Epigenetic Silencing of the Candidate Tumor Suppressor Gene *Per1* in Non–Small Cell Lung Cancer

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Abstract

Purpose: Epigenetic events are a critical factor contributing to cancer development. The purpose of this study was to identify tumor suppressor genes silenced by DNA methylation and histone deacetylation in non–small cell lung cancer (NSCLC).

Experimental Design: We used microarray analysis to screen for tumor suppressor genes.

Results: We identified *Per1*, a core circadian gene, as a candidate tumor suppressor in lung cancer. Although Per1 levels were high in normal lung, its expression was low in a large panel of NSCLC patient samples and cell lines. Forced expression of Per1 in NSCLC cell lines led to significant growth reduction and loss of clonogenic survival. Recent studies showed that epigenetic regulation, particularly histone H3 acetylation, is essential for circadian function. Using bisulfite sequencing and chromatin immunoprecipitation, we found that DNA hypermethylation and histone H3 acetylation are potential mechanisms for silencing Per1 expression NSCLC.

Conclusions: These results support the hypothesis that disruption of circadian rhythms plays an important role in lung tumorigenesis. Moreover, our findings suggest a novel link between circadian epigenetic regulation and cancer development.

Lung caner is the leading cause of cancer-related death in the United States (1, 2). Prevention, screening, and treatment of this cancer are all problematic, emphasizing the need for the development of new diagnostic and therapeutic strategies. Epigenetic events are an important normal cellular function and, as evident from recent research, are a critical force driving initiation and progression of cancer (3, 4). Recent studies show that silencing of tumor suppressor genes, resulting from epigenetic alterations, are an early event in many human malignancies, including non–small cell lung cancer (NSCLC; refs. 3, 4). Epigenetic interventions, particularly those targeting histone deacetylase are among the most promising therapies for cancer, and histone deacetylase inhibitors are already being used in the clinic (5, 6). Moreover, because epigenetic changes occur early in tumorigenesis and are associated with distinctive cancer types, they could represent targets for chemoprevention and early diagnosis. These recognitions have prompted extensive research aimed at discovering silenced tumor suppressors.

In the present study, we used a combined treatment of NSCLC cells with 5-aza-2′-deoxycytidine (5-Aza-dC) that reverses DNA methylation and suberoylanilide hydroxamic acid (SAHA) that inhibits histone deacetylases followed by microarray analysis to identify additional tumor suppressor genes in lung cancer. After screening over 22,000 genes, we focused on Per1 for additional studies. Reduced expression of Per1 was found in a large collection of NSCLC samples. Epigenetic silencing of Per1 promoter was detected in NSCLC cell lines and overexpression of Per1 was associated with growth inhibition in these cells. The results suggest that Per1 is an epigenetically silenced tumor suppressor in lung cancer.

Materials and Methods

Patients and samples. Under an existing approved Institutional Review Board, lung cancer tissues and adjacent normal lung tissues were obtained from lung cancer surgical specimens. Tissue samples were collected immediately after surgical resection, quick frozen in liquid nitrogen, and then stored in −80°C until their use.

Cell culture and transfections. Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in the recommended medium and conditions. Per1 expression vector (pCDNA3.1-Per1) was described previously (7). Transfections were done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA).

Western blot analysis. Cell lysates were prepared using the lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40]. Immunoblots were incubated with the following antibodies: Per1 and PARP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin antibody was from Sigma-Aldrich (St. Louis, MO). Western blots were stripped between hybridizations with stripping buffer [10 mmol/L Tris-HCl (pH 2.3), 150 mmol/L NaCl].

