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

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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.

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-azacytidine, 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 acid (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 (CDKIs) p21 and p16 (6-9), as well as genes whose products can induce differentiation, such as retinoic acid receptor β2 (RARβ2) (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 DNMT1, 5a, 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 quantification 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...
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

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.
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 G1 phase population (Figure 1C). The G1 arrest observed is typical of the effect of HDACi on cell cycle progression (8, 9). We, and other investigators, have previously demonstrated that this G1 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 CDK1 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.

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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16UM</td>
<td>TTATTAGGGTGGGGTGAATTGT</td>
<td>CAACCCCAAACCAACACATAA</td>
</tr>
<tr>
<td>p16M</td>
<td>TTATTAGGGTGGGGGCGGATCGC</td>
<td>GACCCGAAACCGGACCGCTAA</td>
</tr>
<tr>
<td>p21M</td>
<td>TTTTAGATGTGTTGAGGTTTTG</td>
<td>AACACAACTCAACACACCTTA</td>
</tr>
<tr>
<td>RARB2</td>
<td>TGGGACGCGGACCGATCG</td>
<td>GACCAATCCACCGAAACGA</td>
</tr>
</tbody>
</table>

UM=methylated, M=unmethylated.

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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARB2</td>
<td>AGGGAAAAGGGAAGGGCAAG</td>
<td>AGAAAAACACATCCAGGTCG</td>
</tr>
<tr>
<td>DNMT1</td>
<td>AGGGAAAAGGGAAGGGCAAG</td>
<td>AGAAAAACACATCCAGGTCG</td>
</tr>
<tr>
<td>DNMT3a</td>
<td>CAGGGTCAACAGAAGCATATCC</td>
<td>GTGCTCTACCATTTGTGAACTTG</td>
</tr>
<tr>
<td>DNMT3b</td>
<td>CCTGGCTGAAATTCTCAACGCCCC</td>
<td>GTCCTGTATGTCAGACAGGAAA</td>
</tr>
<tr>
<td>Actin</td>
<td>CGGACCCACACGACATAG</td>
<td>GGACACCGAGGCCAGAA</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARB2</td>
<td>AAAAAAGGGAAGGGCAAG</td>
<td>GGGGAAAAGGCCCTACATCC</td>
</tr>
<tr>
<td>p21</td>
<td>CTGGGAGACCTCAAGGTCG</td>
<td>AGATTAGGGCTTTCTTCGGA</td>
</tr>
<tr>
<td>p16</td>
<td>CATAGATGCCGCCGGAAGGT</td>
<td>CAGAGCTCTCTTTGTGTTCTTCAA</td>
</tr>
<tr>
<td>Actin</td>
<td>CTGGCACCACGACAAATG</td>
<td>GGACACCGAGGCCAGAA</td>
</tr>
</tbody>
</table>
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, 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 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 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.
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, RARB2, 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 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,
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 DNMTs (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 G0/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 G0/G1 phase, but cancer cells maintain higher methylation levels compared to normal cells even in the G0/G1 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. Takahshima 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, disenable 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.

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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


