carlsbad, CA, USA). The LNCaP prostate cancer cell line was obtained from ATCC (Manassas, VA, USA). Antibodies for immunoblot analyses were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). DNMT1 antibody was purchased from Abcam, Cambridge, MA, USA. PD98059 was purchased from CalBiochem (La Jolla, CA, USA). ERK, AKT and p38 MAP kinase antibodies were from Santa Cruz Biotech. Primers were obtained from Invitrogen. *DNMT1, MEK1* and *MEK2* siRNA Accell Smart Pools were obtained from Dharmacon (Lafayette, CO, USA). AZA, SB and SAHA, were purchased from Sigma (St Louis, MO, USA)

Cell culture. LNCaP cells were obtained directly from ATCC (Manassas, VA, USA), which validates cell line characterizations (ISO Guide 34 accredited), and passaged in the laboratory for fewer than 3 months after receipt. LNCaP prostate cancer cells were grown in RPMI-1640 media containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 ml fetal bovine serum.

Cell survival assays. Viable cell enumeration was performed using a trypan blue exclusion assay. The percentage viable cell counts were plotted against time of incubation.

Methylation-specific PCR (MS-PCR). MS-PCR was performed with bisulfite-treated genomic DNA from treated and untreated cells grown in 100 mm plates. Genomic DNA was isolated using reagents from Stratagene (La Jolla, CA, USA) according to the manufacturer's protocol and treated with bisulfite according to a published protocol (12). MS-PCR was performed with primers specific for either methylated or bisulfite-modified unmethylated DNA. The PCR reactions were carried out in a Hybaid PCR Sprint Thermal Cycler (Hybaid, Franklin, MA, USA). PCR amplifications without genomic DNA were performed as controls. Each PCR product was run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Total RNA was prepared from pelleted cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. PCR was performed with cDNA using primers specific for DNMTs 1, 3a, 3b and actin, as described below. PCR amplifications without DNA were performed as controls. PCR products were also separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination, for quality control.

Real-time quantitative PCR was performed with the cDNA prepared as described above for quantitation of *RARB2, p21* and *p16* transcript expression. The results were normalized with the expression level of β-actin transcripts in each sample. The PCR was performed in triplicate for each sample and the means are presented with the standard deviations. Primers used for PCR are shown in Tables I-III.

Cell cycle analyses. For cell cycle analyses, cells were treated with SB or SAHA for the indicated times and stained with propidium iodide (PI) as described elsewhere (13) and then analyzed for DNA content by flow cytometry.

siRNA knock-down. *DNMT1, MEK1* and *MEK2* siRNA Accell Smart Pools and non-specific siRNAs were obtained from Dharmacon. LNCaP cells were transfected twice at 24 h intervals with siRNAs, according to the protocol provided by the manufacturer. Briefly, LNCaP cells were seeded in 6-well plates. At 40-50% confluency, the medium was aspirated, the cells were washed and 1 ml of siRNA (1 mM) in Accell delivery medium was added to each well. At 24 h, the medium was aspirated and the process was repeated. After 72 h, the medium was aspirated, cells were washed with PBS, trypsinized, washed again, and lysed for immunoblot analysis. In parallel experiments, the cells were washed, trypsinized, and washed with PBS. The cell pellet was then subjected to genomic DNA preparation, followed by bisulfite treatment for MS-PCR.

Immunoblot analyses. Cells were lysed in 1% NP-40 lysis buffer, containing vanadate and a protease inhibitor cocktail as described (14). Protein concentration in each sample was estimated by using a

