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RNA Interference-Mediated Knockdown of DNA Methyltransferase 1 Leads to Promoter Demethylation and Gene Re-Expression in Human Lung and Breast Cancer Cells

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ABSTRACT

DNA methyltransferase 1 (DNMT1) is required to maintain DNA methylation patterns in mammalian cells, and is thought to be the predominant maintenance methyltransferase gene. Recent studies indicate that inhibiting DNMT1 protein expression may be a useful approach for understanding the role of DNA methylation in tumorigenesis. To this end, we used RNA interference to specifically down-regulate DNMT1 protein expression in NCI-H1299 lung cancer and HCC1954 breast cancer cells. RNA interference-mediated knockdown of DNMT1 protein expression resulted in >80% reduction of promoter methylation in *RASSF1A*, *p16^{ink4A}*, and *CDH1* in NCI-H1299; and *RASSF1A*, *p16^{ink4A}*, and *HPP1* in HCC1954; and re-expression of *p16^{ink4A}*, *CDH1, RASSF1A*, and *SEMA3B* in NCI-H1299; and *p16^{ink4A}*, *RASSF1A*, and *HPP1* in HCC1954. By contrast, promoter methylation and lack of gene expression was maintained when these cell lines were treated with control small interfering RNAs. The small interfering RNA treatment was stopped and 17 days later, all of the sequences showed promoter methylation and gene expression was again dramatically down-regulated, indicating the tumor cells still were programmed for these epigenetic changes. We saw no effects on soft agar colony formation of H1299 cells 14 days after DNMT1 knockdown indicating that either these genes are not functioning as tumor suppressors under these conditions, or that more prolonged knockdown or other factors are also required to inhibit the malignant phenotype. These results provide direct evidence that loss of DNMT1 expression abrogates tumor-associated promoter methylation and the resultant silencing of multiple genes implicated in the pathogenesis of human lung and breast cancer.

INTRODUCTION

Tumor acquired, aberrantly methylated CpG dinucleotides in the promoter regions of tumor suppressor genes (TSGs) is a hallmark and major means of TSG inactivation in human cancer (1, 2). Substantial evidence indicates that promoter methylation is associated with loss of TSG expression in lung and breast cancers (3, 4). The repressed state conveyed by the presence of DNA methylation in TSG promoters can be reversed by administration of the nucleotide analog 5-Aza-2′-deoxycytidine (5-Aza-CdR; Ref. 5). However, this drug is cytotoxic even at low concentrations, which may lead to expression changes not directly related to DNA methylation (6, 7). To address this concern, genetic approaches have been used to analyze DNA methylation in cancer.

At present three active DNA methyltransferases (*DNMT1*, *DNMT3A*, and *DNMT3B*) and one candidate gene, *DNMT2*, have been identified in mammals (8). *DNMT1*, the first DNA methyltransferase to be cloned, is responsible for maintaining DNA methylation patterns during DNA replication (9). Fournel *et al.* (10) showed recently that ablation of DNMT1 expression with antisense oligonucleotides resulted in loss of promoter methylation, re-expression of *p16^{ink4A}*, and inhibition of cell proliferation in the bladder cancer cell line, T24. In contrast, Rhee *et al.* (11) demonstrated that targeted deletion of *DNMT1* by homologous recombination in the colon cancer cell line HCT116 was not sufficient to cause promoter demethylation and gene re-expression. In these experiments, *DNMT1* deletion resulted in only a small decrease (~20%) in overall genomic methylation, and imprinted genes were not re-expressed. Rhee *et al.* (12) additionally showed that deletion of both *DNMT1* and *DNMT3B* reduced overall genomic methylation by >95% as well as promoter methylation of specific genes, and caused the re-expression of multiple genes (*p16^{ink4A}* and *TIMP-3*), resulting in substantial growth suppression of HCT116 cells. Paradoxically, a more recent publication by Robert *et al.* (13) showed that DNMT1 depletion using either antisense or small interfering RNA (siRNA) techniques led to demethylation of *p16^{ink4A}* and *MLH1* promoters, and re-expression of p16^{ink4A} in the same HCT116 cells. Therefore, it is still unclear how the different *DNMT* genes act alone or in concert to maintain or establish DNA methylation patterns in individual types of human cancers.

To address this issue, we used RNA interference (RNAi) technology to knock down DNMT1 protein expression in the non-small cell lung cancer cell line, NCI-H1299, and the breast cancer cell line, HCC1954. Using quantitative assays for DNA methylation and mRNA expression, we found that DNMT1 knockdown led to a dramatic loss of methylation (>80%) compared with nontreated controls at the promoters of *RASSF1A*, *p16^{ink4A}*, *CDH1*, and *HPP1*, and re-expression of *RASSF1A*, *p16^{ink4A}*, *CDH1*, *HPP1*, and *SEMA3B* in lung and breast cancer cells. These findings provide quantitative evidence of the role of DNMT1 activity in both lung and breast cancer cells.