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Table 1. Candidate tumor suppressor genes epigenetically silenced in H520 cells

Fold change	Gene	Accession no.
3.9	*Per1*; *period homologue 1 (Drosophila)*	NM_002616.1
3.2	*RAI3*; *retinoic acid induced 3*	NM_003979.2
2.6	*ATF3*; *activating transcription factor 3*	NM_001674.1
2.5	*SNN*; *stannin*	AF070673.1
2.4	*GADD45β*; *growth arrest and DNA damage–inducible, β*	AF087853.1
2.2	*DSIPI*; *δ sleep-inducing peptide, immunoreactor*	AL110191.1
2.1	*NDRG1*; *N-myc downstream regulated way*	NM_006096.1

Table 2. Expression of candidate tumor suppressor genes in NSCLC tissue samples

Gene	Total no. samples	Expression level (tumor/normal)		
		Fold change, ≤0.5, no. samples (%)	Fold change, 0.5-2.0, no. samples (%)	Fold change, ≥2-fold, no. samples (%)
Per1	77	47 (61)	19 (25)	11 (14)
RAI3	25	12 (48)	9 (36)	4 (16)
ATF3	25	15 (60)	6 (24)	4 (16)

NOTE: Expression of the listed genes was examined in a panel of NSCLC and matched normal tissues by real-time PCR.

Microarray analysis. H520 cells were cultured either in the presence of 5-Aza-dC (1 μmol/L 72 h) in combination with SAHA (2.5 μmol/L for the last 24 h) or left untreated. The experiments were done in triplicates. Biotinylated cRNAs were prepared and hybridized to Human U133A microarrays (Affymetrix, Santa Clara, CA), which contains ~22,000 genes. Array hybridization and scanning were done at the University of California at Los Angeles Microarray Core Facility (Los Angeles, CA). Data analysis was done with the GeneSpring software version 5.0 (Silicon Genetics, San Carlos, CA).

Real-time reverse transcription-PCR analysis. Total RNA from tissue and cultured cells was extracted using Trizol reagent (Invitrogen). Real-time reverse transcription-PCR was done in triplicates using gene-specific primers with an iCycler iQ system (Bio-Rad, Hercules, CA). Expression levels of glyceraldehyde-3-phosphate dehydrogenase and 18S were used as internal controls.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, cell cycle, apoptosis assays, and clonogenic assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Roche Diagnostics, Almeda, CA) were done according to the manufacturer's protocol. For cell cycle analysis, transfected cells were fixed in cold ethanol, stained with 50 μg/mL propidium iodide, and analyzed by FACScan and CellFit programs (Becton Dickinson, San Jose, CA). Apoptosis studies were done with Annexin V-FITC apoptosis detection kit I (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. For clonogenic assays, cells (3×10^4 per well) were plated into 12-well plates using a two-layer soft agar system. After 14 days of incubation, the colonies were counted. Experiments were done in triplicates and repeated at least twice.

Bisulfite sequencing. Bisulfite modification of DNA was done with the EZ DNA Methylation kit (Zymo Research Corp., Orange, CA). The following primers were used for PCR (−1670 to −1475): TTGGGAA-GAGATTTTTAGTTAAT and CCACAAAAATACCTACCTAATC. PCR products (195 bp) were cloned into the pCR2.1-TOPO vector (Invitrogen), and two clones from each sample were sequenced.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) was used