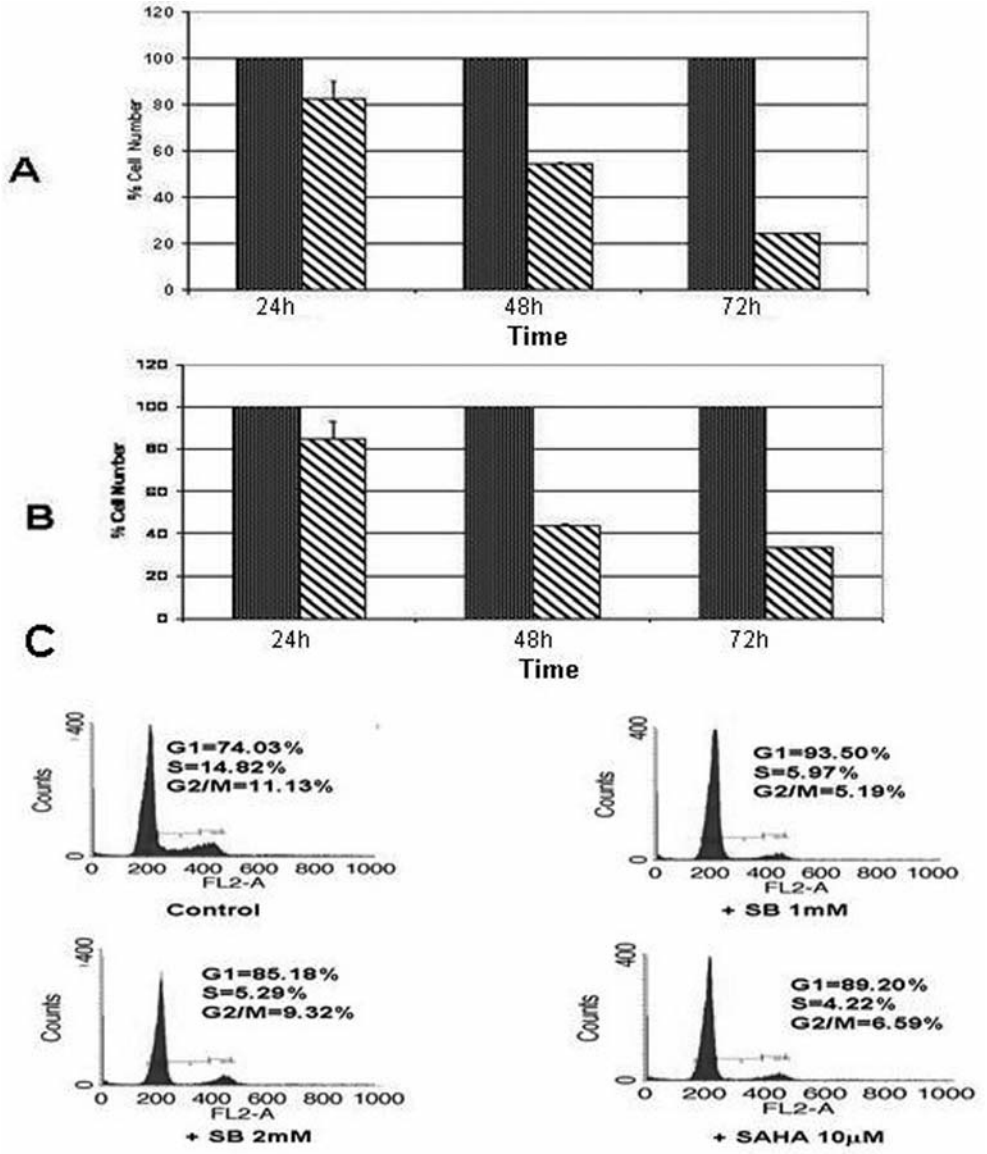

Figure 1. *Effects of HDAC inhibitors on the growth of LNCaP cells. LNCaP cells were exposed to either sodium butyrate (SB) (1 mM) (A) or suberoylanilide hydroxamic acid (SAHA) (10 μM) (B). After 24 h, 48 h, and 72 h, cells were washed, trypsinized, and the viable cells enumerated. Results are expressed relative to the numbers of vehicle-treated (control) cells, arbitrarily assigned a value of 100%. The solid black columns represent the control cells and the striped columns represent the cells exposed to HDAC inhibitors. (C) Cell cycle profiles of LNCaP cells exposed to HDAC inhibitors. LNCaP cells were treated with SB (1 mM or 2 mM) or SAHA (10 μM). After 72 h, cells were washed, trypsinized, and stained with PI. Cell cycle profiles were analyzed by flow cytometry. Results are expressed as the percentage of cells in a particular phase of the cell cycle.*

protein assay reagent (Bio-RAD) with bovine serum albumin as the standard. Equal amounts of protein from each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes blocked with either 5% BSA for phospho-protein blots, or with 3% non-fat milk in Tris-buffered saline/Tween (TBST) (15) and then incubated with the primary antibody overnight at 4˚C. After 3 washes with TBST, the membrane was incubated with the secondary antibody at optimal concentrations for 1 h at room temperature, washed 3 times with TBST (each wash for 10 minutes), developed with chemiluminescence reagent (16) and photographed.

Statistical analysis. *t*-Test was performed to determine the *p*-values to evaluate statistical significance.

Results

HDACi cause cell cycle arrest in prostate cancer cells. HDACi are well-established as having the ability to slow or stop the growth of cells, including tumor cells, by inducing a cell cycle arrest. Two structurally distinct HDACi, SB and

Table I. *List of primers used for MS-PCR.*

Primer name	Forward (5'→3')	Reverse (5'→3')
p16UM	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA
p16M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA
p21M	TTTTTGTAGTATGTGAGGTTTTGG	AACACAACTCAACACAACCCTA
RARB2	TCGAGAACGCGAGCGATTCG	GACCAATCCAACCGAAACGA

UM=unmethylated, M=methylated.

Table II. *List of primers used for RT-PCR analysis of DNMT1, DNMT3a, DNMT3b and actin.*

Primer name	Forward (5'→3')	Reverse (5'→3')
RARB2	AGGGAAAAGGGAAGGGCAAG	AGAAAACACATCCAGGGTCCG
DNMT1	AGGGAAAAGGGAAGGGCAAG	AGAAAACACATCCAGGGTCCG
DNMT3a	CAGCGTCACACAGAAGCATATCC	GGTCCTCACTTTGCTGAACTTGG
DNMT3b	CCTGCTGAATTACTCACGCCCC	GTCTGTGTAGTGCACAGGAAAA
Actin	CTGGCACCCAGCACAATG	GGACAGCGAGGCCAGGAA

Table III. *List of primers used for real-time quantitative PCR.*

Primer name	Forward (5'→3')	Reverse (5'→3')
RARB2	CAAACCGAATGGCAGCATCGG	GCGGAAAAAGCCCTTACATCC
p21	CTGGAGACTCTCAGGGTCGAA	GGATTAGGGCTTCCTCTTGGA
p16	CATAGATGCCGCGGAAGGT	CAGAGCCTCTCTGGTTCTTTCAA
Actin	CTGGCACCCAGCACAATG	GGACAGCGAGGCCAGGAA

SAHA (12), were studied here. Approximately 75% and 70% growth inhibition was observed at 72 h after exposure of cells to SB and SAHA, respectively (Figures 1A and B). The results of two- and three-day treatments showed statistically significant inhibition ($p<0.01$). Live-dead analysis of the cells using trypan-blue exclusion indicated that this growth inhibition was not due to cytotoxicity in the cell groups exposed to the HDACi (data not shown).

Cell cycle analysis after 72 h exposure to SB or SAHA showed that each produced a decreased proportion of cells in S phase and a reciprocal increase in the G_1 phase population (Figure 1C). The G_1 arrest observed is typical of the effect of HDACi on cell cycle progression (8, 9). We, and other investigators, have previously demonstrated that this G_1 arrest is dependent upon the induction of specific tumor suppressor genes, including the CDKIs *p21* and *p16* (6-9). The *p21* and *p16* gene promoters are reported to be methylated in prostate cancer and the genes and gene products are not expressed (17, 10).

