

Carcinogen-Induced Gene Promoter Hypermethylation Is Mediated by DNMT1 and Causal for Transformation of Immortalized Bronchial Epithelial Cells

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Abstract

A better understanding of key molecular changes during transformation of lung epithelial cells could affect strategies to reduce mortality from lung cancer. This study uses an *in vitro* model to identify key molecular changes that drive cell transformation and the likely clonal outgrowth of preneoplastic lung epithelial cells that occurs in the chronic smoker. Here, we show differences in transformation efficiency associated with DNA repair capacity for two *hTERT/cyclin-dependent kinase 4*, immortalized bronchial epithelial cell lines after low-dose treatment with the carcinogens methyl-nitrosourea, benzo(a)pyrene-diolepoxide 1, or both for 12 weeks. Levels of cytosine-DNA methyltransferase 1 (DNMT1) protein increased significantly during carcinogen exposure and were associated with the detection of promoter hypermethylation of 5 to 10 genes in each transformed cell line. Multiple members of the cadherin gene family were commonly methylated during transformation. Stable knockdown of *DNMT1* reversed transformation and gene silencing. Moreover, stable knockdown of DNMT1 protein before carcinogen treatment prevented transformation and methylation of cadherin genes. These studies provide a mechanistic link between increased DNMT1 protein, *de novo* methylation of tumor suppressor genes, and reduced DNA repair capacity that together seem causal for transformation of lung epithelial cells. This finding supports the development of demethylation strategies for primary prevention of lung cancer in smokers. [Cancer Res 2008;68(21):9005–14]

Introduction

Lung cancer accounts for 30% of all cancer deaths in both men and women in the United States, and 1.5 million deaths are expected worldwide by 2010 (1). The high mortality from this disease stems from the lack of an effective screening approach for early diagnosis and the refractiveness of advanced cancers to conventional chemotherapy, substantiating the need to develop more effective targeted therapies and chemoprevention. Both these strategies to reduce mortality would benefit from a better understanding of the key molecular changes that are driving cell transformation and the clonal outgrowth of preneoplastic cells.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Malignant transformation occurs after years of chronic DNA damage to the pulmonary epithelial cells by the carcinogens in tobacco. Both genetic and epigenetic changes in oncogenes and tumor suppressor genes are clearly important in the development of lung cancer. However, promoter hypermethylation now rivals gene mutation with the identification of >60 genes as being epigenetically silenced in lung tumors (2). Gene silencing through methylation can occur at the earliest stages of lung cancer development, both in histologic precursors to adenocarcinoma and squamous cell carcinoma and in the bronchial epithelium of smokers. Studies by our laboratory showed that methylation of the *p16* gene occurs in alveolar hyperplasia and basal cell hyperplasia, early precursors to adenocarcinoma and squamous cell carcinoma, respectively, and in the bronchial epithelium of cancer-free smokers (2). Moreover, a nested, case-control study of incident lung cancer revealed that methylation of a panel of genes detected in epithelial cells exfoliated into sputum was associated with a 6.5-fold increased risk for lung cancer (3). In contrast, *p53* mutation is a relatively late event in lung cancer, occurring in severe dysplasia and carcinoma *in situ*, whereas *K-ras* mutation is restricted to a subset of adenocarcinomas (4, 5). Therefore, inactivation of genes by promoter methylation is likely one of the major factors contributing to the development of premalignant cells throughout the respiratory epithelium.

The cytosine DNA methyltransferases (*DNMT*) 1, 3a, and 3b have been implicated to different extents in initiating gene silencing through *de novo* methylation and recruitment of chromatin remodeling proteins (6–9). *DNMT1* has both maintenance and *de novo* methyltransferase activity, associates with chromatin, and is responsible for ~90% of methyltransferase activity in mammalian cells (10). *DNMT1* binds the histone methyltransferases *G9a*, *SUV39H1*, *heterochromatin protein 1 α* (*HPI1/α*), and they are recruited to heterochromatic regions of nucleoli before replication (11, 12). *DNMT1* is also rapidly recruited to sites of DNA damage where it participates in *de novo* methylation and is overexpressed in several cancers including lung (13–16). Together, these studies support the hypothesis that *DNMT1* plays a major role in aberrant gene methylation, and its altered expression may contribute to malignant transformation. *DNMT3a* and *3b* are also overexpressed in tumors and can cooperate with *DNMT1* to spread methylation in the genome (16, 17). Moreover, depending on the experimental strategy, reducing *DNMT1* levels did in some and not in other studies result in re-expression of silenced tumor suppressor genes in cancer cell lines, whereas genetic disruption of both *DNMT1* and *3b* led to gene demethylation (10, 18–20). The relationship between DNA damage induced by tobacco carcinogens, gene methylation, and the role of the *DNMTs* in transformation could be more accurately defined with a comprehensive and robust *in vitro* model.

Current models studying transformation have limitations that make it difficult to precisely chronicle the key events leading to transformation. Primary bronchial epithelial cells have a finite life span, whereas *SV40* immortalized bronchial epithelial cells (BEAS2B) are genomically unstable, and the *p53* gene that regulates many pathways has been inactivated (21). Recently, human bronchial epithelial cell lines (HBEC) were immortalized by insertion of the *telomerase* (*hTERT*) catalytic subunit and *cyclin-dependent kinase 4* (22). HBECs can be passaged indefinitely, have an intact *p53* checkpoint, are genomically stable, and do not grow in soft agar or nude mice (22).

The purpose of this study was to develop an *in vitro* cell transformation model to identify the critical mediators of premalignancy. HBECs were exposed chronically to low doses of tobacco carcinogens to establish the role of DNA repair capacity (DRC), epigenetic and genetic alterations, and the DNMTs in cell transformation.

Materials and Methods

Detailed methods are provided as Supplementary Methods.

Cell culture and carcinogen exposures. HBECs (received from Drs. Shay and Minna, Southwestern Medical Center, Dallas, TX) were established from two different people (HBEC1, smoker without lung cancer; HBEC2, smoker with cancer; ref. 22). Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. HBECs were exposed to benzo(a)pyrene-diolepoxide (BPDE) at concentrations of 0.05, 0.1, or 0.25 $\mu\text{mol/L}$, or methylnitrosourea (MNU; 0.5, 1, 2.5, or 5 mmol/L) or vehicle (DMSO).

Soft agar and nude mice tumorigenicity assays. Colony formation in soft agar was determined for HBECs after exposure to carcinogens for 12 wk. Male, 6-wk-old athymic BALB/c nude mice were injected s.c. with 2.5×10^6 cells in Matrigel diluted 1:1 in PBS in the flank and monitored over 90 d for tumor growth.

Real-time and semiquantitative reverse transcription-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed with the ABI PRISM 7900HT (Applied Biosystems). Experiments were normalized to *PCNA* and β -*actin*. Gel-based RT-PCR was also conducted for *DNMT1*, *DNMT3A*, and *DNMT3B* and *MAD2*.