MATERIALS AND METHODS

Lung and Breast Cancer Cell Lines, and 5-Aza-2′-Deoxycytidine (5-Aza-CdR) Treatment. The non-small cell lung cancer cell line NCI-H1299 and the breast cancer cell line HCC1954 were established by us (14, 15) and deposited in the American Type Culture Collection. Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum, and incubated in a humidified atmosphere and 5% CO_2 at 37°C. NCI-H1299 and HCC1954 were incubated in culture medium with 4 μM 5-Aza-CdR (Sigma, St. Louis, MO) in DMSO for 6 days, with medium changes on days 1, 3, and 5, and cells harvested and total RNA extracted on day 6 using TRIzol (Invitrogen, Carlsbad, CA).

Preparation and Transfection of siRNAs. siRNAs targeting *DNMT1* were designed and prepared as described previously (16). The two siRNA sequences against *DNMT1* were 5′-CGGUGCUCAUGCUUACAACTT-3′ (sense) and 5′-GUUGUAAGCAUGAGCACCGTT-3′ (antisense), and 5′-

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Table 1 *Primer and TaqMan probe sequences for MSP[a] and mRNA expression studies*

Gene	Type	Forward primer	Reverse primer
RASSF1A	M	5′-GTGGTTTCGTTCGGTTCGC-3′	5′-CGATACCCCGCGCGA-3′
	M-probe	6FAM-5′-CCGACATAACCCGATTAAACCCGTACTTCG-3′-TAMRA	
	RT	5′-GCTCGTCTGCCTGGACTGTT-3′	5′-TGGGCATTGTACTCCTTGATCTT-3′
	RT-probe	6FAM-5′-TGTGGAGTGGGAGACACCTGACCTTTCT-3′-TAMRA	
p16^{ink4A}	M	5′-CGCAACCGCCGAACG-3′	5′-TTTTTTCGTTAGTATCGGAGGAAGA-3′
	M-probe	6FAM-5′-CGCGATCCGCCCCACCCT-3′-TAMRA	
	RT	5′-TTCGGCTGACTGGCTGGCCA-3′	5′-AGCTCCTCAGCCAGGTCCAC-3′
CDH1	M	5′-AATTTTAGGTTAGAGGGTTATCGCGT-3′	5′-TCCCCAAAACGAAACTAACGAC-3′
	M-probe	6FAM-5′-CGCCCACCCGACCTCGCAT-3′-TAMRA	
	RT	5′-TTTCTTGGTCTACGCCTGGGACTC-3′	5′-CACCTTCAGCCATCCTGTTTCTC-3′
HPP1	M	5′-GTTATCGTCGTCGTTTTTGTTGTC-3′	5′-GACTTCCGAAAAACACAAAATCG-3′
	M-probe	6FAM-5′-CCGAACAACGAACTACTAAACATCCCGCG-3′-TAMRA	
	RT	5′-TGCTTTCCCTACCTCCTTAAGTGA-3′	5′-CTGTCATCATAACCAGAGCAATTCC-3′
	RT-probe	6FAM-5′-TGCCAAACGCCCACCGGC-3′-TAMRA	
SEMA3B	RT	5′-CTGGCTCAATGAGCCCAAGT-3′	5′-CTACCGCCGTCTCACGAAAG-3′
	RT-probe	6FAM-5′-AGGTATTTTGGATCCCGGAGAGCGAGAATA-3′-TAMRA	
MYOD1	M	5′-CCAACTCCAAATCCCCTCTCTAT-3′	5′-TGATTAATTTAGATTGGGTTTAGAGAAGGA-3′
	M-probe	6FAM-5′-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC-3′-TAMRA	
GAPDH	RT	5′-GACCACAGTCCATGCCATCACT-3′	5′-GCTTCACCACCTTCTTGATGTCA-3′
TBP	RT	5′-TGCTGCGGTAATCATGAGGAT-3′	5′-TGGAAAACCCAACTTCTGTACAAC-3′
	RT-probe	6FAM-5′-AGAGAGCCACGAACCACGGCACTG-3′-TAMRA	

[a] MSP, methylation-specific PCR; M, primer for real-time MSP; M-probe, TaqMan probe for real-time MSP; RT, primer for RT-PCR; RT-probe, TaqMan probe for real-time RT-PCR; TAMRA, 6-carboxytetramethyl rhodamine; 6FAM, 6-carboxyfluorescein.

CGAGUUGCUAGACCGCUUCTT-3′ (sense) and 5′-GAAGCGGUCUAGCAACUCGTT-3′ (antisense). The siRNA sequences against the human T-cell leukemia virus gene (*Tax*) and *Lamin A/C* were as reported previously (16, 17). The siRNA target sequences were tested in a basic local alignment search tool search of GenBank (National Center for Biotechnology Information database) to ensure that only the corresponding gene is the target. RNA oligonucleotides were obtained from the core facility in University of Texas Southwestern Medical Center (see website for details).[6] The sense and antisense oligonucleotides were annealed to make siRNA (18) and stored at −20°C before use. One day before transfection, cells were seeded such that they were 30–50% confluent the next day. Cells were transfected with 100 nM of siRNA using Oligofectamine transfection reagent (Invitrogen) in Opti-MEM I reduced serum medium (Invitrogen) at 37°C in a 5% CO_2 atmosphere for 6 h. The medium was removed and replaced with fresh RPMI 1640 supplemented with 5% fetal bovine serum. Control cells were treated with Oligofectamine alone or with *Tax* and *Lamin A/C* siRNA. Transfection was repeated at 2, 4, and 6 days for a total of 4 treatments. Cells were grown and harvested at 3, 5, 7, 9, 14, and 23 days after the initial transfection for additional analysis.

