DNA Methyltransferase Inhibition Reverses Epigenetically Embedded Phenotypes in Lung Cancer Preferentially Affecting Polycomb Target Genes

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Abstract

Purpose: Cancer cell phenotypes are partially determined by epigenetic specifications, such as DNA methylation. Metastasis development is a late event in cancerogenesis and might be associated with epigenetic alterations.

Experimental Design: An in vivo selection approach was used to generate highly aggressive non–small cell lung cancer (NSCLC) cell lines (A549 and HTB56) followed by genome-wide DNA methylation analysis. Furthermore, the therapeutic effects of the epigenetic agent azacytidine on DNA methylation patterns and the in vivo phenotypes were explored.

Results: Widespread changes of DNA methylation were observed during development of highly aggressive cell lines. Up to 2.5% of the CpG-rich region was differentially methylated as identified by reduced representation bisulfite sequencing compared with the less aggressive parental cell lines. DNA methyltransferase inhibition by azacytidine reversed the prometastatic phenotype; this was highly associated with the preferential loss of DNA methylation at sites that were hypermethylated during the in vivo selection. Of note, polycomb (PRC2) binding sites were particularly affected by DNA methylation changes after azacytidine exposure that persisted over time.

Conclusions: We could show that metastatic capability of NSCLC is closely associated with DNA methylome alterations. Because inhibition of DNA methyltransferase reversed metastasis-prone phenotype, epigenetic modulation seems to be a potential therapeutic approach to prevent metastasis formation.

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Introduction

Epigenetic changes are a hallmark of cancer (1). Altered DNA methylation and histone modifications may also impact on cancer phenotype and disease aggressiveness (2, 3). Epigenetic changes and especially DNA methylation can be drug targeted, holding promise for modulating cancer cell behavior in clinical practice (4, 5). Recent reports have shown that DNA methyltransferase (DNMT) inhibitory drugs such as azacytidine and decitabine can alter cellular cancer phenotypes over prolonged times even after the end of drug exposure (6). These data are consistent with clinical findings in hematologic malignancies (5). Azacytidine and decitabine can induce DNA hypomethylation at specific gene loci, which can lead to sustained gene reactivation (7). However, genome-wide analysis of DNA methylation after drug-induced DNMT inhibition is lacking, and the actual mechanisms of action of these epigenetic modulators remain poorly defined (8).

Metastases are the most frequent cause of cancer-related death after complete tumor resection. Gene expression profiles of metastatic cancer cells are similar to gene expression profiles of cancer stem cells (9, 10). These expression profiles are not likely to result from a large number of additional genetic mutations, but might rather indicate epigenetic alterations that predispose for metastasis (11).
**Translational Relevance**

Cancer genomes are frequently mutated at genomic sequence level and often show chromosomal aberrations. On top of this, epigenetic changes are instrumental in determining cellular phenotypes. Here, we demonstrate that development of prometastatic phenotypes in non–small cell lung cancer is associated with genome-wide changes in DNA methylation. These epigenetic changes as well as the phenotypes were reversible by azacytidine. DNA hypomethylation was preferentially induced at polycomb binding sites. These data suggest that metastatic capability is associated with DNA methylation changes. Possibly, DNA methyltransferase inhibitors are able to reverse metastasis-prone phenotypes as a novel therapy approach to prevent metastasis in an adjuvant therapy situation.

In line with this, metastasis formation in most cases occurs in a rather short time span compared with primary tumor initiation and growth until diagnosis that takes many years. Consequently, a metastasis-associated epigenetic state could potentially be targeted by DNMT inhibitory drugs. In this study, we analyzed the profile of DNA methylation changes occurring during the transition from a cellular state with low metastatic capacity toward a highly aggressive state and whether these changes were reversible by DNMT inhibition.

**Materials and Methods**

**Cell culture and 5-azacytidine treatment**

A549 and HTB56 lung adenocarcinoma cells were cultured and maintained as described (12, 13). Cell line identity was confirmed by STR marker analysis. A549 and HTB56 cells were exposed to 5-azacytidine (Aza; Sigma) at a concentration of 100 nmol/L to 1 μmol/L. The cells were grown under these conditions for 6 days, and every 48 hours, cells were supplemented with fresh medium. After 6 days of exposure with 5-azacytidine, cells were washed 3 times with PBS and released for additional 7 days in regular medium.