Western blot analysis. Cells were harvested and protein extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents (Pierce). DNMT1, proliferating cell nuclear antigen (PCNA), and β -actin proteins were detected by chemiluminescence and by exposure to autoradiography film, double emulsion (ISC Bioexpress). To detect the rare DNMT3A and 3B proteins, the Amersham enhanced chemiluminescence Advance Western blotting detection kit was used.

Stable shRNA transfection. ShRNA sequences to *DNMT1*, *3a*, and *3b* (available upon request) were ligated into the pSilencer 2.1 (Ambion). Stable shRNA transfections in HBECs were performed using electroporation and hygromycin-resistant colonies were selected.

***P16* exon 2 deletion, *K-ras*, and *p53* mutation assays.** Exon 2 of *p16* was amplified using primers and PCR conditions as described (23). The absence of an exon 2 PCR product in treated compared with the parent HBEC lines was scored as a deletion. The BstN1 mutant allele enrichment method was used to screen for mutations in codon 12 of the *K-ras* gene. Exons 5 to 9 of the *p53* gene were screened for mutation using denaturing gradient gel electrophoresis (24).

Methylation-specific PCR. Nested, methylation-specific PCR (MSP) was used to screen for promoter methylation of 30 genes in transformants and during carcinogen exposure as described (3). Genes were called positive for methylation if a methylated PCR product was detected. Primer sequences and PCR conditions are available upon request.

Immuno-slot-blot for N7-methyldeoxyguanosine. DNA (1 μg) from cultured cells or a standard containing a known amount of N7-methyldeoxyguanosine (N7-meG) were denatured in 200 μL TE buffer plus

20 μL 2N NaOH. This treatment generates 2,6-diamino-4-hydroxy-5-*N*-methylformamido-pyrimidine (imidazole ring-opened 7-meG) from 7-meG. Samples were processed as described (25) and washed filters were treated with peroxidase-labeled polymer conjugated to goat anti-rabbit and goat anti-mouse immunoglobulins (Daco) diluted 1:1,000 in hybridization buffer. The enzymatic activity on the membrane was visualized by chemiluminescence (Amersham) after exposure to X-ray film.

DRC. DRC was measured by the cytokinesis-block micronucleus assay (26). One thousand cells were assessed for the presence of micronuclei as described (26).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was done using the ChIP Assay kit (Upstate). Antibodies for acetyl-H3K9, dimethyl-H3K4, trimethyl-H3K9, trimethyl-H3K27, HP1 α , and IgG input control were purchased from Upstate Chemicon and used to capture protein-DNA complexes. ChIP PCR analysis was performed using 2 to 3 μL of DNA and primers spanning the region -200 to $+1$ (with respect to ATG) of both the E-cadherin and H-cadherin promoters were used.

Results

Optimization of carcinogen dose and exposure frequency.

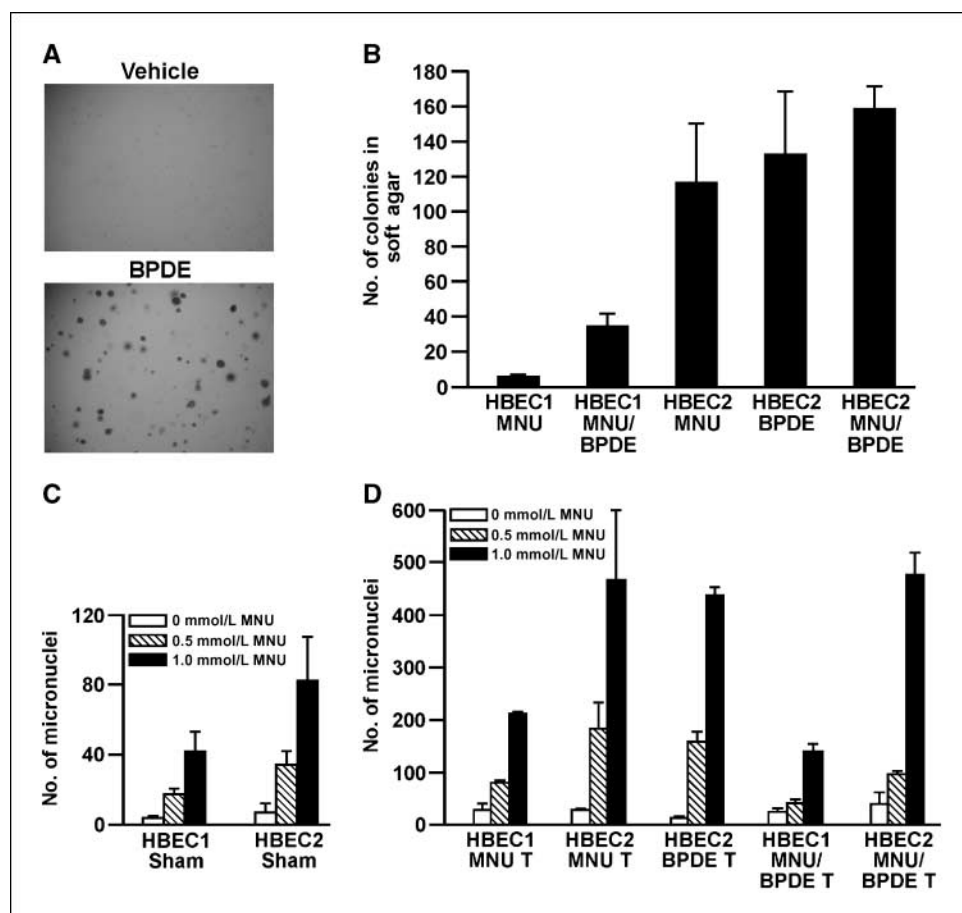
The development of an *in vitro* model system to study factors underlying transformation of epithelial cells by tobacco carcinogens should use a dose and treatment protocol that induces DNA damage in the absence of overt toxicity. These conditions more closely reflect the situation occurring in the bronchial epithelium of the smoker. The carcinogens chosen, BPDE and MNU, are direct-acting carcinogens derived from, or that generate reactive intermediates formed from tobacco carcinogens [BPDE, an active metabolite from benzo(a)pyrene, and MNU is an alkylating agent like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)], respectively. HBEC1 and 2 were exposed to BPDE (0.05–0.5 $\mu\text{mol/L}$) and MNU (0.5–5 mmol/L), and cell viability was measured by the MTT assay. BPDE (0.1 $\mu\text{mol/L}$) and MNU (1 mmol/L) did not effect cell viability, whereas 0.25 $\mu\text{mol/L}$ BPDE and 2.5 mmol/L MNU reduced viability by $\sim 30\%$ compared with vehicle-treated cells (data not shown). The doses were reduced 50% (0.05 $\mu\text{mol/L}$ of BPDE; 0.5 mmol/L MNU) when treatment with BPDE and MNU was done in combination to obviate any effects on cell viability.

