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Class I HDACs are mediators of smoke-carcinogen induced stabilization of DNMT1 and serve as promising targets for chemoprevention of lung cancer

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Abstract

DNA methylation is an early event in bronchial carcinogenesis and increased DNA methyltransferase (DNMT)1 protein expression is a crucial step in the oncogenic transformation of epithelia. Here, we investigate the role of class I histone deacetylases (HDACs) 1–3 in the stabilization of DNMT1 protein and as a potential therapeutic target for lung cancer chemoprevention. Long-term exposure of immortalized bronchial epithelial cells (HBEC-3KT) to low doses of tobacco-related carcinogens led to oncogenic transformation, increased HDAC expression, cell cycle independent increased DNMT1 stability and DNA hypermethylation. Overexpression of HDACs was associated with increased DNMT1 stability and knockdown of HDACs reduced DNMT1 protein levels and induced DNMT1 acetylation. This suggests a causal relationship among increased class I HDACs levels, upregulation of DNMT1 protein, and subsequent promoter hypermethylation. Targeting of class I HDACs with valproic acid (VPA) was associated with reduced HDAC expression and a profound reduction of DNMT1 protein level. Treatment of transformed bronchial epithelial cells with VPA resulted in reduced colony formation, demethylation of the aberrantly methylated SFRP2 promoter and de-repression of SFRP2 transcription. These data suggest that inhibition of HDAC activity may reverse or prevent carcinogen induced transformation. Finally, immunohistochemistry on human lung cancer specimens revealed a significant increase in DNMT1, HDAC1, HDAC2, and HDAC3 expression, supporting our hypotheses that class I HDACs are mediators of DNMT1 stability.

In summary, our study provides evidence for an important role of class I HDACs in controlling the stability of DNMT1 and suggests that HDAC inhibition could be an attractive approach for lung cancer chemoprevention.

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Conflict of Interest: none

Introduction

Epigenetic alterations have been identified as key events in the pathogenesis of NSCLC carcinogenesis(1). Aberrant methylation is common in lung cancer precursor lesions such as dysplasia and atypical adenomatous hyperplasia(2, 3). Detection of aberrant methylation in the sputum of either current or former smokers can serve as a marker for increased lung cancer risk(4). The DNA methyltransferase (DNMT)1 mediates the transfer of acetyl-groups from S-adenosyl-methionine to cytosine residues in the DNA and is required for maintenance of DNA methylation(5). Smoke carcinogen exposure leads to increased DNMT1 protein expression and subsequent de-novo methylation(6–8). Inhibition or knockdown of DNMT1 leads to decreased colony formation of transformed bronchial epithelial cells (7) and decreased tumor counts in mouse models of carcinogen induced lung cancer (9), implicating DNMT1 as a critical mediator of early smoking related bronchial carcinogenesis.

DNMT1 functions during S-phase and its protein levels are tightly regulated throughout the cell cycle(10). DNMT1 turnover is regulated by posttranslational modifications such as acetylation(11–13), phosphorylation (14) and methylation(15, 16). The dominant mechanism for increased DNMT1 protein expression after carcinogen-exposure has not yet been determined (6–8).

HDACs were originally identified as transcriptional repressors, counteracting the activity of histone acetyltransferases that activate transcription by acetylation of histone tails, thereby loosening the DNA/core histone interaction and providing a permissive chromatin state for transcriptional machinery. To date several classes of HDACs have been identified: Class I HDACs (HDAC-1,-2,-3,-8) are mostly localized in the nucleus and target proteins involved in the regulation of gene transcription. HDAC3 is unique in this list because it is also found in the cytoplasm. Here, it is involved in src-signaling and has been found to act as a chaperone for the TR2 receptor in promyelocytic leukemia (17).

In lung cancer, increased mRNA levels of HDAC1 have been associated with higher stage and worse prognosis (18, 19). Similar findings have been reported for HDAC3 (20). Recently, genome-wide analyses of copy number changes, DNA methylation patterns and gene expression changes in a large set of lung cancers of adeno-(AC) and squamous cell (SCC) origin revealed important differences in gene expression patterns between these two histologic subtypes. Further *in silico* compound screens suggested that the pattern of gene expression signature associated with SCC in particular, may be altered by HDAC inhibition (21).