**Functional in vitro assays**

Proliferation assays with [3H]-thymidine incorporation (14), migration assays (12, 14), cell viability assays (15), and human tumor cloning assays (16) were performed as described.

**Analysis of genome-wide DNA methylation levels by reduced representation bisulfite sequencing**

A total 0.3 to 1 μg of DNA was used for reduced representation bisulfite sequencing (RRBS) library preparation using published protocols with minor modifications (17, 18; see also Supplementary Methods). Sequencing data can be downloaded from NCBI Gene Expression Omnibus (GEO) platform (GSE44390).

**Illumina methylation bead arrays**

We used the Infinium Human Methylation 27 BeadChip according to the manufacturer’s instructions (see also Supplementary Methods). Data can be downloaded from GEO platform (GSE44390).

**Gene expression analysis**

We used the human Gene 1.0 ST Array (Affymetrix) using parental and highly metastatic A549 cell lines according to the manufacturer’s instructions (see also Supplementary Methods). Data can be downloaded from GEO platform (GSE44390).

Detailed methodologic information about single-nucleotide polymorphism (SNP) analysis, exome capture, and high-throughput exome-seq. Processing-read mapping, variant calling and effect determination, as well as global DNA methylation analysis and analysis of double-strand break by H2AX staining were described in the Supplementary Methods.

**Mouse in vivo experiments**

Animal experiments were carried out in strict accordance with the relevant regulations. Eight- to 10-week-old NOD.CB17-Prkdc-scid/l [nonobese diabetic/severe combined immunodeficient (NOD/SCID)] mice were used. To establish a more aggressive cell line by in vivo selection, 2 NOD/SCID mice per cell line were irradiated with a single dose of 3.5 Gy from a cobalt-60 unit 1 day before injection. A total number of 2 × 10⁶ parental cells (A549_R0 or HTB56_R0) were injected intravenously into the tail vein of 2 different mice. Mice were sacrificed after 8 weeks for the first round of selection, after 6 weeks for the second round of selection, and after 4 weeks for the third round (R3) of selection. Nodules of both lungs were obtained and pooled after 30 minutes of digest with an adequate amount of Trypsin (approximately 5 × 10⁴ cells) in cell culture in the indicated media. A maximum of 7 doubling times was needed to reach the cell count for the next round of selection in mice. To analyze lung nodule formation after intravenous tumor cell injection, NOD/SCID mice were irradiated with a single dose of 3.5 Gy from a cobalt-60 unit 1 day before injection. A total of 2 × 10⁶ (A549) or 1 × 10⁶ (HTB56) cells either highly or low metastatic and either exposed or nonexposed were injected intravenously into the tail vein (12, 14). Mice were followed up for 4 weeks. At this time, mice were sacrificed, and lung nodules were counted. In all experiments, treatment groups were randomized to prevent cage effects.

**Statistical analysis**

All data are shown as mean ± SD if not indicated otherwise. Statistical analyses were done with SPSS, version 18 (IBM). Statistical significances of overall differences between multiple groups were analyzed by Kruskal–Wallis test. Differences between 2 groups were analyzed by Student t test. A P-value of <0.05 was considered significant.
Results