Western Blot Analysis. Cells were grown and harvested at 80–90% confluency, and cellular proteins were extracted with lysis buffer [40 mM HEPES-NaOH (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl] containing Complete Mini, a mixture of protease inhibitors (Roche, Indianapolis, IN). Total protein was electrophoresed on SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated with the mouse monoclonal anti-DNMT1 (Imgenex, San Diego, CA), the rabbit polyclonal anti-DNMT3B (a kind gift from Dr. A. Robert MacLeod, MethylGene Inc., Montreal, Quebec, Canada), the rabbit monoclonal anti-p16^{ink4A} (Santa Cruz Biotechnology, Santa Cruz, CA), or the mouse monoclonal anti-actin (Sigma) antibodies. The membranes then were developed with peroxidase-labeled antibodies (Amersham Pharmacia, Piscataway, NJ) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL). Actin protein levels were used as a control for equal protein loading.

Quantitative Methylation-Specific PCR (MSP) Assay. Genomic DNA was obtained from cell lines by digestion with proteinase K (Life Technologies, Inc.), followed by phenol:chloroform (1:1) extraction. One μg of genomic DNA was denatured with 2 N NaOH and modified with sodium bisulfite, as described previously (19). The modified DNA was purified using the Wizard DNA purification kit (Promega, Madison, WI), treated with 3 N NaOH, precipitated with ethanol, and resuspended in water. Sodium bisulfite-treated genomic DNA was amplified by fluorescence-based real-time MSP (Perkin-Elmer Corp., Foster City, CA) as described previously (20). For the internal reference gene, *MYOD1*, the primers and probe were designed to avoid CpG nucleotides. The methylation ratio is defined as the ratio of the fluorescence emission intensity values for the target PCR products to those of the *MYOD1* PCR products, multiplied by 100. The ratio is then divided by the ratio of the nontreated sample and multiplied by 100 to yield a percentage. The sequences of the primers and probes are shown in Table 1. Quantitative real-time MSP assays were performed in a reaction volume of 25 μl by using components supplied in a TaqMan PCR Core Reagent kit (Perkin-Elmer Corp.). Each assay was performed in triplicate. The final reaction mixtures contained the forward and reverse primers at 300 nM each; the probe at 100 nM; 200 μM each of dATP, dGTP, dCTP, and dTTP; 5.5 mM $MgCl_2$; 1× TaqMan Buffer A; 1 unit of HotStarTaq DNA polymerase (Qiagen Inc., Valencia, CA); and 2 μl bisulfite-converted genomic DNA. PCR was performed under the following conditions, 95°C for 12 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. We performed quantitative real-time MSP with the Gene Amp 5700 Sequence Detection System (Perkin-Elmer Corp.). DNA from lymphocytes of a healthy volunteer treated with SssI methyltransferase (New England BioLabs, Beverly, MA) was used as a positive control. The same untreated, unmethylated DNA was used as a negative control for methylated alleles. Water blanks were included with each assay.

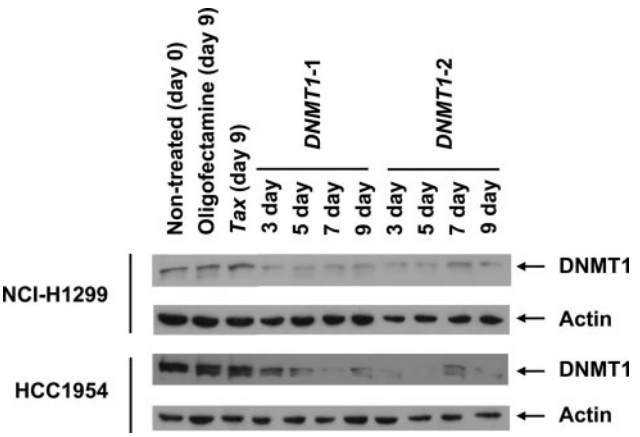

Fig. 1. RNA interference-mediated knockdown of DNMT1 protein expression in NCI-H1299 non-small cell lung cancer and HCC1954 breast cancer cell lines. NCI-H1299 and HCC1954 cells were untreated, or treated with Oligofectamine alone, *Tax* small interfering RNA (siRNA), or two different sequences of siRNA targeted to *DNMT1* (*DNMT1*-1 and *DNMT1*-2) four times (on days 0, 2, 4, and 6). Western blots were performed on lysates from untreated cells at day 0, and Oligofectamine-treated cells and *Tax* siRNA treated cells at day 9, and *DNMT1* siRNA-treated cells at 3, 5, 7, and 9 days. Twenty μg of total protein were loaded per lane.

[6] Internet address: http://cbi.swmed.edu/pages/oligonet_index.htm.