Clinically, HDAC inhibitors have shown promise for the treatment of advanced NSCLC either in combination with chemotherapy (22) or in combination with the demethylating agent 5-azadeoxycytidine (23). These studies focused on advanced cases of lung cancer and show a somewhat limited efficacy of HDAC inhibition in treating lung cancer in this setting. Here, we focused on the role of HDACs in early lung carcinogenesis. We used a model of long-term exposure of human bronchial epithelial cells to smoke carcinogens and examine the relationship between HDACs and DNMT1 during, and to investigate strategies to target, this critical early event in bronchial carcinogenesis.

We identified a biochemical interaction between DNMT1 and class I histone deacetylases (HDAC)s after carcinogen exposure as the primary mechanism responsible for DNMT1 protein stabilization and up-regulation. Furthermore, we find a significant increase in DNMT1 and class I HDACs in primary lung tumor samples, and characterize the effects of

HDAC inhibitors in overcoming tobacco-induced epigenetic changes. This study points to a potential role for HDAC inhibition in chemoprevention of aerodigestive carcinogenesis.

Materials and Methods

Constructs and transfection

DNMT1 (NM_001130823.1), HDAC1 and HDAC3 cDNAs were obtained from Open Biosystems (Waltham, MA). An N-terminally truncated DNMT1 (encoding aa 121–1616) cDNA and shSET7 and control vectors were gifted by Dr. Paula Vertino(24). HDAC2 cDNA was purchased from DNASU (ORFeome consortium). cDNAs were cloned into pDEST51-vectors expressing a C-terminal H6-V5 tag (Invitrogen) and into pDEST-27 vectors expressing an N-terminal GST-tag. Vectors were transfected using Lipofectamine-2000 (Invitrogen).

Cell lines

Human Bronchial Epithelial cells immortalized with hTERT and cdk4 (3KT)(25) were exposed to 500uM Methyl-nitroso-urea (MNU) and 50nM Benz(a)pyrene (BaP) for 24 hrs per week with 6 days of out-growth post exposure(7). 3KT cells treated in this manner for 31 weeks are designated T31. 3KT cells were exposed to vehicle control and cultured in parallel for the same duration to account for possible changes in epigenetic gene regulation induced by long-term cultures. Stable lines expressing DNMT1 were established with treatment of transfected T31 cells with 10uM Blasticydin. Stable HDAC3 overexpressing cells were established by treating transfected 3KT cells with 10uM Blasticydin. In some experiments VPA was added at 0.1–1mM for indicated time. MG-132 was added at 2.5uM for 16hrs or 25uM for 3 hours as indicated. All cell lines used were tested for Mycobacterium contamination by Bionique testing labs. 3kt and T31 were authenticated through STR analysis by biosynthesis Inc.

Soft Agar Assay

3KT and carcinogen exposed cells were seeded at 1000 cells per well in 0.35% agarose containing growth medium into 6well cell culture dishes coated with 0.5% bottom agar containing RPMI/10%FBS/1%pen-strep. Feeding medium was added to the top agar once solidified and replenished 3 times weekly. Sodium Valproate (VPA) was added in the top agar and feeding medium as indicated. Cells were allowed to grow for 21 days, stained with crystal violet and scored on a low-power objective light microscope.

Protein Precipitations

Cells expressing H6-tagged truncated-DNMT1 were lysed in NTA lysis buffer (50mM Sodium Phosphate, 300mM NaCl, 10mM Imidazole, 0.05% Tween-20 pH8) containing PMSF, protease and phosphatase inhibitors. After sonication and clarification by centrifugation, DNMT1 was affinity purified using nickel-nitrilotriacetic acid (NiNTA) magnetic agarose (Qiagen). Cells expressing GST-tagged HDAC and V5-tagged DNMT1 proteins were lysed in 1%NP40 buffer (50mMTris pH 8, 150mM NaCl, 0.5mM EDTA, 1%NP40) containing PMSF, protease and phosphatase inhibitors. GST-tagged proteins were isolated using a glutathione sepharose resin (GE healthcare) while V5-tagged constructs were immunoprecipitated using V5 antibodies (Invitrogen) and eluted with V5 peptide (Alpha Diagnostic). In Co-immunoprecipitation experiments, DNaseI (900U/ml) (Invitrogen) was added before precipitation to rule out the possibility that a protein-protein interaction may be mediated by chromatin.

Flow Cytometry

Cells were synchronized with 5uM aphidocholine for 24 hrs, released for the indicated time (0, 6, 12, 24hrs) and fixed in 70% ice cold Ethanol. Cells were stained with 7-Aminoactinomycin D (7AAD) 250ng/mL in the presence of 100ug/mL RNase A. Cells were counted on a BD-FACSCANTO II instrument and analyzed on DIVA and FLOW-Jo software.