Reversal of CpG methylation in tumor suppressor gene promoter regions by HDAC inhibitors. To investigate whether cell cycle inhibitory proteins are silenced by methylation in LNCaP cells and whether their methylation status is reversed by exposure to HDACi, the promoter methylation status of the CDKI genes *p21* and *p16* and the tumor suppressor *RARB2* gene was determined. LNCaP cells were incubated with SB for 24 h, and genomic DNA was isolated, treated with bisulfite, and MS-PCR was performed. Vehicle-treated cells served as a control. The *RARB2, p21,* and *p16* gene promoters were methylated in LNCaP cells (Figure 2A). As a loading control, amplification of all *p16* promoter sequences present in the DNA preparation, regardless of methylation status, was also performed. Exposure to SB reversed the methylation of the *p21* and *RARB2* gene promoters, and *p16* promoter methylation was reduced. At lower, suboptimal concentrations of SB (0.5 mM), the reversal of *RARB2* methylation was complete, and *p21* methylation was markedly reduced, but *p16* methylation was not affected, indicating some gene-selective, concentration-dependent effects of SB. Exposure to the structurally distinct HDACi SAHA reversed the promoter methylation of these same genes in a similar pattern.

These results suggest that the methylation of silenced tumor suppressor genes could be reversed by exposure to different classes of HDACi.

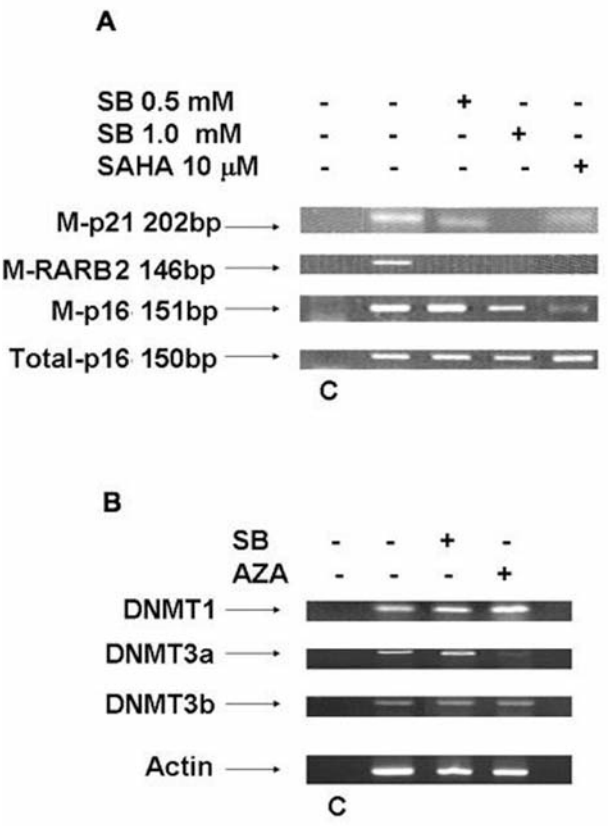

Figure 2. *(A) DNA methylation analysis of the p21, RARB2, and p16 promoters. LNCaP cells were treated with sodium butyrate (SB) (0.5 mM or 1 mM) or suberoylanilide hydroxamic acid (SAHA) (10 μM) for 24 h. Genomic DNA was isolated and treated with bisulfite. After purification, MS-PCR was performed. Lane C represents a negative PCR control. The bottom panel shows total (methylated and unmethylated) p16 in each sample, as a loading control. (B) Transcript levels of DNMTs in cells exposed to HDAC or DNA methylase inhibitors: LNCaP cells were treated for 48 h with SB (1 mM) or 5-aza-2'-deoxycytidine (AZA) (25 μM). Total RNA was isolated and cDNA was prepared. PCR was performed for DNMTs 1, 3a, and 3b, and β-actin. Lane C represents a negative PCR control. The bottom panel shows the total actin transcript in each sample, as a loading control.*