Once the dose of carcinogen was established, the time interval between exposures was defined. HBEC1 and 2 were treated with MNU (1 mmol/L), and the formation and removal of N7-meG adducts was determined. Similar rates of adduct formation were seen in both cell lines 4 and 24 hours after treatment and then adducts declined with a $t_{1/2}$ of ~ 48 hours (data not shown). Studies were not conducted with BPDE due to the lack of a sensitive assay for detection of low levels of this adduct; however, transformation studies in BEAS2Bs used a weekly dosing protocol for this carcinogen (27). Therefore, to avoid any cumulative toxicity, cells were exposed to carcinogen weekly for 12 weeks.

Transformation of HBECs and association with DRC. HBEC1 and 2 were plated in soft agar after 12 weeks of carcinogen treatment. Colony formation, indicative of cell transformation, was apparent in both cell lines (Fig. 1A). Significant differences in transformation efficiency were seen (Fig. 1B). Four- to 16-fold fewer colonies developed from carcinogen exposed HBEC1 compared with HBEC2, and no transformation was evident in HBEC1 treated with BPDE. However, the transformed cells did not form tumors in nude mice.

One factor that could account for the difference in transformation efficiency is DRC. This hypothesis was evaluated by quantitating the generation of micronuclei after exposure of the nontransformed (parent) cell lines to carcinogen. A dose response

Figure 1. Transformation and DRC in immortalized bronchial epithelial cell lines. **A**, growth in soft agar of HBEC2 exposed to vehicle (DMSO) or BPDE for 12 wk. **B**, comparison of colony formation between HBEC1 and 2 after individual or combination carcinogen treatment. **C**, significant differences ($P < 0.01$) in number of micronuclei per 1,000 cells were seen between HBEC1 and 2 after treatment with MNU. **D**, increased number of micronuclei in HBEC1 and 2 transformed with MNU, BPDE, or their combination. Columns, mean from three experiments; bars, SE.



for micronuclei formation was seen in both cell lines treated with either MNU or BPDE (Fig. 1C; data not shown). The number of micronuclei formed in HBEC2 was ~ 2 -fold greater ($P < 0.01$) at both doses of either carcinogen compared with HBEC1, indicating a reduced DRC. The effect of transformation on DRC was also assessed through treatment of the transformed clones with carcinogen. There was a marked reduction in DRC relative to parent cells lines. For example, in HBEC1 transformed by the combination of MNU and BPDE (HBEC1 MNU/BPDE), the number of micronuclei formed increased from 42 to 152 per 1,000 cells after exposure to 1 mmol/L MNU (Fig. 1D). More striking reductions in DRC were seen in the repair deficient HBEC2 with >400 micronuclei observed in the transformants after MNU treatment. Similar increases in micronuclei formation were seen with BPDE treatment (data not shown).

The timing for induction of transformation was defined by quantitating colony formation after 6 and 9 weeks of carcinogen treatment. Colonies were detected in HBEC1 MNU/BPDE-treated cells at 6 weeks and increased in number at 9 weeks, whereas colony formation was only seen after 12 weeks of MNU treatment of HBEC1 (Table 1). Sparse colony formation was seen after 9 weeks of treatment of HBEC2 with MNU or BPDE but not until 12 weeks with the combined treatment (Table 1).

Genetic changes induced during transformation. The increase in micronuclei in transformed cells is an indirect measure of chromosome instability that could lead to deletion and/or gene mutation. Mutation of the *K-ras* and *p53* genes have been ascribed to the DNA damaging properties of MNU and BPDE, and the

deletion of the *p16/p19* locus is frequently observed in carcinogen-induced murine lung cancer (28–30). Mutations were not detected in codon 12 of the *K-ras* gene or in exons 5 to 9 of the *p53* gene in any of the transformants (data not shown). In contrast, exon 2 of the *p16* gene was deleted in HBEC1 transformed by MNU or the combination of MNU and BPDE (data not shown). Deletion of this locus was not observed in any of the HBEC2-transformed cell lines. Because the *p16* and *p14* genes share exon 2, this deletion functionally disrupts both proteins (23). The timing for deletion of the *p16/p14* locus was defined by evaluating HBEC1 cells after 6, 9, and 12 weeks of treatment with MNU or the combination of carcinogens. This deletion was first seen in both treatments at 6 weeks (data not shown).

Increase in DNMT1 protein during carcinogen exposure. Our group has shown that expression of the *DNMT1* gene is increased at the earliest histologic stage (alveolar hyperplasia) of carcinogen-induced murine lung tumor development and increases during progression to adenocarcinoma (15). Quantitative TaqMan assays and gel-based RT-PCR were used to investigate the effect that carcinogen-induced transformation had on expression of *DNMT1*, *DNMT3a*, and *3b* whose expression is also increased in some cancers (16). There were no significant differences in mRNA expression for any of the *DNMTs* in the transformed cell lines or during carcinogen treatment by TaqMan assays (data not shown). This was confirmed for all transformed cell lines compared with passage control cells for each *DNMT* using gel-based RT-PCR (Fig. 2A). Moreover, this approach allowed us to assess expression of the major isoforms for *DNMT3b* and again no differences in

Table 1. Time course for change in DNMT1 protein levels, methylation of gene promoters, and colony formation in HBECs during carcinogen treatment

Carcinogen exposure (wk)*	HBEC2 MNU			HBEC2 BPDE			HBEC2 MNU/BPDE		
	DNMT1 †	Methyl. ‡	Colonies§	DNMT1	Methyl.	Colonies	DNMT1	Methyl.	Colonies
0	1.0	4	0	1.0	4	0	1.0	4	0
6	4.3	4	0	2.5	5	0	1.8	6	0
9	5.1	7	2 ± 0.7	4.5	7	10 ± 3.2	1.5	9	0
12	6.2	9	116 ± 19.3	15.1	12	132 ± 21.0	5.4	13	158 ± 7.6
Transformant	5.0	11	NA	14.3	14	NA	6.3	9	NA
	HBEC1 MNU			HBEC1 MNU/BPDE					
0	1.0	2	0				1.0	2	0
6	0.5	2	0				0.9	2	7 ± 0.3
9	2.3	2	0				4.8	3	14 ± 2.6
12	2.5	3	6 ± 0.7				5.7	5	34 ± 4.3
Transformant	8.6	10	NA				9.5	7	NA

Abbreviation: NA, not applicable.

*HBEC1 and 2 cell lines were treated weekly for 12 wk with MNU, BPDE, or their combination. Transformant represents the expansion of five pooled colonies that grew in soft agar and level of DNMT1 Protein and number of methylated genes in that transformed cell line.

† DNMT1 protein levels were compared with vehicle-treated passage control and normalized to PCNA.