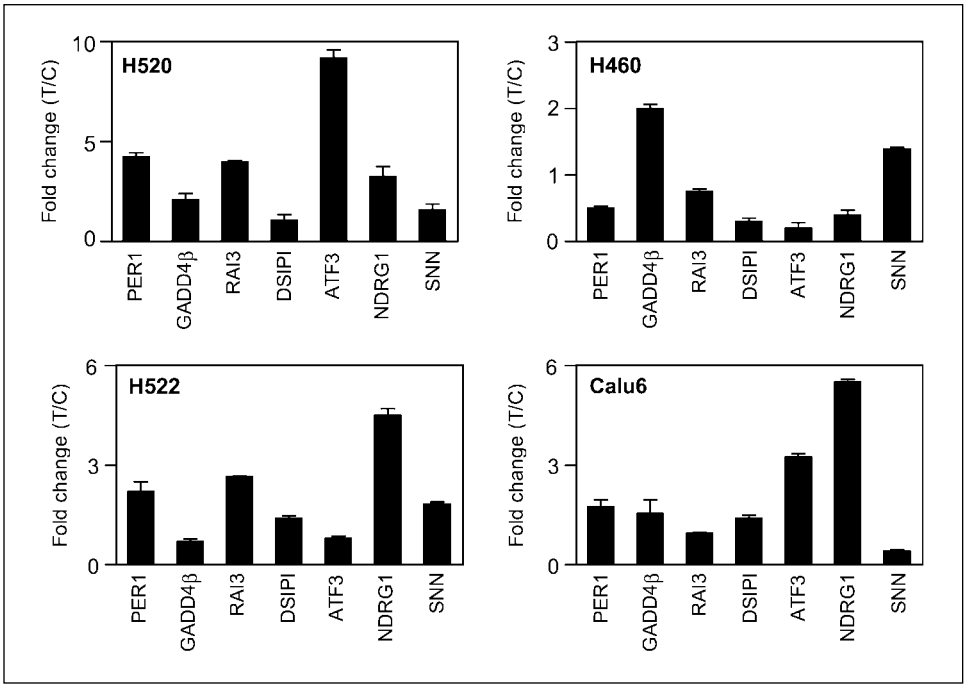

Fig. 1. Verification of microarray analysis. Four NSCLC cell lines, H520 (squamous cell), H460 (large cell), H522 (adenocarcinoma), and Calu6 (anaplastic) were treated with 5-Aza-dC (1 μmol/L, 72 h) and SAHA (2.5 μmol/L, added for the last 24 h). The expression of the indicated genes was measured by real-time PCR. Levels of 18S were used as internal controls. Fold change was calculated compared with nontreated cells, which were considered to be 1. Columns, mean of three measurements of each sample; bars, SD.