Expression Assays for *p16^{ink4A}*, *CDH1*, *RASSF1A*, *HPP1*, and *SEMA3B*. The expression of *p16^{ink4A}* and *CDH1* mRNA was analyzed by reverse transcription-PCR (RT-PCR). cDNA was generated with 4 μg of total RNA with SuperScript II First-Strand Synthesis using the oligodeoxythymidylic acid primer System (Life Technologies, Inc.). The housekeeping gene *GAPDH* was used as an internal control to determine the quality of the resultant cDNA. PCR products were resolved on 2% agarose gels. The expression of *RASSF1A, HPP1,* and *SEMA3B* genes was analyzed by quantitative real-time RT-PCR. We used TATA box binding protein (*TBP*; Ref. 21) as an internal reference gene to normalize the expression of *RASSF1A*, *HPP1*, and *SEMA3B*. Quantitative real-time RT-PCR was performed in a reaction volume of 25 μl including 2 μl of cDNA. The expression ratio is defined as the ratio of the fluorescence emission intensity values for the target PCR products to those of the *TBP* PCR products, multiplied by 100. To obtain a percentage the treated

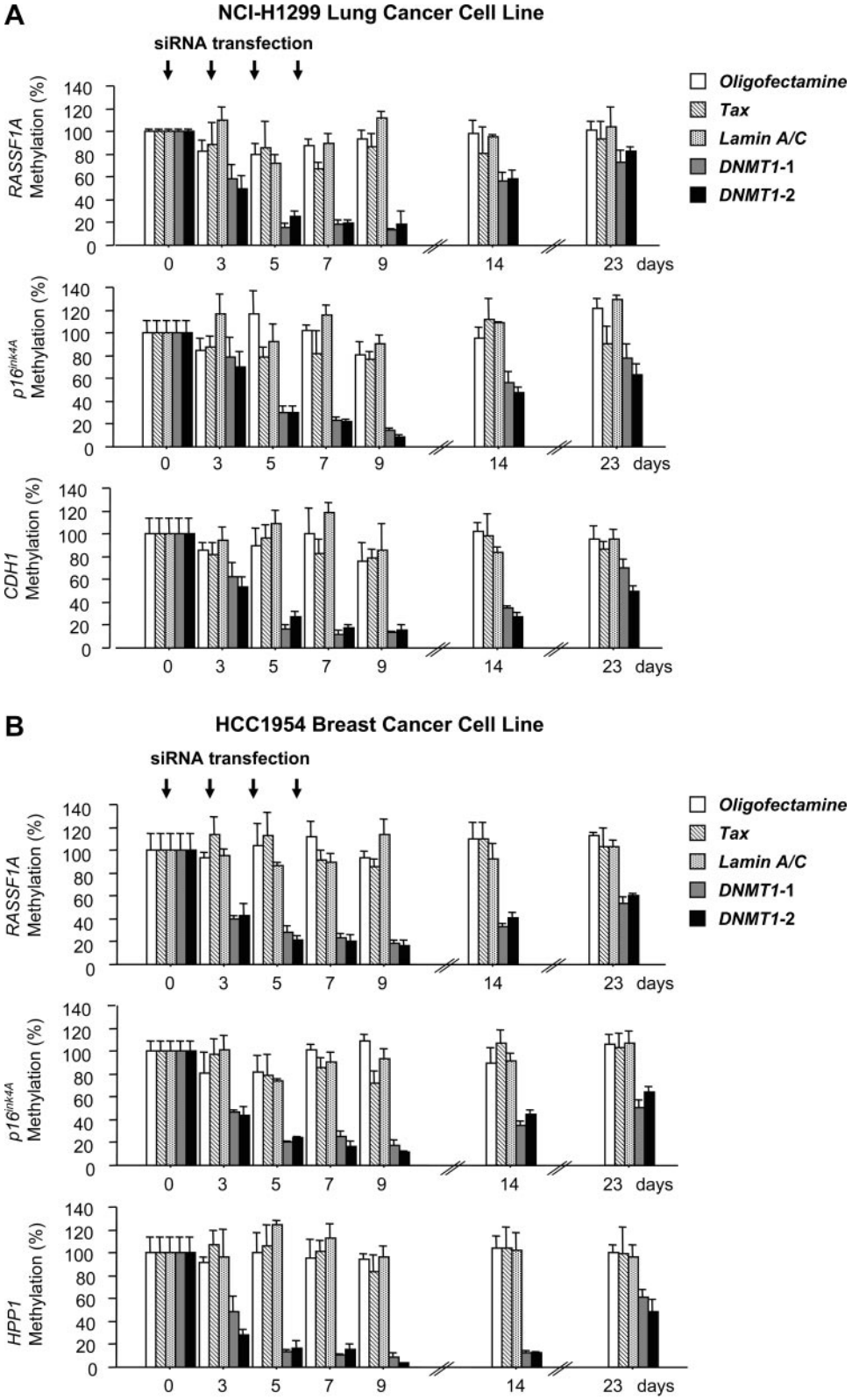

Fig. 2. Time course and kinetics of methylation status of *RASSF1A*, *p16^{ink4A}*, and *CDH1* genes in NCI-H1299, and the *RASSF1A*, *p16^{ink4A}*, and *HPP1* genes in HCC1954 treated with small interfering RNAs, monitored by real-time methylation-specific PCR assay. Cells were treated with Oligofectamine alone (□), *Tax* (▨), *Lamin A/C* (▥), *DNMT1*-1 (▤), or *DNMT1*-2 (■) small interfering RNA four times (on days 0, 2, 4, and 6). Cells were harvested at 0, 3, 5, 7, 9, 14, and 23 days, and DNA was extracted and treated with sodium bisulfite. Real-time methylation-specific PCR was performed as described ("Materials and Methods"). Each ratio was normalized to *MYOD1* and converted to a percentage based on the same ratio in untreated cells. Each point represents averages from three independent experiments; *bars,* ±SE. *A,* NCI-H1299. *B,* HCC1954.