‡ Denotes the number of genes methylated at each time point of a total of 30 genes tested and includes genes methylated in HBEC cell lines before carcinogen treatment.

§ Denotes the mean ± SE colonies per well that grew in soft agar.

|| Deletion of the *p16/p14* exon 2 was first seen in the HBEC1 cells after 6 wk of carcinogen treatment with either MNU or the combination of MNU and BPDE.

expression were seen (data not shown). However, levels of DNMT1 protein were increased 5- to 14-fold in transformed cells with the highest protein levels seen in HBEC2 transformed with BPDE (Fig. 2B; Table 1). In the HBEC1 MNU/BPDE-, HBEC2 MNU-, and BPDE-transformed cells, DNMT1 protein levels increased during carcinogen exposure (Fig. 2C; Table 1) compared with passage control cells (cells treated with vehicle and passed along with carcinogen-treated cells). The increase in protein was significantly different from passage control cells when normalized to the housekeeping gene, *β-actin*, or to the cell cycle-regulated gene, *PCNA* (Fig. 2C). In the HBEC2 MNU/BPDE and HBEC1 MNU-transformed cell lines, the increase in protein levels occurred early in the treatment period but did not change further until 12 weeks of treatment or until selection of the transformed cells, respectively (Table 1). The slight reduction in DNMT1 protein levels during the initial 6 weeks of some of the carcinogen treatment protocols was most likely due to an observed reduction in the rate of cell growth that was then restored by 9 weeks (Table 1).

There also seemed to be increased protein levels of DNMT3a and 3b in some transformed cell lines. The quantitation of this change is difficult due to the extremely low abundance of these proteins in passage control cells; however, after conducting three independent Western blots for each protein, levels of 3a and 3b ranged from no change to 6- to 7-fold greater in the transformed cell lines (Fig. 2B). No change in DNMT3a or 3b protein was detected in cell lines during carcinogen treatment (data not shown).

Increase in expression of *MAD2* during carcinogen exposure. Stabilization of DNMT1 protein mediated through overexpression of *MAD2* that disrupts normal degradation processes

via the NH₂-terminal destruction domain within the DNMT1 protein has been reported in breast cancer cells (31). Consistent with this finding, a 3- to 5-fold increase in *MAD2* mRNA levels was observed in all transformed cell lines (Fig. 2D; data not shown). A progressive increase in expression of this gene was also seen during carcinogen exposure that paralleled or occurred after first detecting increased DNMT1 protein. For example, in HBEC1 exposed to MNU and BPDE, expression of *MAD2* was increased ~30% at weeks 6 and 9, 200% at week 12, and 500% in transformed cells with *β-actin* as the reference (Fig. 2D).

Gene methylation in transformed HBECs. A panel of 30 genes methylated in primary lung tumors at prevalences of 15% to 80% and involved in all major aspects of cell regulation (e.g., apoptosis and cell adhesion) were assessed for methylation in the transformed HBEC1 and HBEC2 cell lines (Supplementary Table S1). Initially, methylation of this gene panel was examined in the parental and passage controls. The *GATA4* and *decoy receptor 1 (DCR1)* genes were methylated in both cell lines, whereas *RASSF2A* and *progesterone receptor (PGR)* were methylated in the parental and passage control HBEC2 (data not shown). All other genes were unmethylated in control cells. Methylation of 5 to 10 additional genes was seen in each carcinogen-induced transformant (Table 2). Methylation of four of eight cell adhesion genes studied (*DALI*, *E-cadherin*, *H-cadherin*, and *protocadherin-10*) was seen, with at least two of these genes methylated in each transformant (Table 2). Other genes methylated in four of five transformants were the transcription factor *PAX5 α* and the *X transporter protein (XT3)* gene, whereas methylation of *Beta 3*, a novel helix-loop-helix protein (32), was seen in the MNU-, BPDE-, and MNU/BPDE-transformed

HBEC2 lines. *Reprimo*, a mediator of *p53* cell cycle arrest at the G₂ phase of the cell cycle (33) and the transcription factor *FOXA2* (34), were methylated in two of five transformed cell lines.

The timing of gene methylation was assessed during carcinogen exposure. Methylation of *protocadherin-10* and *Beta 3* was detected after 6 weeks of carcinogen exposure, whereas methylation of *PAX5*, *XT3*, and *H-cadherin* was seen after 9 weeks of treatment. Most of the genes methylated in the HBEC2-transformed cell lines were detected in the cells treated for 12 weeks before selection of colonies in soft agar. In contrast, only 1 (*E-cadherin*) of 8 additional genes methylated in transformed clones from the HBEC1 line treated with MNU was detected after 12 weeks of treatment (Tables 1 and 2). This is likely due to the very low transformation efficiency

seen in this cell line, and thus, the number of cells harboring methylated alleles of these genes was likely below the detection limit of the MSP assay at the 12-week time point. When comparing across cell lines and carcinogen treatment, a strong correlation ($r = 0.94$) was seen at 12 weeks between the number of genes methylated and the number of colonies in soft agar. Temporally, the increase in DNMT1 protein generally also paralleled detection of gene methylation during carcinogen treatment, followed subsequently by detection of transformed colonies (Table 1).

Field cancerization in smokers is associated with epigenetic and genetic changes throughout the respiratory epithelium that in some persons culminate with the expansion of a clone of cells harboring multiple alterations that ultimately develop into a

Figure 2. Carcinogen-induced transformation is not associated with increased expression of *DNMTs* but with elevated DNMT1 protein that is associated with expression of *MAD2*. **A**, expression of *DNMT1*, *3a*, and *3b* is not changed in HBEC1- and 2-transformed cell lines. β -Actin and PCNA served as controls for RNA integrity and change in rate of cell replication, respectively. **B**, DNMT1, 3a, and 3b protein levels are increased in transformed HBEC1 and 2 cell lines compared with sham with normalization to β -actin and PCNA. Fold change of DNMT1, 3a, and 3b protein levels normalized to PCNA is also shown. Columns, mean from three experiments; bars, SE. **C**, DNMT1 protein levels increase progressively in HBEC1 exposed to MNU/BPDE and fold change of DNMT1 protein levels normalized to β -actin or PCNA is shown. **D**, *MAD2* mRNA levels increase progressively in HBEC1 exposed to MNU/BPDE with fold change of *MAD2* mRNA levels normalized to β -actin or PCNA depicted. BC, HBEC; S, sham; M, MNU; B, BPDE; T, transformant; W, week.