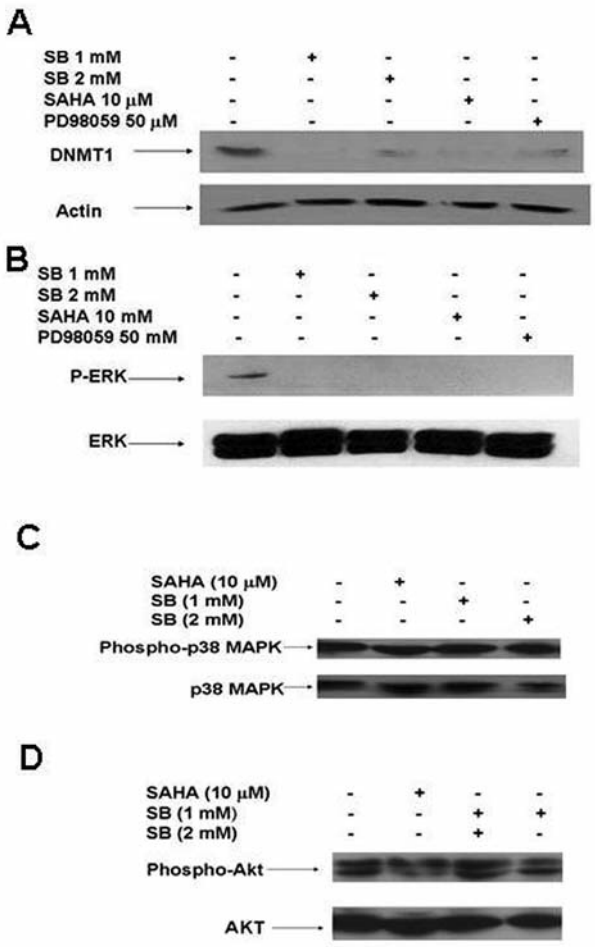

Figure 3. *(A) DNMT1 protein levels after exposure to HDAC inhibitors. LNCaP cells were treated for 24 h with sodium butyrate (SB) (1 mM and 2 mM), suberoylanilide hydroxamic acid (SAHA) 10 μM, or PD98059 50 μM. Cells were washed and lysed, and proteins were separated by a 6% SDS-PAGE, transferred to nylon membrane and blotted with a DNMT 1-specific antibody, then stripped and re-blotted with a β-actin antibody. (B) Phosphorylation status of ERK, (C) p38 MAP kinase (MAPK) and (D) AKT after exposure to HDAC inhibitors. LNCaP cells were treated for 24 h with SB (1 mM or 2 mM) or SAHA (10 μM). Cells were washed, and lysed, and proteins were separated by a 7.5% SDS-PAGE, transferred to nylon membrane and blotted with phospho-ERK, phospho-p38 MAPK or phospho-AKT antibodies. The blots were stripped and re-blotted with antibodies against ERK, p38 MAPK or AKT respectively. The top photographs in each panel show the phospho-protein-specific blots and the bottom photographs show the level of total (phosphorylated and unphosphorylated) protein, which also serves as a loading control.*

HDACi do not affect the levels of DNMT1, 3a or 3b transcripts. As HDACi can alter the expression of genes, the effects of such agents on the expression of *DNMT1*, *DNMT3a* and *DNMT3b*, the three DNA methyl transferases which regulate the methylation status of DNA CpG motifs in gene promoters, was assessed. 5-Azacytidine (AZA), a well-established inhibitor of DNA methylation, inhibits the expression of one or more of these three enzymes (depending upon the cell type studied), and the enzyme activities, and was used as a control. Transcript levels of DNMTs 1, 3a and 3b were assessed in cells exposed to the HDACi SB (or AZA), using quantitative RT-PCR analysis. While exposure to AZA repressed *DNMT3a* transcript levels, SB had no detectable effect on *DNMT1, 3a* or *3b* transcript levels (Figure 2B). Exposure to SAHA similarly had no effect on the transcript levels of any *DNMT* isozyme (not shown).

Down-regulation of DNMT1 protein levels by HDAC inhibitors. A recent report demonstrated that exposure to AZA down-regulated *DNMT1* protein levels *via* enhancement of a

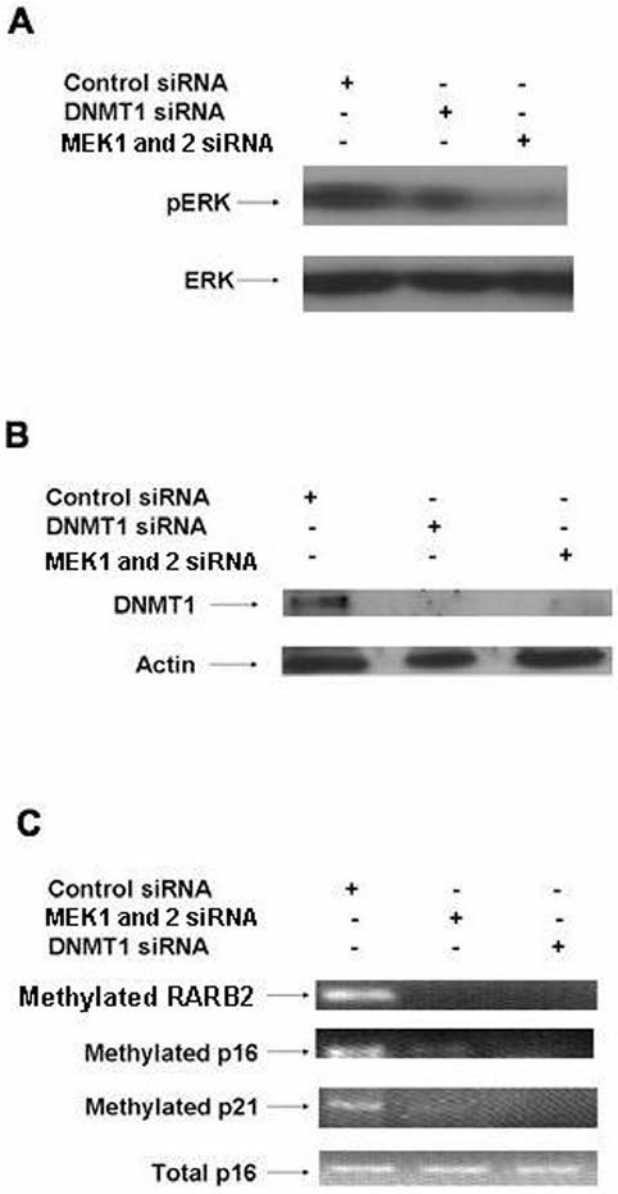

Figure 4. *Deregulation of ERK activity and DNMT1 by siRNA. (A) LNCaP cells were transiently transfected with MEK 1 and 2-specific siRNA or (B) DNMT1-specific siRNA (all at 1 μM). The media were replaced at 24 h and repeat transfection was performed. Cells were washed free of transfection mixture at 72 h. One portion of each of the cultures was lysed and proteins extracted. (A) Proteins were separated by a 7.5% SDS-PAGE, transferred to a nylon membrane and immunoblotted using phosphor-ERK-specific antibody, stripped and re-blotted with an anti-ERK antibody. The top panel shows levels of phosphorylated ERK and the bottom panel demonstrates the level of total ERK in each sample. (B) Proteins were separated by a 6% SDS-PAGE, transferred to nylon membrane and immunoblotted using a DNMT1-specific antibody (top panel), stripped, and re-blotted using a β-actin antibody (bottom panel) which showed loading. (C) Another portion of the treated or control cells at 72 h were used for isolation of genomic DNA. After treating with bisulfite and purification, MS-PCR was performed with each sample, using primers for the genes RARB2, p16, and p21. The bottom panel represents total p16 PCR products, as a loading control.*