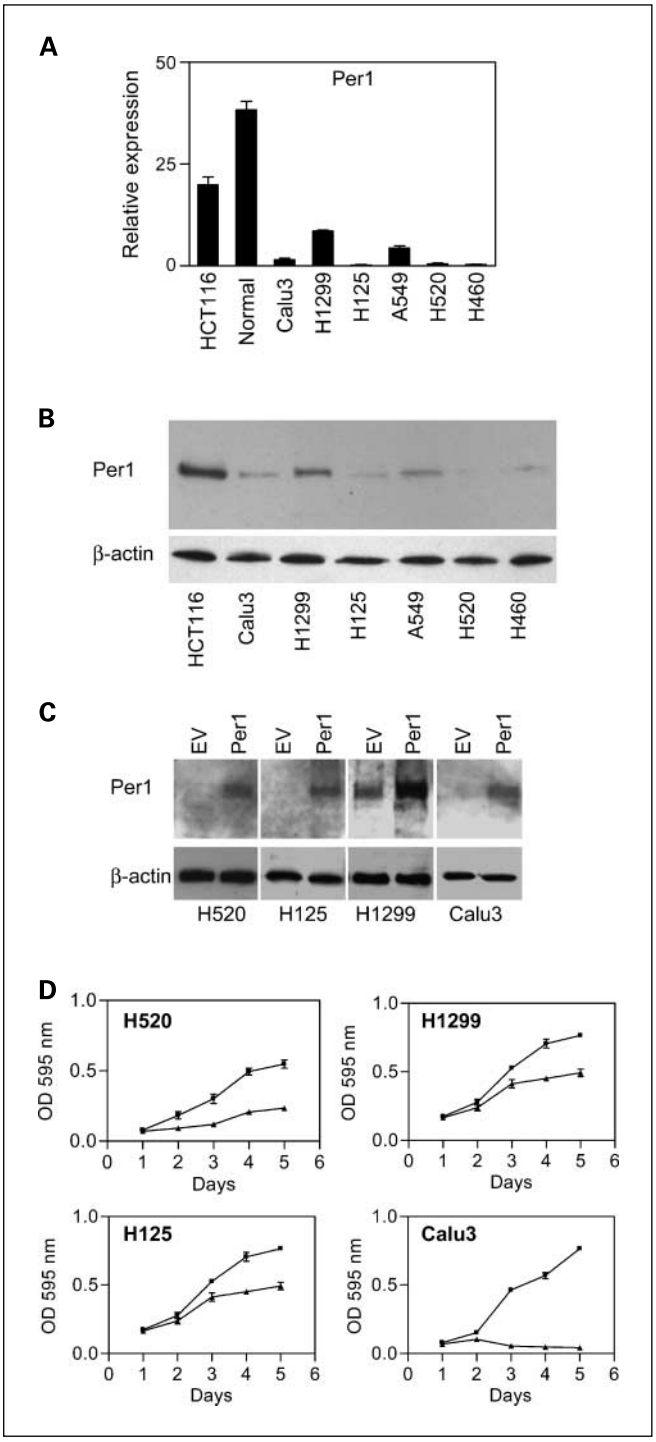

Fig. 2. Per1 is down-regulated in NSCLC cell lines and Per1 expression leads to growth inhibition. *A,* real-time PCR analysis of Per1 expression in normal lung tissue, the indicated NSCLC cell lines, and the HCT116 cell line. Per1 levels are expressed in arbitrary units as a ratio of the Per1 transcripts to glyceraldehyde-3-phosphate dehydrogenase transcripts. Columns, mean of three measurements of each sample; bars, SD. *B,* Western blot analysis of Per1 expression in the indicated NSCLC cell lines. The colon cancer cell line, HCT116, was used as a positive control; β-actin was the control for equal loading. *C* and *D,* NSCLC cell lines, H520 (squamous cell carcinoma), H1299 (large cell carcinoma), as well as H125 and Calu3 (adenocarcinoma) were transfected with either Per1 expression vector (*Per1*) or empty vector (*EV*). *C,* Per1 expression was analyzed by Western blot. β-Actin levels are shown as loading controls. *D,* 2 d after transfection, cells were plated in G418 selection medium and proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Experiments were done in quadruplicate samples and repeated at least twice. Points, mean of representative experiments; bars, SD.