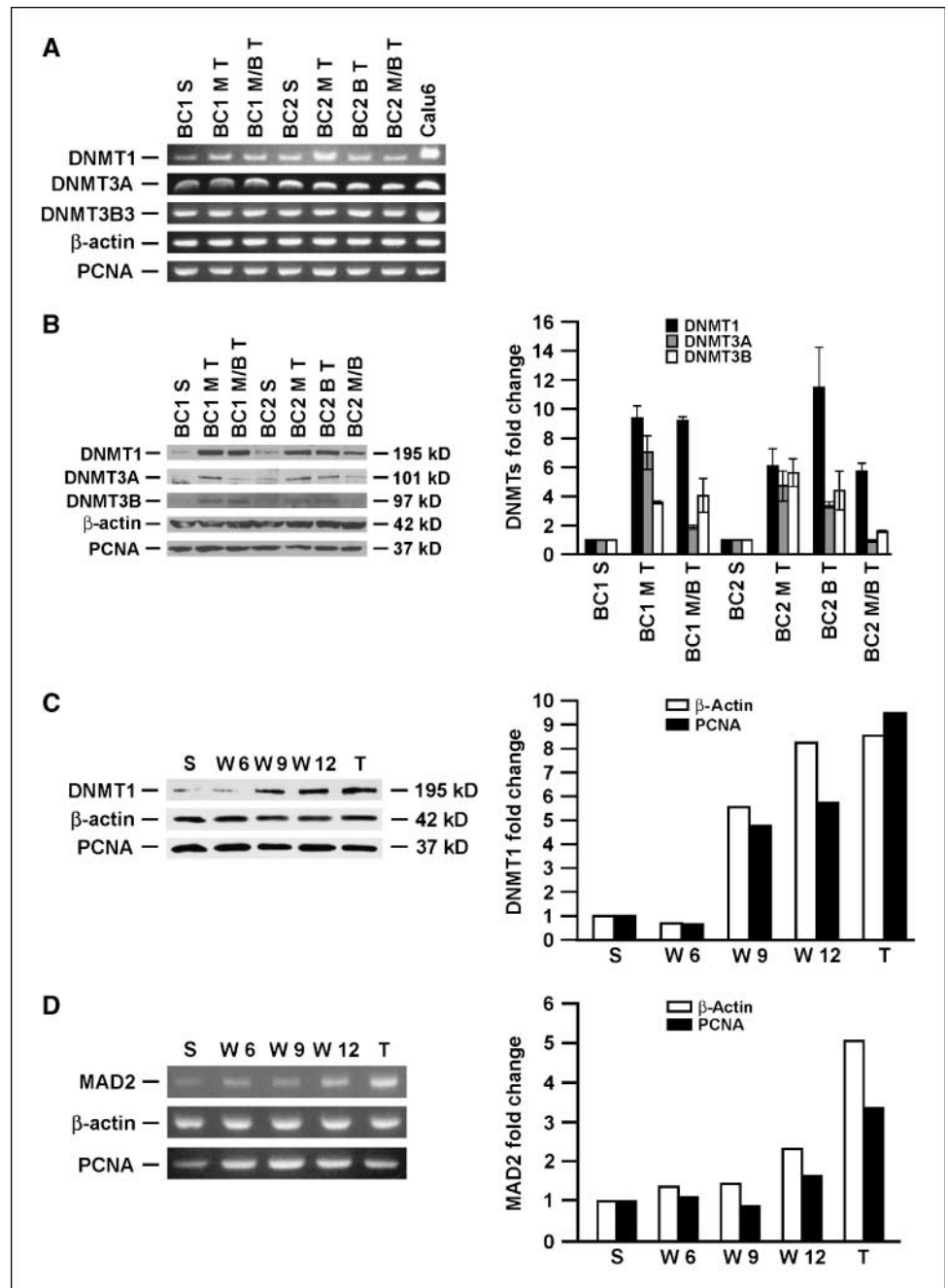


Table 2. Genes methylated in transformed cells after carcinogen treatment

HBEC1	HBEC2	HBEC2	HBEC1	HBEC2
MNU		BPDE	MNU/BPDE	
<i>HCAD</i>	<i>HCAD</i>	<i>HCAD</i>	<i>HCAD</i>	
<i>ECAD</i>	<i>ECAD</i>	<i>ECAD</i>		<i>ECAD</i>
<i>PAX5 α</i>	<i>PAX5 α</i>	<i>PAX5 α</i>		<i>PAX5 α</i>
<i>XT3</i>	<i>XT3</i>			<i>XT3</i>
<i>PCDH10</i>		<i>PCDH10</i>	<i>PCDH10</i>	
<i>PAX5 β</i>		<i>PAX5 β</i>		
<i>RASSF2A</i>			<i>RASSF2A</i>	
<i>IGFBP3</i>				
	<i>Reprimo</i>	<i>Reprimo</i>		
	<i>BETA3</i>	<i>BETA3</i>		<i>BETA3</i>
	<i>DAL1</i>	<i>DAL1</i>		<i>DAL1</i>
		<i>FOXA2</i>	<i>FOXA2</i>	
			<i>DCR2</i>	

NOTE: Genes not listed were unmethylated in all transformed cell lines. Those included *p16*, *MGMT*, *GATA5*, *AK5*, *DAB2*, *APC*, *SFRP1*, *3-OST-2*, *TSLC1*, *LAMC2*, *TUBB4*, *AP2α*, *DAPK*, and *RASSF1A*.

neoplasm (2). If our model recapitulates field cancerization, one would expect to detect methylation of some genes during carcinogen treatment that are not found in the transformed colonies. Assessment of the genes not methylated in the transformants in the primary cells exposed for 12 weeks found evidence for this situation. For example, in the HBEC2 line treated with MNU and BPDE, methylation of *PAX5 β* and *insulin-like growth factor binding protein-3 (IGFBP3)* was seen after 12 weeks of treatment but not in the transformed clones. Other genes that were methylated after 12 weeks but not selected for through the soft agar assay included *GATA5* and *tumor suppressor lost in cancer-1 (TSLC1)*.

Decreased mRNA expression and establishment of heterochromatin is associated with promoter hypermethylation of *H-cadherin* and *E-cadherin*. Gene promoter methylation is

associated with a change from an open to closed chromatin state around the promoter region that is mediated in part, through modification of core histone proteins such as H3 (9). Together with methylation of cytosine, these changes culminate in loss of gene expression. We focused our studies of gene expression and changes in chromatin during carcinogen treatment on the *H-cadherin* and *E-cadherin* genes because they were commonly methylated in the transformed HBEC lines. Methylation of *E-cadherin* was seen after 9 and 12 weeks of BPDE treatment of HBEC2 and in the transformed clones, whereas methylation of *H-cadherin* was first detected after 12 weeks of carcinogen treatment (Fig. 3A). Loss of expression of both genes was first seen in the transformed clones.

Histone marks associated with active open and inactive closed chromatin and the heterochromatic adaptor molecule *HP1α* were assessed at the promoter regions of the *E-cadherin* and *H-cadherin* genes in the HBEC2 BPDE-transformed cells. In the passage control cells, methylation of lysine 4 histone 3, which is associated with an active open state of chromatin (active transcription), was detected at both gene promoters (Fig. 3B). In contrast, in the transformed cells, H3K9me2 and H3K27me3 histone marks associated with heterochromatin and loss of gene transcription were detected at the *E-cadherin* and *H-cadherin* promoters. H3K9me3 and HP1α, indicative of chromatin compaction (12, 35), were also enriched at the *E-cadherin* and *H-cadherin* promoters (Fig. 3B).