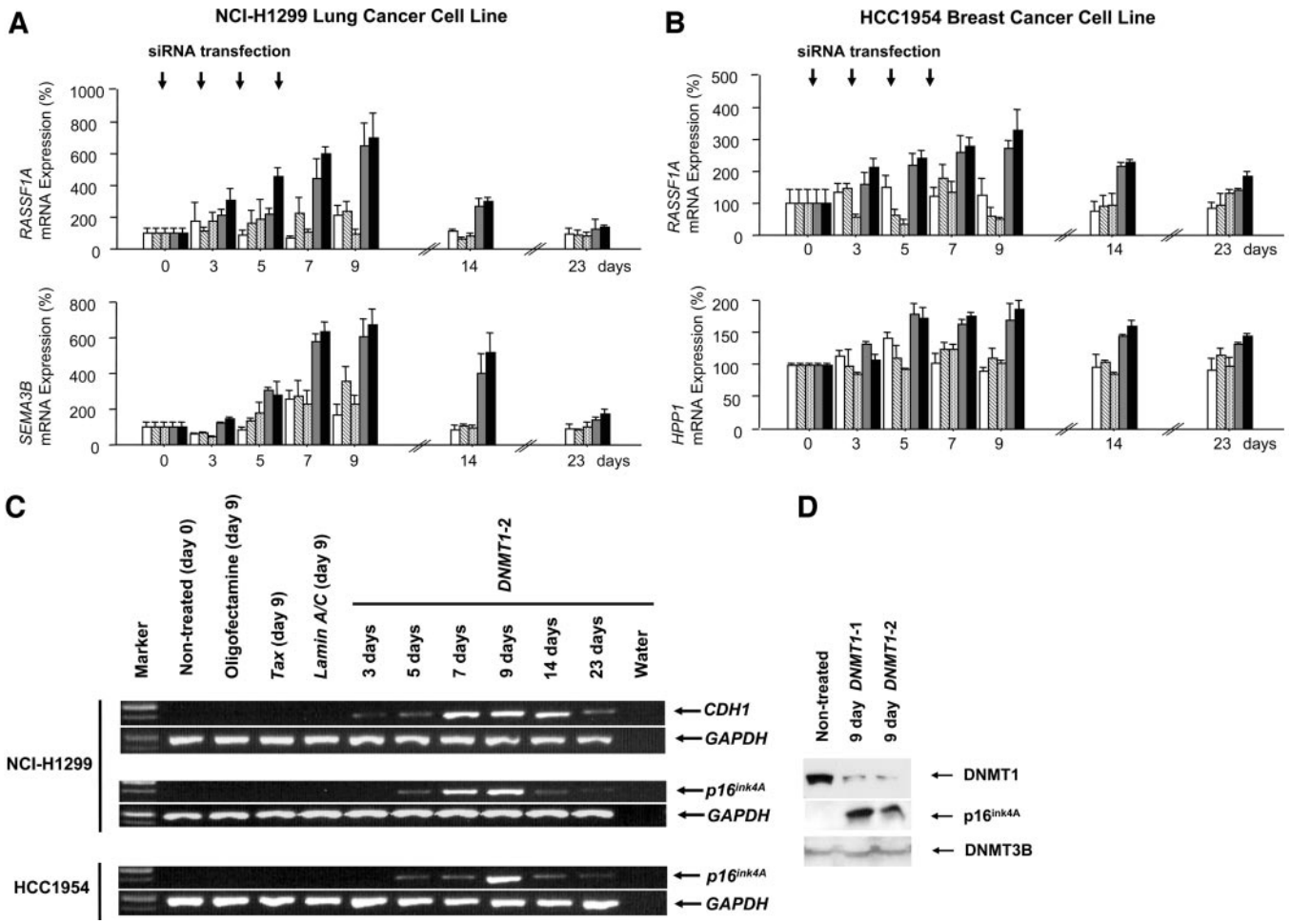

Fig. 3. Time course of mRNA expression of $p16^{ink4A}$ and *CDH1* genes in NCI-H1299, and the $p16^{ink4A}$ gene in HCC1954 by reverse transcription-PCR (RT-PCR); time course and kinetics of mRNA expression level of *RASSF1A* and *SEMA3B* genes in NCI-H1299, and the *RASSF1A* and *HPP1* genes in HCC1954 by real-time RT-PCR. Cells were harvested, RNA extracted, and cDNA synthesized. Real-time RT-PCR was performed as described ("Materials and Methods"). Each ratio was normalized to TATA box binding protein and converted to percentage based on the same ratio in nontreated cells. Each *point* represents averages from three independent experiments; *bars,* ±SE. *A,* NCI-H1299 cells. *B,* HCC1954. *C,* RT-PCR was performed as described ("Materials and Methods") to examine the expression of *CDH1* and $p16^{ink4A}$ in NCI-H1299, and $p16^{ink4A}$ in HCC1954. The PCR products were separated on 2% agarose gel. *GAPDH* was run as a control for RNA integrity. *D,* Western blot was performed to examine the protein expression of $p16^{ink4A}$ and *DNMT3B* in NCI-H1299. *A* and *B*, □, Oligofectamine; ▨, Tax; ▩, Lamin A/C; ■, DNMT1-1; ■, DNMT1-2.