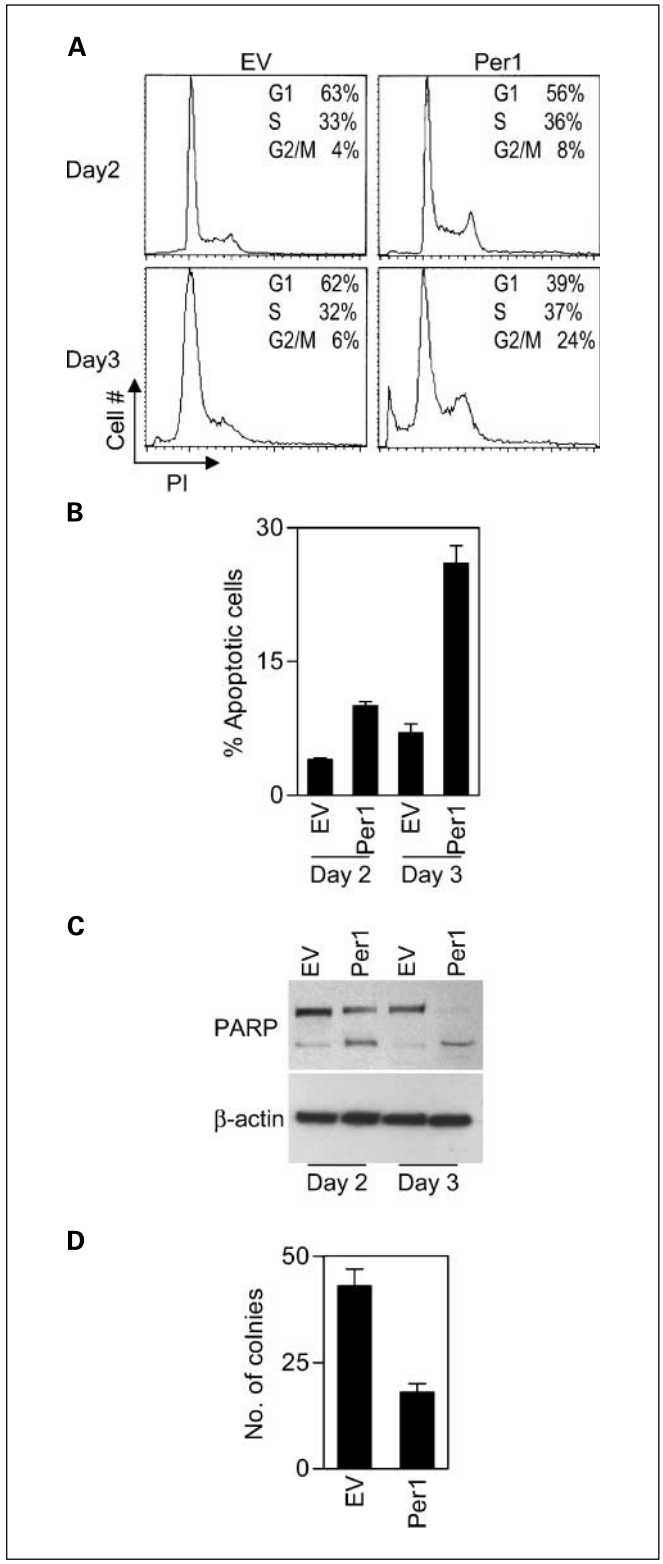

Fig. 3. Per1 expression leads to cell cycle arrest, apoptosis, and reduced clonogenic potential. Calu3 cells were transfected with either the Per1 expression vector or empty vector followed by a brief antibiotic selection. Resistant cells were harvested at days 2 and 3 and used in subsequent assays. *A,* cell cycle analysis with propidium iodide (*PI*) staining. *B,* apoptosis analysis with Annexin/propidium iodide staining. Columns, mean of three experiments; bars, SD. *C,* Western analysis for PARP expression. β-Actin was used as loading control. *D,* clonogenic assays. Cells were cultured in soft agar. Colonies containing ~1,000 cells or more were counted on day 14. Columns, mean of three independent experiments; bars, SD.

according to the manufacturer's protocol. The following Per1 promoter-specific primers were used: TGTCTCTCCCCTCCTCTCAA and AGATACGCTGCGCCTCTTTA.

Results

Identification of genes induced by 5-Aza-dC and SAHA in the NSCLC cell line, H520. To identify candidate tumor suppressor genes silenced by DNA methylation and/or histone deacetylation, we did microarray analysis with H520 cells either untreated or treated with 5-Aza-dC and SAHA. Analyzing the microarray data, we identified 149 genes whose expression increased >2-fold in the treated cells compared with the nontreated cells. To generate a list of genes with potential biological relevance, we used *in silico* analysis to search for genes that are expressed in normal adult lung and not in NSCLC. We also excluded from our list genes that do not have CpG islands in their promoter region (i.e., *FABP4*, *SCYA20*, and *STC1*), established oncogenes (i.e., v-jun, AML1, and EGFR), already identified as potential tumor suppressor genes (i.e., *p19*, *CYR61*, and *CTGF*), or unknown genes (data not shown). Seven genes (*Per1*, *GADD45β*, *RAI3*, *DSIPI*, *ATF3*, *NDRG1*, and *SNN*) met these criteria (Table 1). To confirm the microarray data, we did real-time reverse transcription-PCR using cell lines representing the major histologic subtypes of NSCLC. Following treatment with 5-Aza-dC and SAHA, expression of six of the seven genes increased 2-fold or more in at least one of the cell lines, showing that our microarray analysis was reliable (Fig. 1).

Expression of target genes in NSCLC tissue. To determine the clinical significance of the microarray data, we used real-time PCR to analyze the expression of three of the identified genes (*Per1*, *RAI3*, and *ATF3*) in NSCLC and matched normal tissues. The expression levels of all three genes were low (≥2-fold) in a large percentage (61% *Per1*, $n = 77$; 48% *RAI3*, $n = 25$; and 60% *ATF3*, $n = 25$) of cancer samples compared with matched normal controls (Table 2). These results suggest that the genes identified by our analysis are good candidates to act as tumor suppressor genes in NSCLC. Per1 is one of a set of core clock genes that regulate circadian rhythms. Recent studies suggested that disruption of circadian rhythms may increase susceptibility to cancer development (8, 9). Moreover, deregulation of Per1, as well as other clock genes, has been reported in several malignancies (7, 10–12). Yet, the functional significance of Per1 in lung tissue is mostly unknown. Thus, additional experiments focused on Per1.