Immunoblotting

Cells were lysed in 1× cell lysis buffer (Cell Signaling), containing Complete protease inhibitor and Phostop (Roche) and 1mM PMSF. Cells were sonicated briefly and lysates clarified by centrifugation. Following SDS-PAGE and semi-dry transfer the following antibodies were used: Acetylated Lysine, EZH2, G9a, H3K27Me3, (Cell Signaling), H3K9Me3 (AbCam) H3K4Me2, Acetyl H3K9/K14 (Millipore), HDAC1 (Immunechem and Cell Signaling), HDAC2 (Santa Cruz, Cell Signaling), HDAC3 (Cell Signaling, Abgent), V5 (Invitrogen) DNMT1 (Abcam and EU101 generously provided by Dr. P Vertino). Total H3 (Upstate), Beta Actin and Beta Tubulin (Sigma) GAPDH (Cell Signaling) were used as loading controls depending on the application and molecular weights of target proteins in the experiment.

Methylation Specific PCR

Primers for bisulfite-sequencing, and methylation specific PCR were designed using MSPPRIMER.org(26). Sequences are available upon request. Genomic DNA was bisulfite converted using EZ DNA Methylation Kit (Zymo research) and subjected to nested MSP. Briefly, unbiased primers outside of the CpG island were used to pre-amplify bisulfite converted DNA. The resultant amplicon was further amplified with primers specific for the methylated or unmethylated form of the expected amplicon. Bisulfite sequencing was performed using a nested primer set designed for MSP using bisulfite converted genomic DNA as above. PCR reactions were cloned into pCR2.1topo (Life Technologies) and sequenced by Genewiz Inc.

Quantitative Realtime PCR

RNA was extracted from cell lines by PureLink RNA mini kit (Life Technologies) with DNaseI treatment on column. cDNA was reverse transcribed using Superscript III first strand synthesis kit (Life Technologies). QPCR was performed on a StepOne Plus (ABI) thermocycler unit using either Taq-Man reagents (for SFRP2) or SYBR green mastermix (Biorad or Life Technology respectively) (for HDAC1–3 mRNA, HDAC1–3 pre-mRNA, 18S RNA, DNMT1, G9A, Beta Actin and GAPDH). We observed no significant difference in housekeeping gene levels across treatments and cell types (not shown).

Golden Gate Methylation array

High-throughput methylation profiling was done using the Illumina GoldenGate Methylation Cancer Panel I microarray platform. DNA quality control using picogreen, bisulfite-conversion using the EZ DNA methylation kit (Zymo) and array hybridization according to manufacturer's specifications were performed by Emory Integrated Genomics Core facility.

Statistical analysis of Illumina Golden Gate methylation data was carried out using R. Sample quality checks on the data were carried out using the bioconductor package methylumi(27). Following quality control checks, the replicates were summarized into a composite value using Singular Value Decomposition (SVD)2 algorithm. Significant differentially methylated CpG sites were identified using the nonparametric hypothesis

testing method called Hypothesis based Analysis of Microarrays (HAM)(28, 29). HAM method consists of two steps. In the first step, the significance of a given hypothesis is assessed over the entire dataset. If significant, the next step identified a list of differentially methylated CpG sites. Pathway analysis was carried out on significant differentially methylated genes in comparisons of interest using Metacore from Thomson Reuters.

RNAi

siRNA knockdowns of HDAC1, 2 and 3 were performed with the following reagents: (HDAC1, s73 – life technologies, sc-44208 – Santa Cruz Biotechnology) (HDAC2, sc-29345 - Santa Cruz Biotechnology) (HDAC3, s16878 – Life Technologies) (Control A – Santa Cruz Biotechnology) 40–80pmol of siRNA duplex were added per experiment. Lipofectamine 2000 was used as transfection reagent.

Patient Samples

Archived paraffin embedded samples of 20 early stage lung cancer patients who underwent surgical resection of NSCLC at Emory University affiliated hospitals between May and August 2000 were obtained from the Lung and Thoracic Malignancies Satellite Cancer Tissue Bank at the Winship Cancer Institute of Emory University. Matched tumor and surrounding histologically normal tissue blocks were available on 18 patients.

