DNA Methyltransferase inhibition reverses epigenetically embedded phenotypes in lung cancer preferentially affecting Polycomb target genes

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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 pro-metastatic phenotypes in NSCLC 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.
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 (RRBS) compared to the less aggressive parental cell lines. DNA methyltransferase inhibition by Azacytidine reversed the pro-metastatic 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 which persisted over time. Conclusions: We could show that metastatic capability of NSCLC is closely associated with DNA methylome alterations. Since inhibition of DNA methyltransferase reversed metastasis-prone phenotype, epigenetic modulation appears as a potential therapeutic approach to prevent metastasis formation.
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 behaviour 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 haematological 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). In line with this, metastasis formation in most cases occurs in a rather short time span compared to 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 the current study, we analyzed the profile of DNA methylation changes occurring during the transition from a cellular state with low metastatic capacity towards a highly aggressive state and whether these changes were reversible by DNMT inhibition.
Material 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, St. Louis, MO, USA) at a concentration of 100 nM to 1 µM. 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 three times with PBS and released for additional 7 days in regular medium.

Functional in vitro assays

Proliferation assays with ³[H]-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 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, CA, USA) 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 (GSExxxxx_pending).

Detailed methodological information regarding 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 ten-weeks-old NOD.CB17-Prkdc<scid>/J [non-obese diabetic/severe combined immunodeficient (NOD/SCID)] mice were used. To establish a more aggressive cell line by in vivo selection, two 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 2x10^6 parental cells (A549_R0 or HTB56_R0) were injected i.v. into the tail vein of two 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 thirty minutes of digest with an adequate amount of Trypsin (approximately 5x10^4 cells) in cell culture in the indicated media. A maximum of seven doubling times was needed to reach the cell count for the next round of selection in mice. To analyze lung
nodule formation after i.v. 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 $2 \times 10^5$ (A549) or $1 \times 10^6$ (HTB56) cells either highly or low metastatic and either exposed or non-exposed were injected i.v. 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 plus standard deviation (SD) if not indicated otherwise. Statistical analyses were done with SPSS, version 18 (IBM, Ehningen, Germany). Statistical significances of overall differences between multiple groups were analyzed by Kruskal-Wallis-test. Differences between two groups were analyzed by Student´s 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 NSCLC cell lines with increased propensity to form tumor nodules in murine lungs after i.v. injections (Figure 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 i.v. 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 re-injection led to a rapid increase in metastatic capacity (Figure 1A). A highly aggressive phenotype that was stable over time was evident after three rounds of in vivo selection for both cell lines (Figure 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 (see below). 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 to the parental cells (Supplementary Figure S1A, Supplementary Table 1). One notable exception is the potential amplification of the MET protooncogene on 7q31 which was detected by SNP array only in high aggressive compared to parental cells of HTB56 (Supplementary Table 1B). 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 2). Thus, despite the strong differences regarding 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 Reduced Representation Bisulfite Sequencing (RRBS) (17) protocol to focus on CpG rich regions and, thereby, identify alterations in DNA methylation in the two NSCLC cell lines with increased aggressiveness in vivo. On average, 1.8x10^7 reads were uniquely mapped to the human genome in each cell line (Supplementary Table 3). Based on the spatial density of covered CpG sites (see methods), we defined 19,097 CpG clusters that spanned 7,502,992 base pairs (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 (Figure 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 towards higher CpG methylation in metastatic cells (1.07%) compared to 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 DMRs (differentially methylated regions) 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 two cell lines (Figure 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 (Figure 2A right).

We compared DNA methylation levels derived by RRBS with data generated using the Infinium 27K methylation bead array technology. Overall, we observed highly concordant results (Supplementary Figure S1B, 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 (Figure 2B). Binding sites for Suz12 as an indication for the PRC2 complex in embryonic stem (ES) cells showed an increased likelihood to be DNA hyper- and hypomethylated, respectively, in both cell lines. Moreover, this was observed in two 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 two 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 4). Notably, we observed a high correlation between biological replicates from normal and highly aggressive cell lines from both cell types (Supplementary Figure 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 (Figure 2C and Supplementary Table 4).

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 4).

**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 e.g. 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.
(Figure 3A). After this time, the A549 cell lines did not show increased apoptosis (Supplementary Figure S2A, left). DNA double-strand breaks were present in all NSCLC cells regardless of previous 5-Azacytidine-exposure (Figure 3A, Supplementary Figure S2B). Even at the maximum dose of 5-Azacytidine, DNA double-strand breaks increased only by 20% (Supplementary Figure 2B). 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 (Figure 3B, left). Additionally, in vitro transwell migration was enhanced in the highly aggressive A549 cells and was reversed after 5-Azacytidine-exposure (Figure 3B, middle). Capillary electrophoresis confirmed persistent changes in global DNA methylation after 5-Azacytidine-exposure (Supplementary Figure S2C).