In vivo selection of a highly aggressive phenotype

Epigenetic changes in cancer might be associated with specific phenotypes and properties that can be altered by epigenetic therapy. We generated non–small cell lung cancer (NSCLC) cell lines with increased propensity to form tumor nodules in murine lungs after intravenous injections (Fig. 1A). Extravasation and growth at a distant site are important parts of the metastatic process, and we regarded these parts as surrogate markers for in vivo aggressiveness and potential metastatic capability. Two cell lines were used for this purpose: A549 lung adenocarcinoma cells formed multiple small nodules in NOD/SCID mice after intravenous injection; and HTB56 cells, an anaplastic carcinoma cell line, formed fewer but larger nodules in vivo. Removal of tumor nodules from the lungs and subsequent reinjection led to a rapid increase in metastatic capacity (Fig. 1A). A highly aggressive phenotype that was stable over time was evident after 3 rounds of in vivo selection for both cell lines (Fig. 1B, \( P < 0.001 \) for each cell line). Highly aggressive A549 and HTB56 cells showed enhanced clonogenic growth in vitro and...
additional in vitro features associated with metastatic potential such as accelerated proliferation. On the genomic level, only few additional changes were observed using high-resolution SNP array analysis in the highly metastatic A549 cells, as well as HTB56 cells compared with the parental cells (Supplementary Fig. S1A and Table S1). One notable exception is the potential amplification of the MET protooncogene on 7q31, which was detected by SNP array only in high aggressive compared with parental cells of HTB56 (Supplementary Table S1B). Whole exome sequencing verified a high number of potential driver mutations in the parental cells (A549_R0: n = 244; HTB56_R0: n = 178). Of note, only 4 (A549_R3) or 7 (HTB56_R3) additional mutations were observed in the highly aggressive cell lines (Supplementary Table S2). Thus, despite the strong differences about metastatic capacity in the in vivo phenotypes, only 1.6% (A549) or 3.9% (HTB56) of the mutations were newly acquired during in vivo selection process.

Genome wide changes in DNA methylation in the highly metastatic state

Functionally relevant DNA methylation changes in human cancer occur preferentially at CpG islands and the associated shores (19). We used a modified RRBS (17) protocol to focus on CpG-rich regions and, thereby, identify alterations in DNA methylation in the 2 NSCLC cell lines with increased aggressiveness in vivo. On average, $1.8 \times 10^7$ reads were uniquely mapped to the human genome in each cell line (Supplementary Table S3). Based on the spatial density of covered CpG sites (see Materials and Methods), we defined 19,097 CpG clusters that spanned 7,502,992 bp of the human genome. The length of these clusters ranged from 34 to 2,025 bp (median: 334 bp). The CpGs in CpG clusters were usually free from DNA methylation (Fig. 1C). The median methylation level was 0.74% for CpGs in the parental A549_R0 cells and 1.03% for CpGs in the metastatic A549_R3 cells. Similar numbers were obtained in HTB56 cells with a small increase toward higher CpG
methylation in metastatic cells (1.07%) compared with the parental cells (0.85%). A total of 1,197 regions (0.8% of the tested genomic region) were found to be differentially methylated in highly metastatic A549_R3 cells and 2,405 regions (2.5% of the tested genomic region) in HTB56_R3 cells. In highly metastatic A549_R3 (37.8%) and in HTB56_R3 (20.8%) cells, DNA hypermethylated differentially methylated regions (DMR) were less frequent than DNA hypomethylated ones. A total of 170 (14.2%) of A549_R3 DMRs overlapped and were similarly altered in metastatic HTB56_R3 cells. Concordant DNA hypermethylation was observed in 38 regions, whereas hypomethylation in both cell lines was observed for 132 regions. A few regions (n = 43) were discordantly affected in the 2 cell lines (Fig. 2A, left). In both cell lines, the changes occurred throughout the genome. Most of the altered regions were promoters and gene bodies/exons followed by first introns. DNA hypermethylation and DNA hypomethylation were observed. The fraction of DNA hypermethylated regions as a percentage of all tested regions in this regard ranged from 29% (promoters) to 38% (gene bodies) in A549 cells. Equivalent results with a lower fraction of de novo methylated regions were observed in HTB56 cells (Fig. 2A, right).