Immunohistochemistry (IHC)

Sectioning of the tissue samples and IHC was performed by the Cancer Tissue and Pathology Shared Resource of the Winship Cancer Institute using the following antibodies and dilutions: DNMT1 (abcam) (1:1000), HDAC1 (1:1000), HDAC2 (1:1000) (Cell Signaling), HDAC3 (1:1000) (Abgent) PCNA(1:1000) (Cell Signaling). Staining occurred on a fully automated stainer after standard antigen retrieval steps as previously described. A horseradish-peroxidase labeled secondary anti-rabbit antibody was used in 1:1000 dilution. Staining was evaluated by two independent observers and quantified using a weighted index (intensity (scale 0–2) × % stained)(30).

Statistics

Differences between continuous variables were analyzed by Student's t-test.

Results

Carcinogen-induced bronchial epithelial cell transformation is associated with epigenetic changes

In order to model early events in smoke-carcinogen driven transformation of bronchial epithelia, we exposed immortalized human bronchial epithelial cells (here after 3KT) with low levels of the smoke-related carcinogens MNU and B(a)P for 1 day per week followed by 6 days of outgrowth. After 16 weeks of exposure, immortalized bronchial epithelial cells acquired the ability to grow as anchorage-independent colonies, a hallmark of oncogenic transformation [Fig1A]. This transformation was accompanied by an increase in protein levels for several epigenetic repressors: the DNA methyltransferase DNMT1, the histone methyltransferases G9A (responsible for histone H3 dimethylation on lysine 9 (H3K9me2)), as well as the class I histone deacetylases (HDACs)1–3. Associated with these changes were profound reductions in global levels of histone marks associated with active gene transcription such as H3-acetylation (H3-Ac) [Fig1B]. Together, these changes indicate that tobacco-carcinogen exposure is associated with a more repressive chromatin pattern.

To determine the mechanism by which expression levels of the epigenetic repressors are controlled, we first used qRT-PCR to investigate steady state mRNA levels. We observed that the increase in DNMT1 protein expression levels is not accompanied by an increase in steady-state mRNA levels. In contrast, we observed a profound increase in steady state mRNA levels for HDACs1–3 and G9A, suggesting that either transcription or changes in mRNA stability are responsible for the observed increase in the expression of these genes [Fig. 1C]. To differentiate between transcriptional regulation vs. posttranscriptional mRNA stabilization, we also analyzed the unspliced primary transcripts of HDAC1–3 [Fig S1]. After smoke carcinogen exposure, an increase in the primary transcripts for HDACs 1–2 were observed, indicating increased levels of these two HDACs are transcriptionally mediated. For HDAC3 however, no increase in primary transcript RNA was observed in T31 cells compared to 3KT cells, indicating that its increase in steady state mRNA levels is likely regulated post-transcriptionally.

The observed increase in protein levels of these epigenetic repressors raised the question if these changes are sufficient to lead to permanent epigenetic reprogramming in carcinogen exposed cells. We therefore analyzed a set of candidate genes for DNA hypermethylation by MSP. We chose a set of candidate genes known to be epigenetically silenced in cancer, and examined the methylation status of their promoters in both long-term carcinogen-exposed cells and their vehicle exposed counterparts. Aberrant DNA hypermethylation was found in 6 of 15 (40%) previously unmethylated promoters. In non-carcinogen exposed 3KT cells, only the GATA4 promoter was methylated [Table 1]. As expected, aberrant DNA methylation of the SFRP2 promoter was associated with transcriptional repression [Fig.1D].

We next performed an unbiased methylation analysis of 1,505 CpG dinucleotides representing 807 target genes using the Illumina GoldenGate microarray platform. After adjusting for false positive discoveries (FDR<0.05), we found statistically significant increased methylation in 42 genes after carcinogen exposure for 31 weeks vs. long-term cultured but not carcinogen exposed cells (T31 cells versus 3KT). In contrast, no significant hypomethylation was observed at any gene [Fig S2 and Table S1]. Since the GoldenGate array is enriched for probes for genes whose promoters have been shown to be hypermethylated in cancer, the lack of detectable hypomethylation on this array may be due to this bias. Pathway analysis revealed that hypermethylated candidate genes are mostly involved in cell proliferation and cell cycle control. [Table S2]. Together these data suggest that tobacco-carcinogen exposure mediates aberrant *de novo* DNA methylation through upregulation of DNMT1.