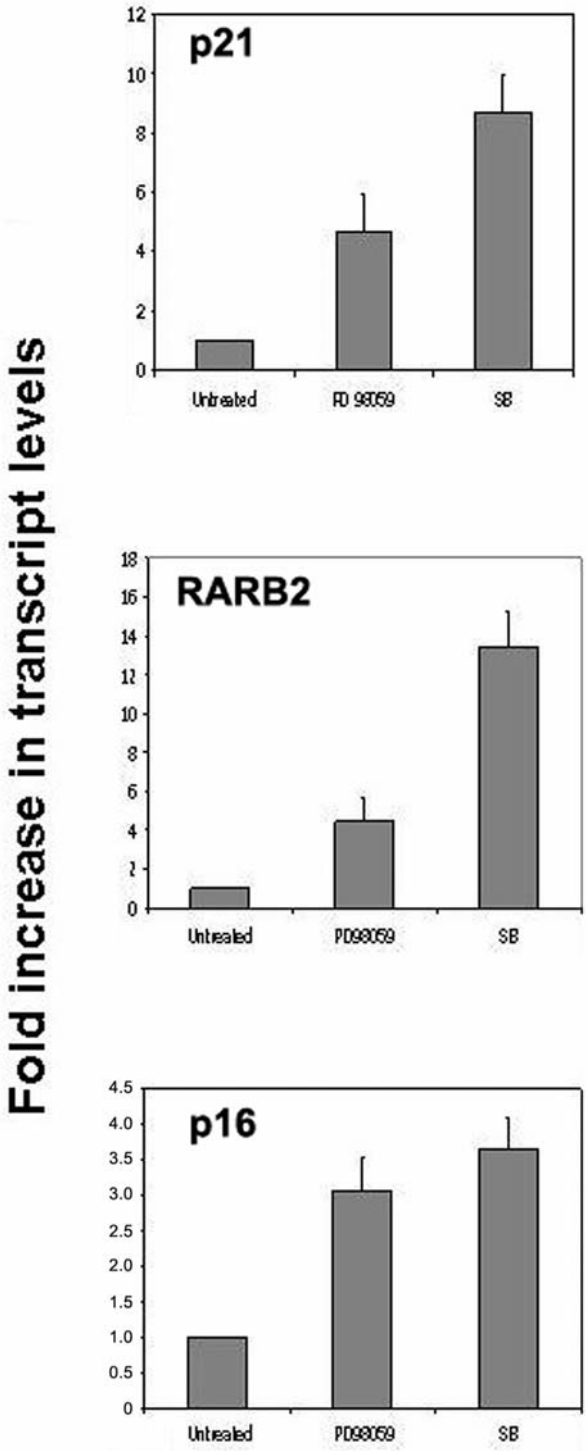

Figure 5. *Gene expression assessed via transcript levels of p21, RARB2, and p16 after exposure to HDAC inhibitors. LNCaP cells were treated for 48 h with sodium butyrate (1 mM) or PD98059 (50 μM). Total RNA was isolated and cDNA prepared. Real-time PCR for p21, RARβ2, and p16 transcripts was performed. Results for each gene in each sample were normalized to the level of β-actin transcripts. The results are expressed as fold increase over the untreated control. The results are the average of 3 sets of independent experiments. Bars indicate the standard deviation.*

proteosome-mediate degradation pathway in HeLa cells (18). We therefore next assessed whether HDACi similarly alters the levels of *DNMT1* protein in LNCaP cells. Lysates of LNCaP cells treated with HDACi and/or *ERK* activation inhibitor PD98059 were immunoblotted with an antibody specific for the *DNMT1* protein. Levels of *DNMT1* protein were barely detectable after 24 h of exposure to SB, SAHA, or PD98059 (Figure 3A).

HDAC inhibitors suppress activating phosphorylation of ERK. A recent study suggested that DNA methylation in colon cancer cells and in NIH 3T3 cells may be regulated by *ERK* activity (4). To determine if HDACi might be exerting effects on DNA methylation through *ERK*, we first looked for effects of HDACi on *ERK* activity, as assessed by activating phosphorylation of *ERK*. LNCaP cells were exposed to SB, SAHA, or vehicle for 24 h. Immunoblotting of the cell lysates was carried out using antibodies against *ERK* and phospho-*ERK*. *ERK* phosphorylation was completely inhibited by exposure to the HDACi, while total levels of *ERK* protein were unaffected (Figure 3B). In contrast, there were no consistent effects of HDACi on the levels of total or phospho-p38 MAP kinase or total or phospho-AKT (Figure 3C and 3D). These results raised the possibility that HDACi might be regulating DNA methylation and gene expression through *ERK*.

If HDACi regulate *DNMT1* levels through their effects on *ERK1*, then repression of ERK1 by independent means should produce the same effects on *DNMT1* levels. *MEK1* and *MEK2* are upstream signaling kinases, which uniquely activate *ERKs* by phosphorylation, but do not alter levels of total *ERK* protein. To repress *ERK1* activity, *MEK1* and *MEK2* transcripts and protein were suppressed by specific siRNAs. Immunoblotting for phospho-ERK and total ERK levels demonstrated efficient suppression of *ERK* activity (as assessed by phospho-ERK levels) by *MEK1* and *MEK2* siRNAs (Figure 4A). Total ERK protein levels were not changed. Immunoblot analysis of cell lysates after suppression of *ERK* activity by *MEK1* down-regulation revealed that *DNMT1* protein levels were profoundly suppressed (Figure 4B), indicating that *ERK1* is upstream of *DNMT1* in this regulatory process.