We compared DNA methylation levels derived by RRBS with data generated using the Infinium 27 K methylation bead array technology. Overall, we observed highly concordant results (Supplementary Fig. S1B and S1C). Furthermore, we analyzed the association between published transcription factor binding sites (TFBS) determined by ChIP-Seq (20) and the DNA methylation changes in the cell lines (Fig. 2B). Binding sites for Suz12 as an indication for the PRC2 complex in embryonic stem cells showed an increased likelihood to be DNA hyper- and hypomethylated, respectively, in both cell lines. Moreover, this was observed in 2 different published Suz12 binding site profiles (20, 21). TFBS for cancer and proliferation-associated factors such as MYC, HDAC2, and CEBPB were significantly underrepresented in both hypo- and hypermethylated regions for the 2 cell lines. Most TFBS are generally protected from DNA methylation changes. The findings for TFBS association with DNA methylation changes were comparable between A549 and HTB56 cells. The reproducibility among different cell lines suggests a general phenomenon. In this light, the stability of DNA methylation might primarily depend on transcription factor binding such that regions without active transcription factor binding are more prone to undergo changes in DNA methylation.

Next, we sought to verify the DNA methylation findings and to specifically identify regions with a stepwise increase or decrease of DNA methylation during in vivo selection. For this purpose, independent samples at each step of selection were RRBS analyzed. (Supplementary Table S4). Notably, we observed a high correlation between biologic replicates from normal and highly aggressive cell lines from both cell types (Supplementary Fig. S3). The majority of changes in both directions were confirmed, and hundreds of regions (particularly those with DNA hypomethylation) followed the pattern of step-wise alterations in DNA methylation (Fig. 2C and Supplementary Table S4).

To evaluate the association between DNA methylation and gene expression, we performed microarray analyses in parental and highly aggressive A549 and HTB56 cells. DNA hypomethylation of promoters was frequently associated with increase in mRNA expression (Supplemental Table S4).

**DNMT inhibition by 5-azacytidine reversed the metastatic-prone phenotype in vivo**

Our findings suggested that altered DNA methylation patterns were associated with the selection of a more aggressive phenotype. About 30% of the observed changes in methylation consisted of DNA hypermethylation. DNA methylation can be targeted, for example, by 5-Azacytidine, which traps and inhibits DNMTs. Exposure to DNA hypomethylating drugs can reactivate silenced tumor suppressor genes. However, 5-azacytidine is toxic at high concentrations and it might be difficult to distinguish epigenetic from toxic effects. Consequently, epigenetic effects that target the phenotypic memory of tumor cells should persist longer than toxic effects. We set up experiments in which the highly metastatic cells were exposed to 5-azacytidine for 6 days. After 6 days, the drug was washed out and cells were released into normal media for 7 additional days (Fig. 3A). After this time, the A549 cell lines did not show increased apoptosis (Supplementary Fig. S2A, left). DNA double-strand breaks were present in all NSCLC cells regardless of previous 5-azacytidine exposure (Fig. 3A and Supplementary Fig. S2B). Even at the maximum dose of 5-azacytidine, DNA doublestrand breaks increased only by 20% (Supplementary Fig. S2B). Next, we analyzed the phenotypes. Proliferation was increased in the highly aggressive A549 cells, but was reversed to the proliferation levels of the parental cells after exposure to 5-azacytidine (Fig. 3B, left). In addition, in vitro transwell migration was enhanced in the highly aggressive A549 cells and was reversed after 5-azacytidine exposure (Fig. 3B, middle). Capillary electrophoresis confirmed persistent changes in global DNA methylation after 5-azacytidine exposure (Supplementary Fig. S2C).

Furthermore, we analyzed the in vivo growth at a distant site as a surrogate for metastatic capacity of tumor cells exposed to 5-azacytidine. Seven days after the end of 5-azacytidine exposure, A549 cells were injected into NOD/SCID mice. Mice inoculated with the aggressive A549_R3 cells without drug exposure developed multiple lung nodules, whereas absent metastases were observed in R3 cells previously exposed to 5-azacytidine (Fig. 3B, right). Similar experiments were performed with HTB56 anaplastic carcinoma cells. Seven days after release from a 6 days exposure of highly concentrated 5-azacytidine (1 μmol/L), increased apoptosis was still present (Supplementary Fig. S2A, right). As a consequence, release experiments (Fig. 3A) were performed at concentrations as low as 250 nmol/L. A 6 days exposure of 5-azacytidine with subsequent release prevented lung nodule formation at a dose as low as 250 nmol/L (Fig. 3C). Effects of 5-azacytidine depended on a minimal...
drug concentration because 100 nmol/L drug exposure only partially prevented lung nodule formation in A549 and HTB56 cells (Supplementary Fig. S2E).