Per1 inhibits growth in NSCLC cell lines. We used real-time PCR to determine the expression of Per1 in lung cancer cell lines from different histologic subtypes of NSCLC. Although Per1 was highly expressed in normal lung, its expression was low in the cell lines examined (Fig. 2A). Western blotting showed a correlation between Per1 mRNA expression and protein levels (Fig. 2B). Next, we assessed the consequence of Per1 expression on the growth rate of NSCLC cell lines. Four NSCLC cell lines were transfected with either the Per1 expression vector (pcDNA3.1-Per1) or a control empty vector (pcDNA3.1). Per1 expression was determined by Western blot analysis (Fig. 2C). Two days after transfection, cells were placed in selection medium for 5 days, and cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Fig. 2D). Per1 expression substantially decreased the growth of all four cell lines, although the range of inhibition varied. The most profound effect was seen in the Calu3 cells. Two days after starting antibiotic selection, no additional increase in cell numbers occurred; by day 4, no viable cells were present. We, therefore, chose the Calu3 cell line for additional experiments.

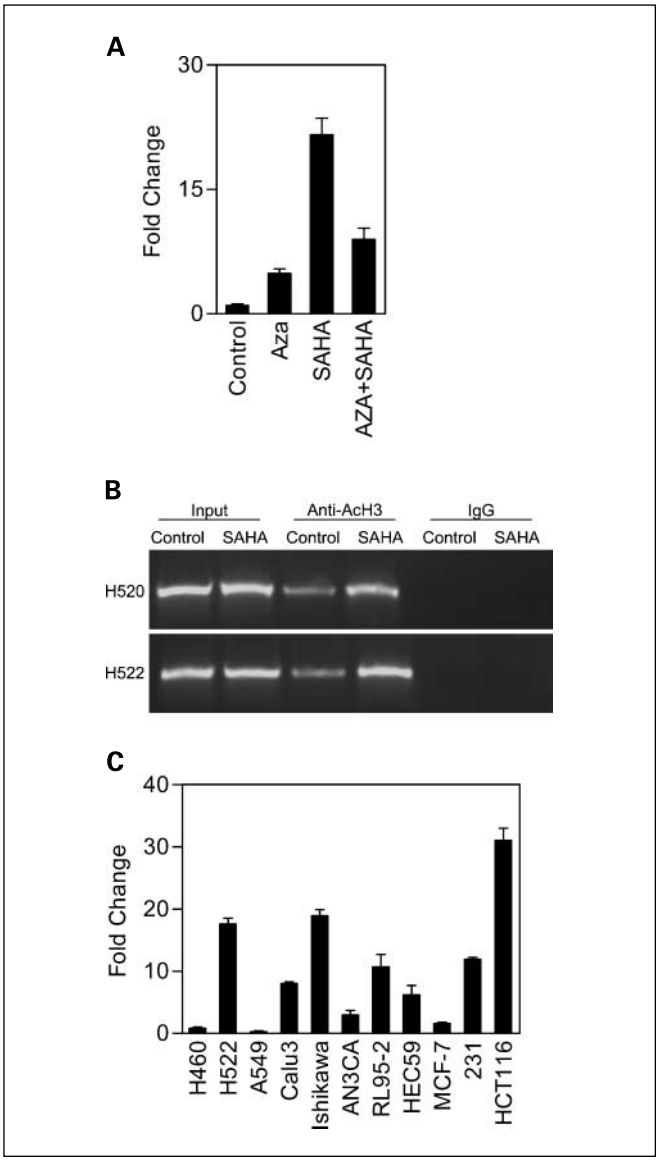

Fig. 4. Induction of Per1 expression and increased acetylation of histone H3 in the Per1 promoter by SAHA. *A,* real-time PCR analysis of Per1 expression in H520 cells after culture with either 5-Aza-dC (1 μmol/L, 72 h), SAHA (2.5 μmol/L, 24 h), both drugs, or no treatment (control). *B,* chromatin immunoprecipitation was done using H520 and H522 cells cultured either without (control) or with SAHA (2.5 μmol/L, 24 h) using acetylated histone H3 (*AcH3*) antibody. Samples were analyzed by PCR with Per1 promoter-specific primers. Input chromatin was included as a positive control; immunoprecipitations with IgG antibody were the negative control. *C,* cancer cell lines were cultured with SAHA at the indicted concentrations for 24 h. NSCLC: H460 (2.5 μmol/L), H522 (2.5 μmol/L), A549 (5 μmol/L), and Calu3 (2.5 μmol/L); breast: MCF-7 (2.5 μmol/L) and MDA-231 (5 μmol/L); endometrial: Ishikawa (2.5 μmol/L), AN3CA (2.5 μmol/L), RL95-2 (2.5 μmol/L), and HEC59 (2.5 μmol/L); and colon: HCT116 (5 μmol/L). Per1 expression was measured by real-time PCR. 18S levels were used as internal controls (*A* and *C*). Fold change was calculated compared with nontreated cells, which were considered to be 1. Columns, mean of three measurements of each sample; bars, SD.