The interaction of DNMT1 with HDACs 1–3 stabilizes DNMT1 in a cell cycle independent manner

DNMT1 expression is usually increased after DNA replication and reaches peak levels in S and G2 phase, where DNMT1 physiologically mediates maintenance methylation of the newly synthesized daughter strand(10). Since deregulated cell-cycle expression of DNMT1 has been linked to cellular transformation, we examined DNMT1 expression in 3KT and T31 cells at various stages of the cell cycle after release from aphidocholin block. Interestingly, a cell cycle independent increase in DNMT1 expression was observed in carcinogen transformed T31 cells but not in regular 3KT cells. In T31 cells, high levels of DNMT1 expression were observed in all stages of the cell cycle starting with G0. This increase in cell cycle independent DNMT1 expression was mirrored by a similar increase in cell cycle independent HDAC3 protein expression [Fig 2A]. In order to determine if a direct correlation exists between HDAC3 and DNMT1 expression, we engineered a 3KT cell line which either stably expressed empty vector or HDAC3. HDAC3 expression was sufficient to induce the previously observed cell cycle independent increase of DNMT1 expression [Fig

2B], suggesting that HDAC3 may stabilize the DNMT1 protein. To further test this hypothesis, we performed co-immunoprecipitation experiments after overexpressing HDAC 1–3 and DNMT1 constructs. We found that DNMT1 associates with HDAC 1 and 3 [Fig2C] and that overexpression of these HDACs stabilized both the V5- tagged DNMT1-protein as well as native DNMT1 [Figs 2B, S3, S4]. Moreover, siRNA knockdown of HDAC2 and 3 led to a profound reduction in DNMT1 protein levels. Although knockdown of HDAC1 alone had little effect on DNMT1 levels, it potentiated the effects of HDAC2 knockdown on DNMT1 levels [Fig2D]. These findings indicate not only that HDACs mediate DNMT1 protein stability, but also that there is a certain redundancy in the activity of these three class I HDACs to stabilize DNMT1.

In order to determine if the carcinogen-induced upregulation of DNMT1 is due to increased de-novo synthesis or decreased protein turnover, we exposed 3KT and T31 cells to the protein synthesis inhibitor cycloheximide (2ug/ml). Cycloheximide exposure was associated with an increase in steady state DNMT1 protein levels indicating that *de novo* peptide synthesis of destruction signals like ubiquitin may be required for DNMT1 protein degradation [Fig S5]. While inhibition of class I HDACs with VPA led to almost complete suppression of DNMT1 levels, DNMT1 degradation after VPA exposure was fully inhibited after co-exposure with the proteasome inhibitor MG-132, suggesting that the primary mechanism for DNMT1 stabilization through interaction with HDACs is by protection from proteasomal degradation. [Fig 3A].

We next sought to determine whether DNMT1 is a direct target for deacetylation by class I HDACs. A degradation resistant DNMT1 construct lacking the N-terminal 110 amino-acids (24) was C-terminally His6- and V5- tagged, and stably expressed in T31 cells. HDACs 1 and 2 were subsequently knocked down by siRNA either individually or in combination. After purification of DNMT1 via a NTA-column DNMT1 acetylation status was determined by immunoblot using a pan acetyl-lysine antibody. Individual knockdowns of either HDAC1 or HDAC2 alone led to only a marginal increase in DNMT1 acetylation, which was much less pronounced than that induced by the class I HDAC inhibitor VPA. Combined HDAC1 and HDAC2 knockdown, however, resulted in greater levels of DNMT1 hyperacetylation comparable to that of VPA treatment; again showing that different class I HDACs have redundant roles in regulating DNMT1 acetylation and expression. Furthermore, knockdown of HDAC1 and/or HDAC2 in conjunction with VPA exposure increased the ubiquitination of DNMT1 [Fig 3B], leading to the conclusion that the predominant mechanism of DNMT1 stabilization in carcinogen exposed cells is deacetylation and prevention of ubiquitination and proteasomal degradation.

In order to rule out additional indirect effects of VPA treatment on other mechanisms, we investigated if DNMT1 degradation could be mediated indirectly a) by either inhibition of HSP90, whose deacetylation by HDAC1 has been previously shown to alter DNMT1 stability(12) or b) be dependent also on DNMT1 methylation, which has been previously reported to be a degradation signal similar to DNMT1 acetylation(16). First, we exposed 3KT and T31 cells to either VPA or the HSP90 inhibitor 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) or a combination of both. In 3KT cells both VPA and 17-AAG induced almost complete reduction of DNMT1 expression. In T31 cells, however, only VPA induced loss of DNMT1 expression. These findings demonstrate that VPA induced induction of DNMT1 degradation is HSP90 independent carcinogen transformed cells [Fig 3C]. Likewise, VPA induced DNMT1 degradation was also observed in T31 cells transfected with shRNA against the methyltransferase SET-7, which usually methylates DNMT1 at lysine-147(16), proving that DNMT1 methylation is not required as intermediary step for VPA induced degradation [Fig 3D].