RRBS was used to analyze the effects of DNMT inhibition by 5-azacytidine on the NSCLC methylome. After 6 days of exposure with 5-azacytidine, widespread loss of DNA methylation was observed at most of the methylated CpG loci (Fig. 4A, left). Almost 7,000 DMRs were identified in each 5-azacytidine exposed cell line with an absolute change in methylation of at least 30% (Supplementary Fig. S4A and S4B). In virtually all (97%) of the altered regions, DNA methylation was decreased (Fig. 4B, left and Supplementary Fig. S4C). In the A549 cells, the higher dose of 5-azacytidine (1 μmol/L instead of 250 nmol/L) did not further increase DNA hypomethylation indicating a saturation effect (Fig. 4B, middle and Supplementary Fig. S4C). More than 80% of...
Figure 4. 5-Azacytidine induced changes in DNA methylation. A, left: distribution of methylation levels in analyzed specimens. The stacked bar plots show the distribution of raw RRBS methylation levels of CpG sites covered in all samples. The raw methylation levels are encoded by colors ranging from green (low methylation close to 0) to red (high methylation close to 1). Right: the bar graph depicts the percentage of DMRs, which were found to be hypermethylated in more aggressive cells and which become hypomethylated upon 5-azacytidine treatment. More than 80% of DNA hypermethylated regions in A549 and HTB56 cells were effectively hypomethylated after a 6-day exposure to 5-azacytidine. B, left: smoothed scatterplot of DNA methylation levels for more aggressive A549 cells versus more aggressive A549 cells after 6 days of 5-azacytidine exposure at 250 nmol/L. Colors represent the density of points ranging from red (high density) to blue (low density). Middle: smoothed scatterplot of DNA methylation levels for more aggressive A549 cells treated with 5-azacytidine at 250 nmol/L versus 1,000 nmol/L doses. Colors represent the density of points ranging from red (high density) to blue (low density). Right: smoothed scatterplot of DNA methylation differences between d6 and d0 in A549 versus HTB56 cells. Colors represent the density of points ranging from red (high density) to blue (low density). (Continued on the following page.)
the genomic regions that were DNA hypermethylated in the more aggressive cells showed reductions in DNA methylation after treatment with 5-azacytidine (Fig. 4A, right). Unsupervised hierarchical clustering revealed that 5-azacytidine induced a specific pattern that was stronger than the cell-specific DNA methylation pattern. Accordingly, 5-azacytidine exposed cell lines showed higher similarity than the untreated cell lines (Supplementary Fig. S5). Different genomic regions, for example, promoter and gene body showed similar changes in loss of DNA methylation (Fig. 4C).

The widespread loss of DNA methylation associated with 5-azacytidine was not entirely uniform. In both cell lines and at both concentrations, we observed a skewing toward the chromosome ends that showed a higher level of demethylation (Fig. 4D). This pattern occurred at most of the chromosomes regardless of the drug concentration (Supplementary Fig. S6). Cell line–specific effects were observed. Multiple sites were hypomethylated only in one but not the other cell line (Fig. 4B, right).

We next analyzed whether specific TFBS as defined by published ChIP-Seq data would influence loss of DNA methylation by the use of 5-azacytidine. These analyses showed that PRC2/Suz12 binding sites in embryonic stem cells were especially susceptible to loss of DNA methylation after 5-azacytidine exposure (Fig. 5A, left). This finding was consistent across both cell lines (A549 and HTB56), as well as for different drug concentrations (Table 1). The observed–expected ratio of genomic regions most strongly hypomethylated after exposure to 5-azacytidine strongly correlated between the 2 different cell lines (Fig. 5A, right).