Table 3. Per1 promoter methylation in NSCLC

Sample*		CpG† no.																	
		01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18
H520	1	○	●	●	●	○	○	○	○	●	●	○	○	○	●	●	●	●	●
	2	○	●	●	●	○	○	○	○	●	○	○	○	●	●	●	●	●	●
N#1	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	2	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○
T#1	1	○	○	●	●	○	●	○	●	●	●	○	●	○	●	●	●	○	●
	2	○	○	●	●	○	○	○	●	●	●	●	●	○	○	●	●	●	○
N#3	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	2	○	●	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○
T#3	1	○	○	○	●	○	●	●	○	●	●	○	○	●	●	●	●	○	●
	2	○	●	○	●	●	●	●	○	●	●	○	●	●	●	●	○	○	●

Abbreviations: N, normal; T, tumor.
*Allelic patterns of CpG sites in the H520 NSCLC cell line and in two NSCLC patient samples. No methylation was detected in four other patient samples.
†Methylated CpG sites are marked as filled circles (●) and unmethylated sites as open circles (○).

Cell cycle and apoptosis analysis were done to determine whether growth inhibition by Per1 is a result of either cell cycle arrest or induction of cell death. Calu3 cells transfected with either Per1 or empty vector were selected with G418 for 3 days. Cell cycle analysis showed that Per1 expression led to a significant increase in the number of cells in the G_2-M phase (Fig. 3A). An increase in the apoptosis rate, measured by Annexin V, was also noted in the Per1-transfected cells (Fig. 3B). Apoptosis was confirmed by cleavage of PARP, a marker of activated caspases (Fig. 3D). Thus, both cell cycle arrest and apoptosis contribute to Per1-mediated growth inhibition in NSCLC cells. Per1 expression also led to a substantial reduction in the colony-forming ability of Calu3 cells (Fig. 3D).

Methylation and acetylation of Per1 promoter. Combined treatment with 5-Aza-dC and SAHA induced Per1 expression in H520 cells (Fig. 1). To determine which compound was most effective, the cells were treated with either 5-Aza-dC or SAHA, and Per1 expression was measured by real-time PCR (Fig. 4A). SAHA treatment markedly increased Per1 levels (22-fold), whereas 5-Aza-dC treatment led to a moderate increase in its levels (5-fold). Surprisingly, combined treatment of both drugs led to an intermediate induction of Per1 expression (9-fold). Most likely, this can be explained by an event downstream of epigenetic modulations, such as reactivation of an inhibitory transcription factor, reduced RNA stability, or an induction of a negative signal transduction pathway.

Deregulation of Per1 expression associated with promoter hypermethylation was reported in breast and endometrial cancers (10, 12). Bisulfite sequencing was done in the lung cancer cell lines H520 and H522 and identified methylation in H520 cells (Table 3). The methylation status of the Per1 promoter was also analyzed in six pairs of NSCLC and matched normal tissues (Table 3). In the tumor tissues, two of four cases with reduced Per1 mRNA expression showed methylation in the Per1 promoter; no methylation was detected in either two other cases where Per1 mRNA levels were not down-regulated or in any of the normal lung samples (Table 4). Appropriate transcription of circadian genes depends on rhythmic changes of histone H3 acetylation in their promoters (13). Chromatin immunoprecipitation assays using the NSCLC cell lines, H520 and H522, showed that the increase of Per1 induced by SAHA is associated with an increase in acetylated histone H3 binding to the Per1 promoter (Fig. 4B). Next, we examined the effect of SAHA on Per1 expression in additional NSCLC cell lines (H460, H522, A459, and Calu3), as well as endometrial (Ishikawa, AN3CA, RL95-2, and HEC59), breast (MCF-7 and MDA-231), and colon (HCT116) cancer cell line. Levels of Per1 were significantly induced by SAHA in 7 of the 11 cell lines (Fig. 4C). These results suggest that hypermethylation and acetylation of the Per1 promoter are putative mechanisms for down-regulation of Per1 in NSCLC.