Stable knockdown of *DNMT1* reverses and prevents transformation and gene silencing. The increase in DNMT1 protein during carcinogen treatment suggested that this cytosine-DNA methyltransferase could be responsible for the observed gene-specific promoter methylation and transformation. This hypothesis was tested by stable integration of a shRNA to *DNMT1* into the HBEC1 line transformed with MNU and BPDE that resulted in an ~70% reduction in protein levels (Fig. 4A). We were unable to select for stable knockdown of this gene in the HBEC2 transformed cell lines because of their resistance to hygromycin. Colony formation in soft agar was reduced 99% in the HBEC1 cells with knockdown of *DNMT1* compared with the scrambled control or the lung tumor-derived cell line, Calu6 (Fig. 4A). The loss of colony formation was associated with loss of methylation and increased expression of the *H-cadherin*, *protocadherin-10*, and *RASSF2A* genes (Fig. 4B). In contrast, reduction of DNMT1 protein did not affect

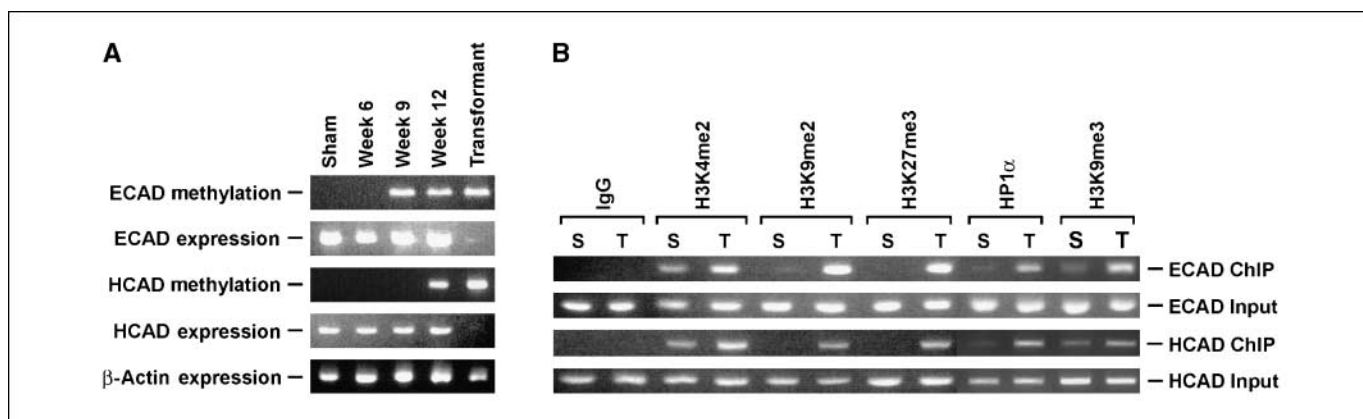
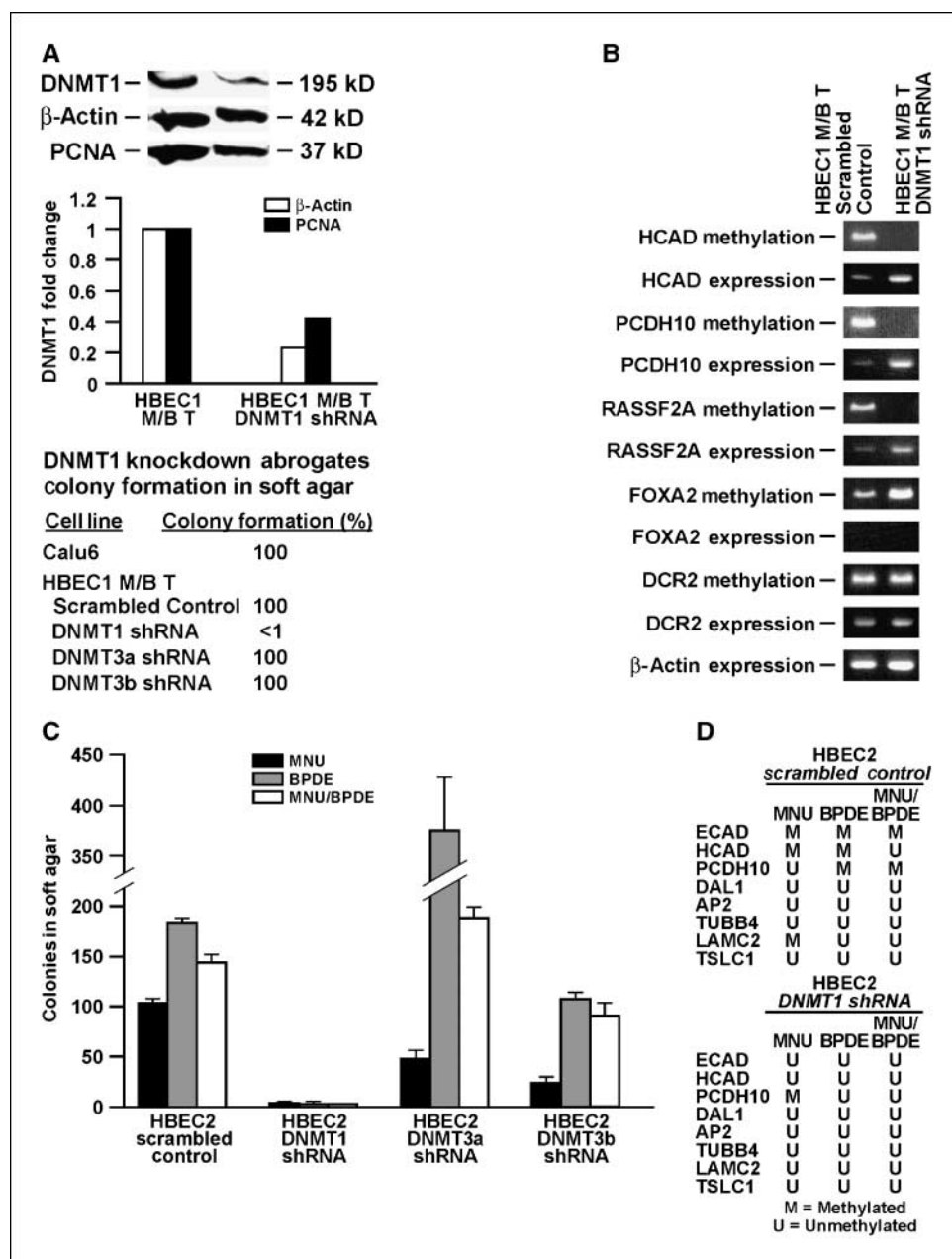


Figure 3. Timing for methylation and loss of gene expression of the *E-cadherin* (*ECAD*) and *H-cadherin* (*HCAD*) genes in HBEC2 exposed to BPDE and the establishment of heterochromatin around the gene promoters. **A**, promoter methylation and expression of *ECAD* and *HCAD* during carcinogen treatment. **B**, detection of heterochromatin marks in the *ECAD* and *HCAD* promoters in the transformed (*T*) cells compared with sham.