Down-regulation of DNMT1 expression by siRNA, or by repression of ERK1, reverses promoter DNA methylation. If HDACi regulate promoter DNA methylation through their suppression of *DNMT1* levels, then repression of *DNMT1* by independent means should be sufficient to produce the same effects on promoter methylation as HDACi. *DNMT1* protein levels were independently suppressed by specific siRNA, and suppression was verified by immunoblotting (Figure 4B). We then determined whether specific repression of *DNMT1* reversed the promoter methylation of the tumor suppressor

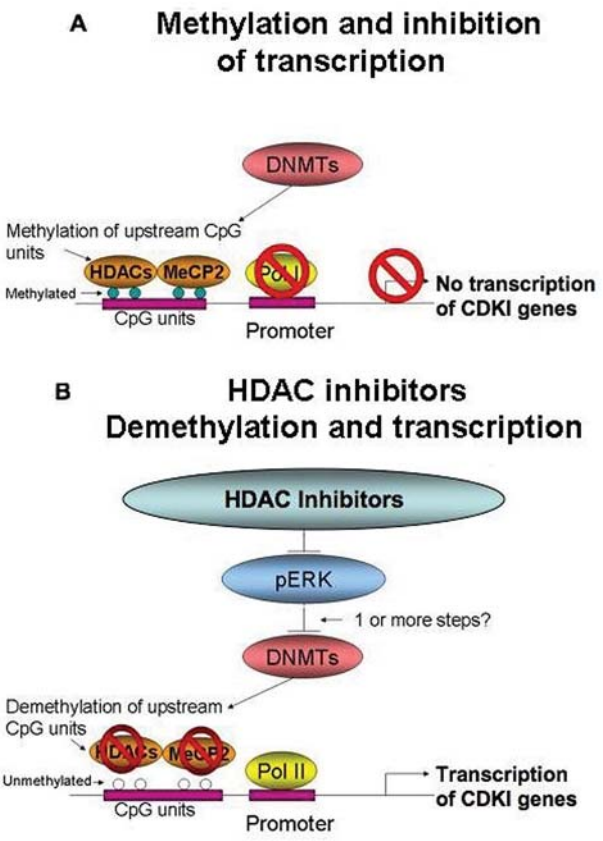

Figure 6. *(A) Model of inhibition of transcription directed by methylation of CpG islands in gene promoter regions. (B) Proposed model of control of gene repression by HDAC inhibitors, wherein HDAC inhibitors reverse DNA methylation and gene silencing.*

genes *p21, p16* and *RARB2*, using MS-PCR on genomic DNA samples obtained from cells treated with *DNMT1*-specific, or control, siRNA. MS-PCR products, indicating CpG methylation, were reduced (*p16, p21*) or absent (*RARB2*) in reactions using genomic DNA from the cells in which *DNMT1* levels had been reduced by *DNMT1* siRNA, but were not altered in untreated or control-siRNA–treated cells (Figure 4C), demonstrating that repression of *DNMT1* alone is sufficient to reverse methylation of these genes. The down-regulation of *DNMT1* by siRNA did not inhibit ERK phosphorylation (Figure 4A), showing that, whereas *ERK* activity influences *DNMT1* levels, *DNMT1* is not an upstream regulator of *ERK* activity.

We therefore next determined whether specific repression of *ERK* activity reversed the promoter methylation of the tumor suppressor genes *p21, p16* and *RARB2*, using MS-PCR on genomic DNA samples obtained from cells treated with *MEK1, MEK2*-specific or control siRNA. MS-PCR products, indicating CpG methylation, were reduced (*p16,*

p21) or absent (*RARB2*) in reactions using genomic DNA from the cells in which *ERK* activity had been reduced by *MEK1* or *MEK2* siRNA but were not altered in untreated or control-siRNA–treated cells (Figure 4C), paralleling the findings obtained with the *DNMT1* siRNA. Thus, repression of *ERK* activity alone, like that induced by HDACi, is sufficient to alter the methylation of these genes. Complementary studies, using the *MEK1* and *MEK2* inhibitor PD98059 instead of siRNA to repress *ERK* activity, confirmed that suppression of *ERK* activity by independent means also reversed methylation of the *p16, p21* and *RARB2* promoters and repressed *DNMT1* levels (data not shown).

HDAC or ERK inhibition induces re-expression of tumor suppressor genes RARB2, p21, and p16. As we have demonstrated that HDACi regulate *ERK* activity, which is sufficient to repress *DNMT1* levels and thereby modulate DNA promoter methylation, we next determined whether HDAC (or *ERK*) inhibition allows re-expression of silenced tumor suppressor genes in LNCaP cells. Cells were treated with SB, the specific *MEK1, MEK2* inhibitor PD98059, or vehicle for 48 h, and transcript levels of *RARB2, p21, p16,* and β-actin were examined by quantitative RT-PCR. Transcript levels of all three tumor suppressor genes were significantly increased after exposure to the inhibitors of HDAC and *ERK* activation, relative to β-actin ($p<0.02$) (Figure 5). The relatively low induction of *p16* transcripts compared to *p21* and *RARB2* transcripts may correlate with the only partial demethylation of the *p16* promoter induced by HDACi or *ERK* inhibition.

Collectively, the findings above demonstrate that HDACi repress *ERK* activity, which is sufficient to repress *DNMT1* protein levels, which, in turn, is sufficient to reverse DNA methylation, thereby resulting in the re-expression of silenced genes.

Discussion

DNA methylation on CpG motifs in gene promoters results in the recruitment of HDACs (with the co-localization of MeCP2 and other regulatory proteins) to the transcriptional regulatory site, altering local chromatin structure and inhibiting transcription complex and Pol II binding, thereby impeding initiation of transcription (18). A simplistic model of this mechanism of inhibition is presented in Figure 6A.