sample ratio was divided by the control ratio and multiplied by 100. Quantitative real-time RT-PCR for both target genes and *TBP* was performed in triplicate.

Soft Agar-Growth Assay. Cells were transfected with siRNAs for a total of four treatments, and 7 days after the initial transfection, cells were replated for soft agar-growth assay. Briefly, 300 viable cells were suspended and plated in 0.33% agar in RPMI 1640 (Life Technologies, Inc.) supplemented with 20% fetal bovine serum and layered over a 0.50% agar base medium in 12-well plates. After 2 weeks, the number of colonies >100 cells were counted in triplicate plates.

RESULTS AND DISCUSSION

RNAi-Mediated Knockdown of DNMT1 Protein Expression in NCI-H1299 and HCC1954. We used RNAi technology to examine the effect of DNMT1 expression on the stability of tumor-associated promoter methylation in lung and breast cancer cells (16, 22, 23). Two siRNAs targeting different sequences of *DNMT1* mRNA were used to verify that our results were a consequence of specific inhibition of DNMT1 expression. In addition, siRNA targeting the human T-cell leukemia virus *Tax* oncogene was used as a negative control, because this viral protein is not expressed in epithelial cells (17). Another negative control involved targeting of the expressed *Lamin A/C* gene, because Lamin A/C protein is nonessential in cultured mammalian cells (24). siRNAs against *DNMT1* (*DNMT1*-1 and *DNMT1*-2), *Tax*, and *Lamin A/C* were transfected into NCI-H1299 and HCC1954 cells every 2 days for a week. Cells were harvested at 3, 5, 7, 9, 14, and 23 days after the initial transfection, and Western Blot analysis was conducted to monitor endogenous DNMT1 protein expression (Fig. 1). Both siRNAs targeted to *DNMT1* mRNA led to substantial down-regulation of DNMT1 expression 3 days after the initial transfection in the NCI-H1299 cell line and HCC1954. These effects continued until at least day 9 (Fig. 1). We routinely observe targeted gene silencing in ~90% of the NCI-H1299 cells transfected with siRNA as detected by immunofluorescent staining of individual cells (data not shown). DNMT1 down-regulation was specific as evidenced by a consistent level of actin protein (Fig. 1) and DNMT3B protein (Fig. 3*D*) in the context of siRNA targeted to *DNMT1*, whereas DNMT1 protein expression was not affected by siRNA targeted to *Lamin A/C* (data not shown).

RNAi-Mediated Knockdown of DNMT1 Expression Led to Demethylation of Tumor Suppressor Gene Promoters in Lung and Breast Cancer Cell Lines. To assess the effect of RNAi-mediated down-regulation of DNMT1 expression on aberrant methylation in the promoter regions of genes thought to be involved in the pathogenesis of

lung and breast cancer in NCI-H1299 and HCC1954 lines, we used a real-time MSP assay to quantitate the degree of methylation before and after DNMT1 knockdown (20, 25). Direct quantitation of the extent of methylation in a particular region of a promoter yields important information about the specificity of DNMT1 activity in terms of the methylation of specific CpGs in the regulatory sequence of particular genes. Several groups have reported that DNMT1 does not have a preference for certain CpG sites or promoters: it appears to act as a general methyltransferase (8, 9). The kinetics of demethylation for the genes we assessed were not significantly different; therefore, our results are consistent with this hypothesis.

To compare the methylation levels of each gene before and after treatment with siRNA, we converted the mean ratio of promoter methylation to a percentage. RNAi-mediated down-regulation of DNMT1 protein expression resulted in a significant decrease in methylation levels at the *RASSF1A*, *p16^{ink4A}*, and *CDH1* promoters in NCI-H1299 (Fig. 2*A*), and similar effects were observed for *RASSF1A*, *p16^{ink4A}*, and *HPP1* in HCC1954 (Fig. 2*B*; $P < 0.001$; all genes examined, repeated measures ANOVA).

Importantly, the kinetics of demethylation correlate with the loss of DNMT1 expression (Fig. 1; Fig. 2, *A* and *B*). The level of methylation for all genes was reduced on day 3, more so on day 5, and reached a nadir on day 7, whereupon maximal demethylation seems to have been reached (Fig. 2, *A* and *B*). As the data indicate, there does not appear to be a significant decrease in methylation levels between day 7 and 9. The methylation level of all of the genes tested was reduced by up to 80% when compared with promoter methylation levels in untreated cells. Reduction of promoter methylation was greatest in the *HPP1* promoter in HCC1954 (Fig. 2*B*) and *p16^{ink4A}* in NCI-H1299 (Fig. 2*A*), yet in neither case was methylation completely lost.