The preferential effect of 5-azacytidine on PRC2/Suz12 targets did not depend on absolute methylation levels. Other genomic regions that are known to show frequent DNA methylation in mammalian cells were consistently underrepresented in loss of DNA methylation. For example, exonic sequences showed a lower number of demethylated sequences than expected (ratio 0.8 in A549 cells at 250 nmol/L, \( P < 0.001 \)).

Changes in methylation levels upon release from 5-azacytidine

We analyzed the changes in DNA methylation patterns directly after treatment with 5-azacytidine, including a recovery phase. Methylation levels predominantly remained reduced after 13 days; in contrast, only minor increases were observed (Fig. 5B and C and Supplementary Fig. S4D). Overall, \( \sim 80\% \) of initially hypermethylated sites remained DNA hypomethylated days after 5-azacytidine were washed out (Fig. 5B). Interestingly, HTB56 cells regained DNA methylation faster than A549 cells, even if the latter ones were preexposed to higher doses of 5-azacytidine (Fig. 5D).

Discussion

Epigenetic alterations might contribute to tumor aggressiveness. Notably, in this study, rapid development of a more aggressive cellular behavior was associated with genome-wide DNA methylation changes. The DNA methylation changes occurred preferentially at embryonic stem cells polycomb target genes, whereas the majority of TFBS were strongly protected from DNA methylation changes. Compared with the hundreds of sites altered in DNA methylation, relatively few potentially relevant genetic changes were observed by either SNP array analysis or exome sequencing.

DNA methylation changes were highly reproducible as indicated by DNA methylation analyses of biologic replicates, and many of the changes occurred in a stepwise manner. The stepwise changes in each round of in vivo selection suggest that more aggressive subclones are continuously selected for, and thus, increase in each round. These findings indicate that the observed changes are nonrandom.

Recently, we have shown that hypermethylated binding sites coincide with actual loss of Suz12 transcription factor binding (18). Therefore, DNA methylation may contribute to silence active polycomb target genes, whereas active TFBS remain unmethylated (22, 23). Epigenetic changes are in principal drug targetable. Here, we demonstrate that 5-azacytidine induced loss of DNA methylation preferentially occurred at polycomb target genes that are known to be often DNA hypermethylated in cancer. Furthermore, DNMT inhibition effects persisted over time and reversed a highly aggressive phenotype in vivo.

DNA methylation changes and cellular aggressiveness

Metastatic cells closely resemble the parental tumor cells in terms of histology, gene expression profiles, and the patterns of somatic mutations. Development of the primary tumor may take multiple years and is associated with acquiescence of multiple somatic mutations. In contrast, metastatic lesions develop in a much shorter time frame and a core set of driver mutations persists in primary tumors and metastases (24). The pattern of somatic mutations in metastases closely resembles the pattern found in the primary tumor. This was analogous to our study with a very low percentage of additional mutations that were acquired...
Table 1. Enrichment of TFBS in genomic region with decreased methylation levels upon 5-azacytidine exposure in lung cancer cell lines

<table>
<thead>
<tr>
<th></th>
<th>HTB56 (250 nmol/L)</th>
<th>A549 (250 nmol/L)</th>
<th>A549 (1 μmol/L)</th>
</tr>
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<tr>
<td></td>
<td>Ratio (obs./exp.)</td>
<td>P value</td>
<td>Ratio (obs./exp.)</td>
</tr>
<tr>
<td>Enrichment</td>
<td></td>
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<tr>
<td>SUZ12_a</td>
<td>2.53 &lt;1 × 10⁻⁵</td>
<td></td>
<td>2.10 &lt;1 × 10⁻⁵</td>
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<tr>
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<td>1.72 &lt;1 × 10⁻⁵</td>
<td></td>
<td>1.57 &lt;1 × 10⁻⁵</td>
</tr>
<tr>
<td>Depletion</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CtBP2</td>
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<td></td>
<td>0.53 &lt;1 × 10⁻⁵</td>
</tr>
<tr>
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<td></td>
<td>0.56 1.12 × 10⁻⁵</td>
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<tr>
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<td></td>
<td>0.55 &lt;1 × 10⁻⁵</td>
</tr>
<tr>
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<td></td>
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<tr>
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<tr>
<td>SP1</td>
<td>0.20 &lt;1 × 10⁻⁵</td>
<td></td>
<td>0.15 4.78 × 10⁻¹⁴</td>
</tr>
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</table>