Figure 4. Stable knockdown of *DNMT1* reverses and prevents transformation and methylation, and restores gene expression. **A**, knockdown of *DNMT1* using shRNA in HBEC1 transformed by MNU and BPDE (HBEC1 M/B T) is associated with marked reduction in colony formation compared with scrambled control, *DNMT3a* and *3b* knockdown, and Calu6 cells. **B**, effect of *DNMT1* shRNA knockdown on methylation and expression of the *HCAD*, *PCDH10*, *RASSF2A*, *FOXA2*, and *DCR2* genes. Gene methylation and expression are compared between HBEC1 M/B transformed and HBEC1 M/B transformed with *DNMT1* knocked down. **C**, ShRNA knockdown of *DNMT1* in HBEC2 blocks carcinogen-induced transformation by MNU, BPDE, or their combination. **D**, ShRNA knockdown of *DNMT1* in HBEC2 blocks carcinogen-induced methylation of cell adhesion genes. Methylation status of 8 cell adhesion genes was determined 12 wk after treatment of scrambled control or *DNMT1* shRNA knockdown cells with MNU, BPDE, or their combination. M, detection of methylated alleles; U, detection of only unmethylated alleles for a gene.



the methylation state or expression of the *FOXA2* and *DCR2* genes. Reducing the expression of the *DNMT3a* and *3b* genes by 65% to 75% through integration of stable shRNA against these transcripts did not affect colony formation or cause demethylation of the genes silenced in these transformed cells (Fig. 4A; data not shown).

These studies were extended to assess the effect of stable knockdown of *DNMT1*, *3a*, or *3b* on carcinogen-induced transformation and promoter methylation. The HBEC2 cell line was used because of its higher efficiency for transformation than the HBEC1. Stable shRNA against *DNMT1*, *3a*, and *3b* were transfected into this cell line and clones were identified with 70% to 75% reduction in expression of these genes (data not shown). Knockdown of these *DNMTs* did not significantly affect growth rate of the HBEC2 lines, a finding previously observed for normal bronchial epithelial cells (36). These cell lines along with a scrambled control were then treated for 12 weeks with vehicle, BPDE, MNU, or the combination.

The soft agar assay revealed transformation efficiencies for the scrambled control that mirror that seen in our original study (Figs. 1 and 4C). In contrast, virtually no colonies were seen in HBEC2 *DNMT1* knockdown cells treated with either carcinogen or the combination. Transformation was not blocked by stable knockdown of *DNMT3a* or *3b* (Fig. 4C). No colonies were seen in vehicle-treated cell lines. We evaluated the methylation status of the 8 genes involved in cell adhesion in scrambled and *DNMT1* knockdown cells treated for 12 weeks. Methylation of *E-cadherin*, *H-cadherin*, and *protocadherin-10* was seen in the scrambled cells treated with the MNU, BPDE, or the combination with the pattern being nearly identical to that seen in our original studies of methylation for these genes in the transformed clones (Fig. 4D; Table 2) and in the *DNMT3a* and *3b* knockdown cells (data not shown). In contrast, methylation of the *E-cadherin* and *H-cadherin* genes was not seen in the *DNMT1* knockdown carcinogen-treated

cells, whereas methylation of *protocadherin-10* was only detected in the MNU-treated cells (Fig. 4D). All other genes were unmethylated in the scrambled and *DNMT1* knockdown cells (exception being methylation of *LAMC2* in scrambled MNU-treated cells).

Discussion

The *in vitro* model developed in these studies mimics the clonal outgrowth of preneoplastic cells that occurs in the chronic smoker and has provided new insight into factors involved in the earliest stages of cell transformation. Our studies show an association between DRC and transformation and identify genes in the cell adhesion pathway as one common target for gene silencing through promoter methylation that culminate in complete loss of transcription and the establishment of heterochromatin. Finally, these studies provide a strong mechanistic link between increased DNMT1 protein, transformation, and the induction of aberrant promoter hypermethylation.

The transformation efficiency of 0.2% to 3% seen in the HBECs after 12 weeks of exposure individually or in combination to the direct acting carcinogens MNU and BPDE likely reflects the situation of field cancerization seen in smokers. In that setting, exposure of the entire respiratory tract to inhaled carcinogens within smoke damages the epithelium and induces heritable genetic and epigenetic changes in some cells. The accumulation of gene alterations in these premalignant clones ultimately leads to the outgrowth of a clone(s) that become the malignant tumor. Our recent *in vivo* studies in which some methylated genes were present in sputum but not in the matched primary tumor from lung cancer patients clearly show the extent of the field defect (37). The soft agar assay selected for cells that had acquired heritable changes, most notably the silencing of genes by promoter hypermethylation, which facilitated growth in the absence of a basement membrane, one of the earliest hallmarks of malignant development. As expected, loss of expression of genes methylated during carcinogen treatment was not seen until selection of the transformed cell population. When transformation efficiency was very low (0.2% in HBEC1 treated with MNU), methylation of most genes was not detected in cells before selection through soft agar. In contrast, in the HBEC2 where transformation efficiency was higher, methylation of some genes was detected in cells after 6 weeks of treatment. Furthermore, cells containing some genes methylated during carcinogen treatment apparently had not acquired sufficient alterations to support growth in soft agar because methylation of these genes (e.g., *GATA5* and *TSLC1*) was not seen in the transformed cells.

The failure of the transformed cells to grow orthotopically on nude mice indicates that additional alterations are required to facilitate tumor formation. Some malignant human tumor-derived cell lines do not readily engraft on subcutaneous skin cells of the nude mouse but require Matrigel as a basement membrane. Expression of a mutant *K-ras* or *EGFR* gene or knocking down the *p53* gene in the HBEC lines also induced growth in soft agar, but the transformed cells did not form tumors in nude mice (38). The ability to confer a complete "malignant phenotype" likely requires the acquisition of additional epigenetic and genetic changes. Mutation of the *K-ras* and *p53* genes, generally late events in tumor development (4, 5), were not observed in our carcinogen-induced transformation model; however, loss of *p14*-mediated regulation of *mdm2* that can disrupt the normal regulation of the *p53* gene was seen in HBEC1 (39). BEAS2B cells exposed continuously to either

cigarette smoke condensate or NNK for 6 months did form tumors 6 months after inoculation into nude mice (40). Finally, exposure of either BEAS2B or primary bronchial epithelial cells to toxic doses of cigarette smoke condensate resulted in the selection of a surviving cell population that formed tumors in nude mice (41). Although these studies achieved the goal of generating a malignant phenotype, it remains to be determined whether the pathways deregulated under this exposure scenario are causal for initiation and development of lung cancer in the smoker.