HDAC1–3 and DNMT1 protein levels are increased in lung cancer compared with surrounding histologically normal lung

The above data suggests that smoke carcinogen-induced transformation *in vitro* is accompanied by alterations in the regulation of epigenetic regulators and that this in turn can impinge upon altered DNA methylation and gene expression profiles. To determine the relevance of these alterations to lung cancer *in vivo*, we analyzed DNMT1 and HDAC1–3 protein expression by immunohistochemistry in surgical specimens of 20 patients with resected NSCLC. All but two patients had tumor and surrounding normal lung tissue specimens available for analysis. While all four genes have a ubiquitous baseline expression in normal lung, we observed a highly significant increase in the staining intensity for all four genes (DNMT1: median WI: 200 (Tumor) vs. 110 (normal), $p=0.0007$; HDAC1: median WI 30 (tumor) vs. 7.5 (normal), $p=0.0002$; HDAC2: median WI: 60 vs. 10 ($p=0.033$); HDAC3: median WI 180 vs. 160 ($p=0.029$)) [Fig4]. These differences are not fully attributable to increases in proliferation as measured by PCNA staining median WI: 158 (Tumor) vs. 148 (Normal) ($p=0.677$) [Fig S7]. These findings strengthen and validate our *in vitro* observations by corroborating that the progression from carcinogen-exposed normal lung to invasive lung cancer is indeed accompanied by an increase in DNMT1 and HDACs 1–3 expression.

Inhibition of class I HDACs partially reverses oncogenic transformation and carcinogen-induced epigenetic changes

The above results point to a role for class I HDACs in stabilizing DNMT1. We therefore investigated the ability of VPA (a class I HDAC inhibitor) treatment to reverse carcinogen-induced alterations in our cell line model. VPA treatment led to a significant reduction in DNMT1, G9A and HDAC1–3 protein levels in T31 carcinogen transformed 3KT cells. Global levels of H3-Ac and H3K4me2, which were markedly reduced by prior carcinogen exposure, were restored, while global levels of the repressive histone modifications H3K9me3 and H3K27me3 were unaffected [Fig5A]. These histone changes are indicative of a potential global reprogramming effect of VPA in the carcinogen transformed bronchial epithelial cell background.

To determine the functional relevance of VPA exposure, we analyzed its effects on anchorage independent growth in soft-agar colony formation assays. Indeed, VPA treatment of carcinogen exposed cells at 0.1 and 0.5 mM concentrations for three weeks led to a statistically significant 30% reduction in anchorage independent colony formation, indicating a powerful although not complete effect on the reversal of oncogenic transformation [Fig 5B]. These concentrations of VPA did not significantly affect cellular proliferation ([Fig S9]), suggesting that its effect is more likely through differentiation rather than cytostasis alone.

Since VPA stimulates the reversal of carcinogen induced transcription and protein stabilization of epigenetic repressors, we next investigated if long-term VPA exposure used in the previous experiment can induce re-programming of target genes subject to carcinogen-induced epigenetic silencing. In analogy to the soft-agar experiment, we exposed T31 cells to low doses of VPA (0.5mM) for 28 days. Bisulfite sequencing revealed the induction of significant hypomethylation of the SFRP2 promoter ($p<0.001$) [Fig 5C]. This promoter hypomethylation was accompanied by an increase in SFRP2 mRNA levels, indicating that VPA is capable of reversing carcinogen-driven hypermethylation, allowing for the re-activation of previously silenced genes [Fig 5D] Similar results were observed for the RASSF1 promoter [Fig S10].