NOTE: Lung cancer cell lines were exposed to 5-azacytidine at either 250 nmol/L (HTB56 and A549) or 1 μmol/L (A549) for 6 days. To determine whether TFBS were over- or underrepresented in hypomethylated regions, we supposed that the number of region centers within DMRs is binomially distributed with n (number of region centers in CpG clusters) and p (sum of DMR widths/sum of CpG cluster widths). P values were derived from a 2-sided binomial test that k = p × n and adjusted for multiple testing. The ratios are defined as the number of observed binding sites divided by the number of expected binding sites. TFBS were obtained from a public ChIP-Seq dataset (23). In addition, a ChIP-ChIP dataset for SuZ12 binding sites was used to confirm the results (24).

During the selection process to the highly aggressive phenotype. About the known function of the affected genes, the vast majority of the few acquired mutations are most probably random with no causative relation to the prometastatic character. One potential exception is the genomic amplification of a 400-kbp region located on 7q31, which was detected only in HTB56_R3 by SNP array. This chromosomal region contains MET protooncogene, which, at least in part, might have contributed to the transition from low- to high-aggressive HTB56.

Metastatic cells also have been found to be linked to the frequency and phenotype of cancer stem cells (25). These findings suggest that tumor subpopulations, for example bulk tumor, cancer stem cells, and metastatic cells are genetically very similar, but mainly differ in epigenetic programs and structure. In line with this, prometastatic changes such as the epithelial–mesenchymal transition (EMT) are known to be inducible and reversible (26).

Evaluating the relation between DNA methylation and gene expression after transformation from parental to the metastatic-prone phenotype, interesting targets could be identified. For instance, ICAM1, SIX2, and GNMT revealed both hypomethylation in the promoter region and increased mRNA expression after conversion to the aggressive state of A549, as well as HTB56. A high expression of ICAM-1 is associated with a more aggressive tumor phenotype, and plays an important role in extravasation of cancer cells, a key step in metastasis (27). SIX2 is homeobox gene, which is targeted by polycomb complex; interestingly, upregulation of SIX2 by overexpression leads to increased migration (28). The GNMT gene encodes for a glycine-N methyltransferase; high-expression GNMT decreases S-adenosylmethionine (which is the cosubstrate of DNMTs) and thereby evoke low levels of global DNA methylation (29).

So far, few studies directly explored the relationship between epigenetic changes and metastatic features (30).
Our data indicated widespread and step-wise changes in DNA methylation patterns in more aggressive lung cancer cells. Epigenetic changes might be a likely explanation of the phenotype based on 3 observations: (i) The step-wise generation of the highly aggressive cells by *in vivo* selection, (ii) the use of bulk cell cultures in all experiments with the prominent changes in DNA methylation, and (iii) the low number of additional genomic alterations and/or somatic mutations in the more aggressive NSCLC cell lines containing almost no gene with a high preponderance for metastases in NSCLC. These findings are in line with recent data, indicating that no metastases-specific mutations are likely to exist (31).

Metastatic spread is an inherently complex process and no models are available that accurately capture the entire process (32). The *in vivo* model that we used here allowed the injection and recovering of relatively high numbers of cells avoiding single-clone effects. All in all, this model recapitulates extravasation, growth at a distant site and proliferation.

Obviously, xenograft models and intravenous injection can integrate only partial aspects of metastatic spread. Processes such as primary invasion as well as immune response in the organism are not reflected in these models. Accordingly, it is likely that the observed DNA methylation changes do not represent a comprehensive feature of metastasis-associated NSCLC methylome features. In contrast, the aim of the study was to provide evidence for the general plasticity, existence, and reversionality of DNA methylation patterns in the development of aggressive phenotypes. For this purpose, a (relatively) simple model with the possibility to recapture a high number of cells was instrumental. As such, this xenograft model captured features of highly aggressive tumors and allowed to investigate epigenetic therapy effects.