A significant association was observed between DRC and transformation efficiency. Interestingly, this association was related to the cancer status of the subjects that provided the bronchial epithelial cells. Greater DRC was seen in HBEC1 derived from a cancer-free smoker compared with HBEC2 derived from a lung cancer patient. Although this association is only from two subjects, lung cancer case-control studies show an association between reduced DRC and risk for cancer (42). For example, lung cancer patients were five times more likely than controls to have reduced nucleotide excision repair capacity (42). Chromosome instability, a hallmark of neoplasia, was also present in the transformants, as indicated by the marked increase in formation of micronuclei in response to carcinogen exposure. Together, these findings corroborate previous studies suggesting that chromosome instability stemming from DNA replication stress in response to DNA damage occurs during the earliest stages of cancer development (43).

The fact that MNU and BPDE both induce single-strand DNA breaks likely accounts for the similarity in genes studied that were silenced by methylation in the HBECs transformed by these exposures (42). The genes silenced have biological plausibility for a prominent role in cell transformation. Specifically, the *cadherins* are a family of calcium-dependent proteins that participate in the maintenance of tight cell-to-cell adhesion. Methylation of *E-cadherin* and *H-cadherin* was detected in four of five transformed cell lines, whereas methylation of *protocadherin-10* was seen in three of five transformed cell lines. Methylation of these genes was seen in a second experiment that compared methylation in the scrambled control to the *DNMT1* knockdown (Fig. 4D), indicating that silencing of the cadherin genes is not a random event. These genes are methylated at prevalences of 34% to 62% in lung tumors (2). Methylation of the *E-cadherin* gene was associated with invasion of cultured breast cancer cells (44). *DALI1*, an actin-binding protein methylated in 57% of primary lung tumors, was also commonly methylated in the transformed HBEC lines (45). Thus, loss of function of these genes is likely a major factor contributing to transformation. This hypothesis is supported by the fact that reversal of transformation by knockdown of *DNMT1* was associated with loss of methylation and re-expression of *E-cadherin* and *protocadherin-10*, and the lack of methylation of these genes in HBEC2 *DNMT1* knockdown cells treated with carcinogen. Studies to carefully assess the timing for silencing of these genes during lung cancer development have not been conducted; however, methylation of *E-cadherin* has been detected in bronchial epithelial cells from smokers (46).

Other genes methylated in the transformed cells from our panel are likely contributing to the preneoplastic phenotype. *PAX5* β encodes for the transcription factor B cell-specific activating protein that, in turn, directly regulates *CD19*, a gene shown to negatively control cell growth (47). Methylation of *PAX5* β was associated with complete abrogation of *CD19* expression in lung cancer cell lines (47). *PAX5* α and *FOXA2* also code for transcription

factor binding proteins, whereas *Reprimo* mediates *p53* cell cycle arrest at the G_2 phase of the cell cycle (33, 34).

The increase in DNMT1 protein seems to be a key factor in *de novo* methylation and silencing of some genes that likely contribute to transformation. Increased DNMT1 protein levels were seen after 6 to 9 weeks of all exposures that resulted in transformation and coincided with detection of gene methylation in the exposed cells. In contrast, increased DNMT1 protein was not seen in the nontransformed HBEC1 treated with BPDE, nor was methylation of any *cadherin* gene observed (data not shown). The increase in DNMT1 protein could be due to stabilization. One mechanism of degradation of the DNMT1 protein is through the anaphase-promoting complex (APC), a multicomponent ubiquitin ligase complex consisting of 12 core proteins along with substrate recognition adaptors CDC20 and FZR1 that can bind to the NH₂-terminal 118-amino acid domain of DNMT1 to facilitate protein degradation. Previous studies showed that overexpression of *MAD2*, an inhibitor of *CDC20*, stabilized DNMT1 protein levels (31). Moreover, a correlation was observed between *MAD2* and DNMT1 protein levels in breast tumors. Similarly, *MAD2* expression was increased in all transformed cell lines, and the increased transcription of this gene during carcinogen treatment largely paralleled that seen for DNMT1 protein. Furthermore, we have observed an association between overexpression of *MAD2* and increased DNMT1 protein in lung tumor-derived cell lines.³ Thus, *MAD2* may be one factor contributing to the increase in DNMT1 protein. Other factors could include changes in APC core proteins in response to carcinogen. Although changes in levels of DNMT3a and 3b protein may contribute to transformation in our model, they do not seem to be major driving factors because knockdown of these genes did not affect gene methylation status, growth in soft agar, or prevent carcinogen-induced transformation.

The link between *DNMT1* and *de novo* methylation during carcinogen exposure is likely due to the important role of *DNMT1* in DNA repair. Whereas most studies have focused on double-

strand break damage, a similar scenario is likely occurring in response to single-strand breaks. *DNMT1*, but not *3a* or *3b*, is rapidly recruited to sites of DNA damage where it functions to restore epigenetic information (14). Le Gac and colleagues (13) found that in cells treated with doxorubicin that induces double-strand breaks, DNMT1 is recruited by activated *p53* and binds within the promoters of the *survivin*, *cdc2*, and *cdc25* genes. The transcriptional repressor HDAC1 and the repressive mark H3K9me2 were found at these promoters after DNA damage (13). *In vitro* studies using a *survivin* reporter construct showed that DNMT1 complexed with *p53* could lead to *de novo* methylation of this reporter (48). In addition, the introduction of a double-strand break in a recombinant gene in the genome of HeLa or mouse embryonic cells led to silencing that was associated with homology-directed repair and DNA methylation mediated by DNMT1 (49). Our *in vitro* model establishes for the first time a link between increased DNMT1 protein, *de novo* methylation of tumor suppressor genes, and reduced DRC that together seem causal for transformation of lung epithelial cells. This finding strongly supports the development of demethylation strategies for primary cancer prevention in smokers.

Disclosure of Potential Conflicts of Interest

S. Belinsky is a consultant to Oncomethylome Sciences. Under a licensing agreement between Lovelace Respiratory Research Institute and Oncomethylome Sciences, nested MSP was licensed to Oncomethylome Sciences and the author is entitled to a share of the royalties received by the Institute from sales of the licensed technology. The Institute, in accordance with its conflict-of-interest policies, is managing the terms of these arrangements. The other authors disclosed no potential conflicts of interest.

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³ Unpublished.

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Carcinogen-Induced Gene Promoter Hypermethylation Is Mediated by DNMT1 and Causal for Transformation of Immortalized Bronchial Epithelial Cells

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