In this study, we determined the effects of HDACi exposure on prostate cancer cell cycle progression. In accordance with our previous findings, we observed cell cycle inhibition after only about 48 h of HDACi exposure, whereas the promoter demethylation and the down-regulation of *DNMT1* were observed within 24 h. Only 15-20% of the cell population showed evidence of cell cycle arrest in the G_0/S phases after 72 h of treatment, indicating that the demethylation process preceded any significant cell cycle arrest. DNMTs may be regulated in a cell-cycle-dependent pattern in some cells (19). The level of DNMT expression is lower during the G_0/G_1 phase, but cancer cells maintain higher methylation levels compared to normal cells even in the G_0/G_1 phase. Others have observed that during stimulation of T-cells, demethylation of the interleukin-2 (IL-2) gene occurs as early as 20 minutes, indicating that methylation and demethylation could be dynamic processes in cells and may operate independently of the regulation of DNMTs during the cell cycle (20). This observation supports the conclusions from our work and the T-cell study that induction of the gene demethylation process can be independent of cell cycle regulated demethylation and is a dynamic process.

Prior to exposure to HDACi, the *p21, RARB2* and *p16* genes were methylated and silenced in prostate cancer cells. This re-expression after exposure to HDACi might be the direct result of their demethylation, or there might also be an additional transcriptional component contributed by the HDACi once the silencing methylation is reversed, at least in the case of the *p21* gene. For example, the *p21* gene (when unmethylated) can be induced by p53 family members, or through 'butyrate-responsive' elements in its promoter (6). In addition, in acute myeloid leukemia cells, the DNA methylation inhibitor AZA reversed the methylation of *p73* gene, allowing expression of its transcripts, which in turn induced *p21* (21). Interestingly, a recent study showed that *p21* can negatively regulate *DNMT1* expression in certain cells (22). Conversely, our results in human prostate cancer cells demonstrated that down-regulation of *DNMT1* by HDACi increased expression of *p21*. Under normal circumstances, *p21* is not likely to regulate *DNMT1* in these prostate cells, because the *p21* gene is silenced by methylation (10, 17). It thus appears that, depending upon the type of cell and the external conditions, *DNMT1* can regulate *p21* expression, and *vice versa*.

DNMT enzyme activity has been reported to be regulated at the level of expression (transcription) or enzymatic activity, as well as post-transcriptionally (18). Our results showed that HDACi had no detectable effect on the transcript levels of any of the three DNMTs, while, in parallel control cultures, exposure to AZA inhibited *DNMT3a* transcript levels.

Exposure to HDACi down-regulated *DNMT1* protein levels within 24 h. This time-frame of *DNMT1* down-regulation corresponds well with the kinetics of promoter demethylation which was observed within 24 h. The down-regulation of *DNMT1* protein in LNCaP cells might be mediated by proteosomal degradation, a regulatory mechanism recently observed in breast cancer cells (23), and this possibility is currently being explored. This repression

of *DNMT1* levels by exposure to HDACi is likely the mechanism by which the silencing of these tumor suppressor genes is reversed, as the down-regulation of *DNMT1* by siRNA was sufficient to demethylate and allow re-expression of all the three genes tested.

We found that HDACi exposure inhibited the activating phosphorylation of ERK. We also observed that exposure to the *ERK* activation inhibitor PD 98059 down-regulated *DNMT1* in LNCaP cells within 24 h. *MEK1* and *MEK2*-specific siRNA treatment (*MEK1* and *MEK2* are the activating upstream kinases of *ERK*) down-regulated *DNMT1* and produced demethylation of *p21, p16,* and *RARB2*. These results are consistent with a model in which *ERK* is an upstream regulator of *DNMT1*. Whereas ERK inhibition down-regulated *DNMT1*, suppression of *DNMT1* did not inhibit *ERK* activity, supporting this model. Studies from colon cancer showed similar results (5). Collectively, our results demonstrated that HDACi inhibited *ERK* activity, which then suppressed *DNMT1* levels within 24 h. Down-regulation of *DNMT1* resulted in demethylation of CDKIs and tumor suppressor genes to cause cell cycle arrest, which was observed at around 48 h. Our model is supported by a previous study which showed that *DNMT1* knock-down caused cell cycle arrest (24). It remains to be determined how HDACi inhibits *ERK* activation, although our preliminary results do not suggest a direct inhibitory effect on *ERK* phosphorylation (A. Takashima and DVF, unpublished data). How *ERK* modulates *DNMT1* protein levels, and whether this is proteosome-mediated, is currently under investigation.

We propose a model, supported by the studies detailed herein, wherein HDACi can influence the initial methylation status of the DNA (Figure 6B). HDACi suppress *ERK* activity, which in turn down-regulates *DNMT1* protein levels, post-transcriptionally. Depending upon the cell type, the down-regulation of *DNMT1* could be a result of either transcriptional inhibition or post-translation degradation. *DNMT1* down-regulation then results in the demethylation of CpG motifs in the upstream regions of silenced promoters, disenabling the recruitment of repressor complexes, and permitting Pol II recruitment and initiation of transcription, with consequent expression of formerly silenced genes.

Our findings therefore suggest that multiple signaling pathways may regulate *DNMT* activity under different circumstances and in different cell types. Furthermore, in light of the wide-spread utilization of DNA methylation for gene silencing and ultimate regulation of diverse cellular processes in normal and malignant cells, and the current intense interest in both general and isozyme-specific HDACi as therapeutics, our findings that HDACi can influence gene expression at levels other than that of histone acetylation may have important and broad implications.

Acknowledgements

This study was supported by the National Institutes of Health (CA112102 [DVF] and DK52962 [SPP] and the Department of Defense [W81XWH-06-1-0408 and W81XWH-07-1-0577 (DVF)] and the Karin Grunebaum Cancer Research Foundation (DVF). Sibaji Sarkar is a recipient of an ACS Grant IRG-72-001-34. We thank Dr. Sam Thiagalingam of Boston University School of Medicine for reagents and for technical help. We also thank Dr. Sarmila Majumder and Dr. Kalpana Ghosal of Ohio State University, Columbus, OH, USA, for their generous gifts of reagents and for technical advice.