It is known that siRNA can be used to specifically knock down target genes, but RNAi never completely eliminates the targeted gene products (26). Thus, the presence of basal amounts of promoter methylation we observed, even with extended siRNA treatment, may result from residual DNMT1 protein or other DNMTs. Other methyltransferases such as DNMT3B or methyl-DNA binding proteins may affect methylation levels in the promoters of TSGs. Rhee *et al.* (12) demonstrated that genetic disruption of *DNMT1* by homologous recombination did not lead to promoter demethylation and re-expression of *p16^{ink4A}* in the colon cancer cell line HCT116, whereas *p16^{ink4A}* was demethylated and re-expressed in HCT116 cells, in which both *DNMT1* and *DNMT3B* were disrupted. Therefore, knockdown of both DNMT1 and DNMT3B or other factors may be required to achieve complete demethylation of genes involved in cancer pathogenesis.

5-Aza-CdR treatment results in global demethylation of genomic DNA in many cancer cell lines. Upon removal of 5-Aza-CdR and continued culture, remethylation occurs slowly and in a sequence-specific manner (27). The propensity of particular regions of DNA to become remethylated may result from selective pressure, such as TSG function, or some cryptic sequence information within loci that are preferentially remethylated. Recent research using 5-Aza-CdR indicates that *de novo* methylation of CpG sites in the *p16^{ink4A}* promoter is not stochastic. Thus, the kinetics of selective CpG island remethyl-ation in the promoters of genes may reflect differences in the contribution individual CpG sites have to gene repression. However, due to the nonspecificity and cytotoxicity of 5-Aza-CdR, it is unclear which DNMT is responsible for the apparent nascent methylation, or whether remethylation is really the result of the expansion of a resistant subclone within the treated population of cells (27).

To address these issues and to determine how persistent loss of promoter methylation was in the context of the specific down-regulation of DNMT1 protein, we maintained the treated cell lines in the absence of any additional siRNA treatment. We then reexamined the methyl-ation level of all genes at day 14 and day 23 after initial treatment. The kinetics of remethylation varied between genes in both cell lines; however, remethylation (returning to 40–80% of starting levels) and loss of gene expression was observed in all cases by day 23 (Fig. 2, *A* and *B*). These results indicate that the appearance of *de novo* methylated CpG sites within multiple gene promoters occurs in tandem with the re-expression of DNMT1 protein. This finding clarifies the results from the 5-Aza-CdR experiments described above, because it suggests that DNMT1, as opposed to DNMT3A and DNMT3B, has important *in vivo*, *de novo* DNA methyltransferase activity. A previous report has demonstrated that DNMT1 has *de novo* methylase activity, but only *in vitro* (28). The variation in remethylation kinetics between the two cell lines may result from differences in their doubling times (NCI-H1299 have a doubling time of 25 h, whereas HCC1954 double every 31 h), because *de novo* methylation has been shown to be dependent on cell division (27).

Demethylation Induced by RNAi-Mediated DNMT1 Knockdown Restored the Expression of Several Tumor Suppressor Genes in Lung and Breast Cancer Cell Lines. To establish whether loss of promoter methylation mediated by *DNMT1* siRNA resulted in the quantitative re-expression of genes, we analyzed the expression status of *RASSF1A* and *SEMA3B* genes in NCI-H1299, and *RASSF1A* and *HPP1* genes in HCC1954 line by real-time RT-PCR (Fig. 3, *A* and *B*). RNAi-mediated DNMT1 knockdown induced the expression of all genes examined ($P < 0.001$, repeated measures ANOVA). The expression levels of all genes in *DNMT1* siRNA-treated cells were 2–8-fold higher than that of untreated cells. We examined the expression status of *SEMA3B* because it is silenced by tumor-associated promoter methylation in NCI-H1299, and is located on 3p21, a known tumor suppressor locus as reported by us and others (29, 30). The expression level of *p16^{ink4A}* and *CDH1* genes in NCI-H1299 and *p16^{ink4A}* in HCC1954 were examined by 37-cycle end point RT-PCR. NCI-H1299 cells treated with *DNMT1* siRNA expressed *p16^{ink4A}* mRNA from day 5 to day 23 and expressed *CDH1* from day 3 to day 23 (Fig. 3*C*). HCC1954 cells treated with *DNMT1* siRNA expressed *p16^{ink4A}* from day 3 to day 23 (Fig. 3*C*).

Because the *p16^{ink4A}* gene locus has a complicated structure, it was not possible to design an isoform-specific TaqMan probe. Thus, we sought to verify gene induction by Western blot. Both of two different siRNAs targeted to *DNMT1* restored p16^{ink4A} protein expression (Fig. 3*D*). Thus, there is a clear inverse relationship between the presence of methyl-CpGs in the promoter of *p16^{ink4A}*, and the expression of *p16^{ink4A}* mRNA and protein. We additionally compared the effect of the *DNMT1* siRNA (*DNMT1*-2) on the restoration of gene expression with that of 5-Aza-CdR treatment in these cell lines. siRNA inhibitors of DNMT1 protein expression are at least as effective at restoring mRNA expression as 5-Aza-CdR treatment (Table 2).