Discussion

Our data provide a compelling link between class I HDAC overexpression in lung cancer and DNMT1 protein stabilization, one of the key mediators of aberrant DNA methylation and widely believed to be an important oncogene in the initiation of cancer. While DNMT1 protein expression is regulated by multiple different mechanisms, we show here that the dominant mechanism of VPA induced DNMT1 degradation is through acetylation, ubiquitination and proteasomal destruction. We have explored the contribution of alternative mechanisms such as DNMT1 methylation, AKT-phosphorylation, which has been reported to induce DNMT1 phosphorylation or through inhibition of HSP90. The differences in HSP90 dependency of DNMT1 stability following VPA between 3KT cells and T31 cells are. One possibility is that after carcinogen exposure other HSPs may be upregulated as well, limiting dependence on HSP90 alone. Finally, it has been reported that DNMT1 translation may be regulated by microRNAs. While we have not studied the contribution of microRNAs specifically, the fact that loss of DNMT1 protein expression after VPA treatment can be inhibited both with cycloheximide and MG-132 point towards the proteasome as primary mechanism of DNMT1 degradation. DNMT1 targeting for cancer prevention has been challenging so far, since the only clinically available DNMT1 inhibitors 5-azacytidine and 5'-aza-deoxycytidine are nucleoside analogs, raising the theoretical risk that incorporation into the DNA could lead to secondary malignancies. Moreover, the need for daily subcutaneous injection of these agents would likely lead to low patient acceptance in the prevention setting. Our observation that VPA not only leads to an increase in histone-acetylation, but also reverses other carcinogen-induced epigenetic changes such as G9A and DNMT1 upregulation and DNA hypermethylation ultimately leading to the re-expression of epigenetically silenced genes opens a new potential avenue for lung cancer chemoprevention. We have recently completed a large cohort study of US veterans with either current or past tobacco exposure, where long-term use of VPA was associated with a significant reduction in smoking-related squamous cell carcinoma of the head and neck and a trend towards reduction of squamous cell carcinoma of the lung(31), supporting the potential clinical application of VPA for chemoprevention of smoking related malignancies of the upper aerodigestive tract. It should be noted that a benefit of VPA was only observed with long-term use of VPA (> 3 years). This long duration is similar to that required in chemoprevention studies of other cancers such as breast cancer prevention with tamoxifen. Moreover, the long duration of exposure required may be an explanation why relatively short courses of HDAC treatment alone are insufficient to prevent lung cancer in carcinogen induced mouse models(32).

The histone methyltransferases G9A and EZH2 are important transcriptional repressors. In particular, the interaction between G9A, H3K9me2, heterochromatin protein 1 (HP1) and DNMT1 has been hypothesized to direct *de novo* DNA methylation to loci previously marked by H3K9me3(33). Demethylation after treatment of cancer cells with nucleoside DNMT inhibitors generally only yields transient de-methylation, followed by gradual remethylation after drug withdrawal (34, 35). Since G9A has been implicated as potential mediator of *de novo* DNA methylation(33), the reduction in G9A protein levels we observed after HDAC inhibition are particularly important, since there might be a lesser tendency for target genes to become remethylated.

In summary, our data support a model [Fig 5F] in which tobacco-related carcinogen induced upregulation of HDAC1–3 mRNA and protein expression leads to increased stability of the oncogenic DNMT1 protein, thus enabling carcinogenic transformation. Moreover, our study provides strong rationale for the potential use of HDAC inhibitors as chemopreventive agents against lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Phenotypic changes associated with passage of HBEC 3kt in tobacco related carcinogens

A: Passage of HBEC 3kt in carcinogen increases clonogenicity by soft agar colony formation assay.

B: Immunoblots of passage 14, 25 and 31 of benz(a)pyrene and MNU exposed or vehicle treated bronchial epithelial cells reveals increases in DNMT1, G9a and HDAC1–3 protein levels, associated with a decrease in histone-H3 acetylation. Histograms indicate quantified values of fold change between carcinogen exposed and mock treated cells normalized to total H3 expression.

C: quantitative rt-PCR for DNMT1, G9A and HDACs 1–3 reveals that DNMT1 protein is not regulated by changes in steady-state mRNA levels, while G9A and HDAC1–3 are. Values are relative expression between T31 and 3kt and normalized to 18S RNA expression.

D: quantitative rt-PCR for SFRP2 shows transcriptional repression in carcinogen exposed cells, normalized to 18s expression.

Figure 2. DNMT1 stability is differentially affected in carcinogen exposed cells

A: Aphidicholine synchronized (5uM for 24hrs) 3kt and T31 cells reveals that DNMT1 is dysregulated throughout the cell cycle post carcinogen exposure (0hrs: G0/G1; 6hrs: G0/G1 12hrs: S/G2; 24hrs: S/G2)

B: Aphidicholine synchronized stably transfected 3kt cells with either control vector (EV) or HDAC3 reveals that HDAC overexpression causes DNMT1 dysregulation throughout the cell cycle

C: Stably expressed Full-length V5-tagged DNMT1 associates with GST-tagged HDAC1, HDAC2 and HDAC3 irrespective of DNaseI pre-incubation

D: siRNA knockdown of HDAC1,2,3 (H1,H2,H3 respectively) decreases levels of DNMT1 in T31 cells, untreated 3kt and non-silencing siRNA (NS) transfected T31 serve as controls. Histograms show change in expression compared to non-silencing siRNA normalized to beta actin.