Further, 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 (Figure 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 µM), increased apoptosis was still present (Supplementary Figure S2A, right). As a consequence, release experiments (Figure 3A) were performed at concentrations as low as 250 nM. A 6 days exposure of 5-Azacytidine with subsequent release prevented lung nodule formation at a dose as low as 250 nM (Figure 3C). Effects of 5-Azacytidine depended on a minimal drug concentration since 100 nM drug exposure only partially prevented lung nodule formation in A549 and HTB56 cells (Supplementary Figure 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 (Figure 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 Figure S4A, B). In virtually all (97%) of the altered regions, DNA methylation was decreased (Figure 4B, left; Supplementary Figure S4C). In the A549 cells, the higher dose of 5-Azacytidine (1 µM instead of 250 nM) did not further increase DNA hypomethylation indicating a saturation effect (Figure 4B, middle; Supplementary Figure S4C). More than 80% of the genomic regions that were DNA hypermethylated in the more aggressive cells showed reductions in DNA methylation after treatment with 5-Azacytidine (Figure 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 Figure S5). Different genomic regions, e.g., promoter and gene body showed similar changes in loss of DNA methylation (Figure 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 towards the chromosome ends that showed a higher level of de-methylation (Figure 4D). This pattern occurred at most of the chromosomes regardless of the drug concentration (Supplementary Figure S6). Cell line specific effects were observed. Multiple sites were hypomethylated only in one but not the other cell line (Figure 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 ES cells were especially susceptible to loss of DNA methylation after 5-Azacytidine-exposure (Figure 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 two different cell lines (Figure 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 nM, 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 (Figure 5B, C; Supplementary Figure 4D). Overall, ~80% of initially hypermethylated sites remained DNA hypomethylated days after 5-Azacytidine were washed out (Figure 5B). Interestingly, HTB56 cells regained DNA methylation faster than A549 cells, even if the latter ones were pre-exposed to higher doses of 5-Azacytidine (Figure 5D).
Discussion

Epigenetic alterations might contribute to tumor aggressiveness. Notably, in the current study, rapid development of a more aggressive cellular behavior was associated with genome wide DNA methylation changes. The DNA methylation changes occurred preferentially at ES cells Polycomb target genes, whereas the majority of TFBS were strongly protected from DNA methylation changes. Compared to 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 biological 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 non-random.

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, while 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. Further, 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 during the selection process to the highly aggressive phenotype. Regarding 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 pro-metastatic 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 \textit{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, e.g. bulk tumor, cancer stem cells and metastatic cells are genetically very similar, but mainly differ in epigenetic programs and structure. In line with this, pro-metastatic 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 co-substrate of DNA methyltransferases) 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 three 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 i.v. 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 reversibility of DNA methylation patterns in the development of aggressive phenotypes. For this purpose, a (relatively) simple model with the possibility to re-capture 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 \textit{in vitro}. Metastatic cells exposed to 5-Azacytidine reverted their phenotype \textit{in vitro}, as well as \textit{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 re-activating only a few specific metastasis suppressor genes. Our data showed that hypomethylation of pro-metastatic genes appeared probably more frequently in NSCLC than hypermethylation of anti-metastatic 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 e.g. those of high metastasis. Consequently, 5-Azacytidine turns out to be a potential drug for NSCLC patients 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 nM or 1000 nM) did not influence this effect. On the other hand, we confirmed the fact that a dose response exists for 5-Azacytidine in vivo by using 100 nM 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 towards 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 ES-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, re-methylation 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 de-methylated binding sites of PRC2 from ES cells.
AUTHORSHIP

A.H. designed and analyzed experiments and wrote the manuscript. A.-K.H. and C.R. performed and analyzed experiments and wrote the manuscript. M.R., D.J., A.W., I.S. and S.O. performed and analyzed experiments. K.H. and H.-U.K. analyzed NGS data. M.S., R.W. M.D. and L.T. reviewed the manuscript. W.E.B., N.H.T. and C.M.-T. designed the study and experiments and wrote the manuscript.

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Figure legends

Figure 1: DNA methylation changes and aggressiveness of lung cancer cell lines.

A) Experimental outline for the generation of highly aggressive lung cancer cell lines. A549 or HTB56 cells were intravenously injected into the tail vein of NOD/SCID mice. After 8 weeks the mice were sacrificed and lung nodules reminiscent of metastases were removed. Tumor cells were expanded in vitro and subsequently i.v. injected. The high metastatic potential cell lines (after three rounds of selection) were compared to the parental cell lines.