This work was presented in part at the Genomics, Gene Regulation and System Biology meeting at Cold Spring Harbor Laboratory, NY in March, 2008, at the MIT Genome Regulation, System Biology and Computational Biology Symposium in October, 2008 and in the Annual Meeting of American Association of Cancer Research, Denver, in April 2009.

Contributions of Authors

DVF, SS and SPP designed the studies and evaluated the results. SS, ALA, LWF and JEL performed experiments. DVF and SS wrote the manuscript.

References

1 Sorcha M, Cusack TT, Rohn RJ, Medeck KM, Irwin RJ, Brown LM and Julia T: Suppression of MeCP2β expression inhibits neurite extension in PC12 cells. Exp Cell Res *299*: 442-453, 2004.

2 Leverson JM and Sweatt DW: Epigenetic mechanisms in memory formation. Nature. Rev Neuroscience *6*: 108-118, 2005.

3 Latham T, Gilbert N and Ramsayoe B: DNA methylation in mouse embryonic stem cells and development. Cell Tissue Res *331*: 31-55, 2008.

4 Lu R, Wang X, Chen ZF, Sun DF, Tian ZQ and Fang JY: Inhibition of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway decreases DNA methylation in colon cancer cells. J Biol Chem *282*: 12249-12259, 2007.

5 Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE *et al*: Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res *66*: 6361-6369, 2006.

6 Vaziri C, Stice LL and Faller DV: Butyrate-induced G_1 arrest results from p21 disruption of retinoblastoma-protein mediated signals. Cell Growth Diff *9*: 465-474, 1998.

7 Chen JS and Faller DV. Histone deacetylase inhibition-mediated post-translational elevation of $p27^{Kip1}$ protein levels is required for G1 arrest in fibroblasts. J Biol Chem *202*: 87-99, 2005.

8 Chen JS, Faller DV and Spanjaard RA: Short-chain fatty acid inhibitors of histone deacetylases: promising anticancer therapeutics? Current Cancer Drug Targets *3*: 219-236, 2003.

9 Mork CA, Spanjaard RA and Faller DV: A mechanistic approach to anticancer therapy: targeting the cell cycle with histone deacetylase inhibitors. Curr Rev Pharmacol *11*: 1091-1104, 2005.

10 Gu J, Zhao X, Spanjaard RA, Chen TC, Flanagan JN, Boosalis M, Perrine SP and Faller DV: Synergistic activity of retinoids and histone deacetylase-inhibitors on growth suppression, differentiation, and apoptosis in human prostate cancer cell lines. J Cancer Mol *2*: 25-36, 2006.

11 Nakayama T, Wantanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, Yatani R and Shiraishi T: The role of epigenetic modifications in retinoic acid receptor β2 gene expression in human prostate cancers. Lab Invest *81*: 1049-1057, 2001.

12 Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA and Baylin SB: Incidence and functional consequences of *hMLH1* promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA *95*: 6870-6875, 1998.

13 Vaziri C and Faller DV: Repression of platelet-derived growth factor beta-receptor expression by mitogenic growth factors and transforming oncogenes in murine 3T3 fibroblasts. Mol Cell Biol *15*: 1244-1253, 1995.

14 Sarkar S, Rooney MM and Lord ST: Activation of integrin-β3-associated syk in platelets. Biochem J *338*: 677-680, 1999.

15 Sarkar S: Tyrosine phosphorylation and translocation of LAT in platelets. FEBS Lett *441(3)*: 357-360, 1998.

16 Sarkar S, Svoboda M, de Beaumont R and Freedman AS: The role of AKT and RAFTK in beta1 integrin-mediated survival of precursor B-acute lymphoblastic leukemia cells. Leuk Lymphoma *43(8)*: 1663-1671, 2002.

17 Jarrard DF, Bova GS, Ewing CM, Pin SS, Nguyen SH, Baylin SB, Cairns P, Sidransky D, Herman JG and Isaacs WB: Deletional, mutational, and methylation analyses of *CDKN2* (*p16/MTS1*) in primary and metastatic prostate cancer. Genes Chromosome Res *19*: 90-96, 1997.

18 Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T and Jacob ST: 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteosomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. Mol Cell Biol *25*: 4727-4741, 2005.

19 Robertson KD, Keyomarsi K, Gonzales A, Velicescu M and Jones PA: Differential mRNA expression of the human DNA methyl transferases (DNMTs) 1, 3a, 3b during the G_0/G_1 to S phase transition in normal and tumor cells. Nucleic Acid Res *28*: 2108-2113, 2000.

20 Bruniquel D and Schwartz RH: Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nature Immunol *4*: 235-240, 2003.

21 Schmelz K, Wagner M, Dorken B and Tamm I: 5-Aza-2'-deoxycytidine induces $p21^{WAF}$ expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia. Int J Cancer *114*: 683-695, 2005.

22 Tan HH and Porter AG: p21 (WAF1) negatively regulates DNMT1 expression in mammalian cells. Biochem Biophys Res Commun *382*: 171-176, 2009.

23 Zhou Q, Agoston AT, Atadja P, Nelson WG and Davidson NE: Inhibition of histone deacetylases promotes ubiquitin-dependent proteosomal degradation of DNA methyl transferase1 in human breast cancer cells. Mol Cancer Res *6*: 873-883, 2008.

24 Milutinovic S, Zhuang, Q, Niveleau A and Szy M: Epigenomic stress response. J Biol Chem *278*: 14985-14995, 2003.

Received May 11, 2011
Revised June 12, 2011
Accepted June 13, 2011