Figure 3. Modulating type I HDAC function alters DNMT1 stability

A: T31 cells were exposed to 1mM VPA for 24h in the absence or presence of the proteasome inhibitor MG-132 (2.5uM ×16h). VPA induced DNMT1 degradation was blocked by MG-132.

B: Pull-down of N-terminally truncated DNMT1 by IMAC in siRNA treated T31 cells. Total Acetylated lysine band runs at same molecular weight as V5H6-DNMT1. Ubiquitination of DNMT1 is increased with HDAC knockdown and VPA exposure. All cells were treated for 16hrs with 2.5uM MG-132.

C: Treatment of 3kt and T31 with 1mM VPA, 10nM 17AAG or a combination reveals that HSP90 driven DNMT1 stabilization is not a major component of DNMT1 stabilization in carcinogen exposed T31 cells.

D: VPA induces loss of DNMT1 in T31 cells independent of Set7 expression. Knockdown of SET7 did not show an appreciable loss of DNMT1. VPA treatment causes a significant reduction in DNMT1 levels with or without Set7 expression

Figure 4. Immunohistochemistry reveals upregulation of DNMT1 and type I HDACs in human lung tumors compared to matched normal tissue

A: Representative images of a single patient's tumor (T) and matched normal tissue (N). B: Weighted indices of DNMT1, HDAC1, 2, or 3 in tumor versus normal tissue. 20 Patients' tumors and matched normal tissues were scored and averaged. 2-tailed student's t test was employed to check for significance, values indicated.

Figure 5. HDAC inhibition restores carcinogen transformed cells to a more normal phenotype
 A: 24hour exposure of T31 cells to 1mM VPA reduced levels of DNMT1 and type I HDACs and increased global histone H3-Acetylation and H3K4 dimethylation.
 B: Soft agar colony formation assay. Carcinogen transformed cells (T30) were treated with 0.1mM or 0.5mM VPA for 3 weeks while seeded in soft agar. Clonogenicity of T30 cells were reduced by VPA.
 C: carcinogen-transformed T31 cells were passaged for 28 days in the presence of VPA. Bisulfite sequencing of the SFRP2 locus was performed on cells derived from day 0 and day 28. Long term VPA treatment of carcinogen transformed cells decreases methylation of SFRP2 promoter CpG island ($p < 0.001$).
 D: Bar graph showing SFRP2 mRNA levels (18S) for T30 cells treated with 0, 0.1, or 0.5 mM VPA for 3 weeks.
 E: Schematic diagram of the HDAC-DNMT1 feedback loop.

D: Promoter demethylation is associated with de-repression of SFRP2 transcription
E: Model of smoke carcinogen induced transformation and reversal by HDAC inhibition. Smoke carcinogen exposure leads to transcriptional upregulation of HDAC1 and 2 and stabilization of HDAC3 mRNA¹. This leads to stabilization of DNMT1. VPA exposure inhibits class I HDAC², leading to DNMT1 degradation by an acetylation dependent pathway³. Smoke carcinogen exposure leads to epigenetic silencing of tumor suppressor genes (i.e. SFRP2)⁴. VPA can reverse this phenomena.⁵

Methylation Specific PCR (MSP) on several gene CpG islands reveals increased methylation with passage in carcinogen.

Table 1

Function	Gene	Passage						
		3kt	T14	T16	T18	T22	T26	T30
DNA Repair	MLH-1	U	U	U	U	U	U	U
	MGMT	U	U	U	U	U	U	U
Cell Signaling	SFRP1	U	U	U	U	U	U	U
	SFRP2	U	M	U	U	M	M	M
	SFRP4	U	U	U	U	U	U	U
	SFRP5	U	U	U	M	M	M	M
	DKK1	U	U	U	U	U	U	U
	APC	U	U	U	U	U	U	U
G2M Checkpoint	Wif-1	U	U	U	U	U	U	M
	RASSF1	U	M	U	U	M	M	M
Cell Cycle	CHFR	U	U	U	U	U	U	U
	p16	U	U	U	U	U	U	U
Transcription Factor	RUNX3	U						M
	GATA4	M	M	M	M	M	M	M
Cell Adhesion	ADAMTS1	U	M	U	M	M	M	M
	CDH1	U	U	U	U	U	U	U

U=unmethylated, M=Methylated