Discussion

In the present study, we did microarray analysis to screen for tumor suppressors silenced in NSCLC. The genes that we identified have been implicated in growth, apoptosis, and/or

Table 4. Per1 promoter methylation and mRNA expression in NSCLC and matched normal tissues

Paired samples		CpG methylation*	Per1 down-regulation†
1	N	–	+
	T	+	
2	N	–	+
	T	–	
3	N	–	+
	T	+	
4	N	–	+
	T	–	
5	N	–	–
	T	–	
6	N	–	–
	T	–	

*Methylation status was determined using bisulfite sequencing. Two clones from each sample were sequenced.
†≥2-Fold change in Per1 expression between NSCLC samples and matched normal controls. Per1 expression was determined by real-time PCR.

differentiation in various tissues, and therefore, they have a high potential to be involved in lung tumorigenesis. Several earlier reports have used microarray approaches to identify tumor suppressor genes in lung carcinoma (14, 15). Together, the information emerging from these studies will provide a comprehensive view of the epigenetic changes characterizing NCLCS; this may yield benefits in earlier detection and in the design of better antitumor interventions. Down-regulation of three genes from our list, *Per1*, *RAI3*, and *ATF3*, was found in a large percentage of NSCLC patient samples. Recently, *RAI3* and *ATF3* have been shown to play a role in breast and prostate epithelial cancers (16, 17). Further studies are needed to evaluate the involvement of these genes in NSCLC and whether their loss occurs independently or as a result of a common pathway dysregulated in the NSCLC cells.

Between 2% to 10% of genes in any given tissue are under circadian control; some of theses genes regulate key steps in metabolic pathways and the cell cycle, showing the significance of the clock system in many physiologic and pathologic conditions, including cancer (18–21). Indeed, disruption of circadian rhythms has been associated with human tumorigenesis and with poor prognosis (8). In the present study, we identified the circadian gene *Per1* as a potential tumor suppressor in the lung. *Per1* levels were low in a large panel of NSCLC patient samples and in NSCLC cell lines compared with normal lung tissue. In addition, ectopic expression of *Per1* in NSCLC cell lines led to growth inhibition, G_2-M cell cycle arrest, apoptosis, and reduced clonogenic potential. Thus, our findings support the hypothesis that the circadian system is involved in tumor suppression. Earlier studies suggested that influence of Per2 on cell proliferation is p53 dependent (9). We found that Per1 inhibited growth of both wild-type (H520) and mutant (H125 and H1299) p53 NSCLC cell lines, suggesting that, in NSCLC, at least some of Per1 activities are p53 independent. In addition, in the cell lines examined, Per1 levels did not correlate with p53 status. Similarly, no association was found between Per1 and p53 in a panel of endometrial carcinoma samples (12). Further experiments are needed to determine the molecular mechanisms by which Per1 inhibits cell growth.

In mammalian cells, circadian rhythms are maintained by transcriptional feedback loops (22, 23). Two transcription factors, Clock and Bmal1, bind E-box motifs in target genes, including *Per* and *Cry*, to activate transcription. *Per* and *Cry* then interfere with Bmal1:Clock activity, thereby inhibiting their own expression. Binding of the Bmal1:Clock complex to E-box motifs correlates with rhythmic changes in acetylation and methylation of the surrounding DNA (24). Moreover, Clock itself displays histone acetyltransferase activity that is essential for the circadian regulation of clock genes, such as *Per1* (25). Our results suggest a role for promoter methylation and histone deacetylation, in the silencing of Per1 expression in NSCLC. Together, these data suggest a model wherein rhythmic epigenetic changes in the promoters of clock genes are a normal cellular function. Variation in this process could disrupt not only the expression of core clock genes but also the web of genes and cellular pathways that are under circadian control. Elucidating the role of clock genes in cancer may help the development of new therapeutic strategies and shed light into chronotherapy as a way to maximize the effectiveness of current therapies.

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