**5-Azacytidine and metastatic phenotype**

We exposed the cells to 5-azacytidine, to test whether we could revert the metastatic phenotype. 5-Azacytidine inhibits DNMT activity by forming covalent complexes with the catalytic domain (33–35). This epigenetic modulator also acts by direct cytotoxic mechanisms and is incorporated into RNA (36–38). These effects may hamper the evaluation of the relevance of DNA methylation changes. Therefore, we performed 5-azacytidine treatment by exposure and subsequent release *in vitro*. Metastatic cells exposed to 5-azacytidine reverted their phenotype *in vitro*, as well as *in vivo*, adapting to the characteristics of the parental cell line.

Our findings indicate that 5-azacytidine can reverse a metastatic memory in NSCLC cells. Of note, 5-azacytidine might act by altering the epigenetic landscape rather than by reactivating only a few specific metastasis suppressor genes. Our data showed that hypomethylation of prometastatic genes seemed probably more frequently in NSCLC than hypermethylation of antimetastatic genes. The effects of 5-azacytidine might therefore fit better with a model of overall epigenetic disturbance. It is tempting to speculate that the resolve of this epigenetic disturbance might also revert specific phenotypes, for example, those of high metastasis. Consequently, 5-azacytidine turns out to be a potential drug for patients with NSCLC with a high risk of metastases development. 5-Azacytidine effects were not entirely random throughout the genome: we observed a preference for the hypomethylation of polycomb targets (see above) and increased activity near chromosome ends. This phenomenon was consistent in both cell lines and was found in 20 of the 23 chromosomes. Differences in 5-azacytidine doses (250 or 1000 nmol/L) did not influence this effect. However, we confirmed the fact that a dose--response exists for 5-azacytidine *in vivo* by using 100 nmol/L in the indicated model, and lung nodules were formed to the same frequency as untreated controls. However, no linear correlation could be observed between dose-dependent antiproliferative effects and the capacity to reverse the epigenetic phenotype. Interestingly, HTB56 cells were more sensitive toward 5-azacytidine than A549 cells. HTB56 cells also showed more changes in DNA methylation upon *in vivo* selection. Most of the changes in HTB56 cells were DNA hypomethylation. It is possible that 5-azacytidine sensitivity might be associated with the degree of methylation and/or the rate of methylation changes in cancer cells.

**Polycomb targets and DNA methylation changes**

In all the analyses that we performed, including the treatment with 5-azacytidine, polycomb sites were consistently overrepresented as sites of changes in DNA methylation. Bivalent (H3K4 and H3K27 methylated) polycomb binding sites in embryonic stem cells are of increased likelihood to be methylated in human cancers (39–41). The stability of this DNA hypermethylation and its accessibility for DNA hypomethylating therapy was previously unknown. Here, we show that in already established cancer cell lines, the polycomb target genes show an increased propensity for DNA methylation changes. With regard to the highly metastatic cell lines, we observed overrepresentation of Suz12 binding sites in hyper- and hypomethylated regions. This finding suggests that these sites have an inherently increased rate of methylation changes. Increased methylation of these target genes has been observed in aging (42). This view was further enforced by the finding that the Suz12 binding sites were most likely to undergo hypomethylation upon exposure to 5-azacytidine. Also, remethylation after the end of treatment with 5-azacytidine was most likely to occur in polycomb sites. Taken together, these data established that an aggressive phenotype in NSCLC cells is associated with profound DNA methylation changes. Treatment of 5-azacytidine reversed the metastasis-prone signature and preferentially demethylated binding sites of PRC2 from embryonic stem cells.

**Disclosure of Potential Conflicts of Interest**

C. Müller-Tidow has commercial research grant from Celgene. C. Müller-Tidow has Honoraria from Speakers Bureau of Celgene and Janssen. C. Müller-Tidow is a consultant/advisory board member of Janssen. No potential conflicts of interest were disclosed by the other authors.
DNA Methyltransferase Inhibition Affecting NSCLC Methylome

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