We found that specific inhibition of DNMT1 expression by RNAi is a useful technique to examine the relationship between DNMT1 activity and aberrant promoter methylation in cancer cells. RNAi-mediated knockdown of DNMT1 expression persisted for >9 days, and was sufficient for achieving the loss of promoter methylation at *RASSF1A*, *CDH1*, *p16^{ink4A}*, and *HPP1*, and re-expression of *p16^{ink4A}*, *CDH1*, *RASSF1A*, *SEMA3B*, and *HPP1* mRNA, which also persisted for 9–14 days, in lung and breast cancer cells. Our findings support and extend the conclusion of Robert *et al.*, who used HCT116 colon cancer cells (13), that DNMT1 siRNA-mediated knockdown alone is sufficient to achieve inhibition of promoter methylation with associated gene

Table 2 *Gene expression ratios in NCI-H1299 and HCC1954 in response to treatment*

Cell line treatment	NCI-H1299			HCC1954		
	Nontreated[a]	DNMT1siRNA[b]	5-Aza-CdR	Nontreated	DNMT1siRNA	5-Aza-CdR
RASSF1A	1 (3.3)	7.0 (23)	8.2 (27)	1 (4.2)	3.1 (13)	6.4 (27)
HPP1	—	—	—	1 (26)	1.8 (46)	1.7 (44)
SEMA3B	1 (1.1)	6.5 (7.1)	5.6 (6.2)	—	—	—

[a] Using quantitative RT-PCR (see "Materials and Methods") the actual levels of gene expression were determined relative to that for TBP mRNA (TSG:TBP) × 100. A ratio for gene:TBP expression was calculated and is given in parentheses for each gene and nontreated sample. The other ratios are then given relative to an untreated ratio of 1.
[b] siRNA, small interfering RNA; 5-Aza-CdR, 5-Aza-2′-deoxycytidine; RT-PCR, reverse transcription-PCR; TBP, TATA box binding protein; TSG, tumor suppressor gene.

re-expression. Our findings, like those of Robert *et al.,* differ from that of Rhee *et al.* (12) who found that in HCT116 colon cancer cells, using recombinant knockout techniques, both *DNMT1* and *DNMT3B* had to be removed to achieve loss of methylation and gene re-expression.

We assessed the effect of the DNMT1 knockdown on *in vitro* growth of NCI-H1299 cells by soft agar growth assay. Surprisingly, there was no significant difference in colony number between treatment of *DNMT1* siRNAs (*DNMT1*-1 and *DNMT1*-2) and that of *Tax* siRNA (means ± SD of colony number by treatments with *DNMT1*-1, *DNMT1*-2, and *Tax* siRNAs were 115 ± 13, 100 ± 14, and 108 ± 9, respectively). Thus, we could not demonstrate an obvious effect of the loss of DNMT1 expression on the *in vitro* tumor growth of NCI-H1299 cells. Whereas this was unexpected, there are several possible explanations for this result. The first is that the genes we monitored (*e.g., RASSF1A* or *SEMA3B*) really do not function as TSGs. The study of *SEMA3B* as a TSG is early, there are multiple methylation and functional studies of the role of $p16^{ink4A}$ and *RASSF1A* strongly implicating them as TSGs in lung and other cancers (29–33). The tumor cells were plated after 7 days and 4 RNAi treatments the colonies were not scored until 14 days later, it is possible that transient re-expression of the tumor suppressor genes by DNMT1 siRNA was not sufficient to inhibit colony formation due to the short-term inhibition of DNMT1 expression. In fact, a recent study showed that prolonged knockdown of DNMT1 by a tetracycline-inducible vector-based siRNA induced growth arrest, whereas growth resumed 1–2 days after the siRNA knockdown was relaxed (34). It is also possible that the tumor cells have developed other ways to bypass these growth regulatory molecules. For example, the p53 null status of H1299 cells (they are homozygously deleted for *p53*) prevents transient re-expression of the proteins from inducing apoptosis. In fact, a previous study showed that adenovirus-mediated exogenous p16 expression alone did not induce apoptosis in H1299 cells, but only exhibited apoptosis after the addition of exogenous p53 expression (35). Finally, it is possible that DNMT1 knockdown led to the expression of proteins (*e.g.,* those involved in the differentiated state), which either made the cells resistant to tumor suppressor function or caused growth arrest, preventing subsequent induction of apoptosis by other re-expressed proteins. All of these mechanisms will require future study. However, the lack of a dramatic effect on growth of H1299 cells by DNMT1 knockdown indicates to us that the use of agents that block methylation may have to be combined with other approaches before being clinically active. Additional studies of single cells and clones after knockdown will be needed to verify that individual cells can undergo DNMT1 knockdown, loss of promoter methylation, and re-expression of genes followed by later promoter remethylation and gene silencing. In this regard, additional investigations using RNAi vectors that can stably suppress the expression of other DNMTs and/or methyl-DNA binding proteins will elucidate how DNA methylation contributes to cancer pathogenesis, and enable us to systematically analyze the DNA methylation machinery as a target for therapeutic intervention of cancer.

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