B) Verification of the aggressiveness of the low and highly metastatic potential cell lines in vivo. Representative photographs of the resected lungs are shown. Black arrows indicate metastases.

C) Genome wide DNA methylation analyses were performed by Reduced Representation Bisulfite Sequencing (RRBS). The smoothed scatterplots indicate changes in DNA methylation between high metastatic (y-axis) and low metastatic cells (x-axis). Colors represent the density of CpG-sites ranging from red (high density) to blue (low density). Note that the majority of CpG-sites were either unmethylated or fully methylated. Also, more changes in DNA methylation were observed in HTB56 cells compared to A549 cells.

Figure 2: Polycomb target genes show preferentially altered DNA methylation in aggressive lung cancer cells.

A) left: Overlap of differentially methylated regions (DMRs) observed between high and low metastatic HTB56 and A549 cells.
A) right: Differentially methylated genomic regions. Bars indicate the numbers of hyper/hypo-methylated regions in the two cell lines HTB56 and A549.

B) Representation of transcription factor binding sites (TFBS) in the DMRs. The plots depict the ratio of the number of TFBS observed and expected in DMRs for TFBS derived from published ChIP-Seq analyses (23). The x-axis indicates statistical significance as expressed by 1/p-value. Observed/Expected diagrams are separately shown for hypermethylated (left) and hypomethylated (right) regions. Two independent datasets (ChIP-Seq & ChIP-CHIP) were used for Suz12 binding sites in Embryonic Stem cells that indicate Polycomb target genes.

C) Methylation increases (decreases) from parental to highly metastatic cells in a step-wise manner. Shown are the boxplots of methylation levels of depicted regions of replicates of 0R and 3R together with samples of 1R and 2R. The selected regions are the detected DMRs between the original parental and highly metastatic cells that comprised at least one CpG site with a minimum coverage of 10 reads. Per region and sample the mean of methylation level was determined. Regions with differences less than 5% for hypermethylated DMRs (or more than -5% for hypomethylated DMRs) were discarded. While in A549 cells 137 out of 320 regions were confirmed to be hypermethylated, in HTB56 cells 171 out of 238 regions were confirmed to be hypermethylated. In both cell lines a higher percentage of regions were substantiated to be hypomethylated (451/595 in A549; 1270/1433 in HTB56) as initially shown.
Figure 3: 5-Azacytidine reverts the metastasis-prone phenotype in lung cancer cells.

A) The experiment outline indicates the time line of 5-Azacytidine-exposure and release. Cells were exposed to 5-Azacytidine for six days at 250 nM and 1000 nM (A549) or at 250 nM only (HTB56). After six days all remaining drug was washed out and cells were released into 5-Azacytidine free media plus FCS for an additional 7 days to overcome any direct toxic effects and RNA methylation effects. Functional analyses and intravenous injection were performed at day 13. RRBS analyses were performed on days 0, 6 and 13 for both cell lines.

B) Left: Proliferation assays were performed in A549 cells not exposed or 5-Azacytidine exposed and released cells. The proliferation rate was 7-fold increased in the high metastatic A549 cell line compared to the low metastatic control (p < 0.05). After 5-Azacytidine-exposure and a period of release the high metastatic cells showed a significant decrease in proliferation.

Middle: Transwell migration assays were performed. The high metastatic A549 cells showed a 2.5 fold increased migration rate. After 5-Azacytidine-exposure and release the high metastatic cells showed a decrease in migration compared to the not exposed controls (p < 0.05). All bars indicate the mean of three independent experiments performed in triplicates. Controls were set to 100 %.

Right: A549 Cells exposed to 5-Azacytidine (1 µM) and released were injected into NOD-Scid mice. No metastasis formation was observed in 5-Azacytidine exposed cells.

C) HTB56 cells were 5-Azacytidine exposed as depicted in A and subsequently injected into NOD-SCID mice. Since cells exposed to 5-Azacytidine at 1 µM still showed enhanced apoptosis, we performed the experiment at varying 5-
Azacytidine levels. Even at 250 nM, which is a concentration that did not induce increased apoptosis, no metastasis formation was observed.

**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 days exposure to 5-Azacytidine.

B) Left: Smoothed scatterplot of DNA methylation levels for more aggressive A549 cells versus more aggressive A549 cells after six days of 5-Azacytidine-exposure at 250 nM. 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 of 250nM versus 1000nM dosis. 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). For this plot only CpGs with at least 30% methylation level were analyzed. Many CpGs were similarly hypomethylated in both cell lines (high density of points along the diagonal line in the third quadrant). In both cell lines multiple CpGs were identified that were hypomethylated in one cell line but not the other (indicated by arrows).

C) The mean of the relative methylation of CpG clusters is plotted. CpG clusters were grouped according to their genomic association with genes, promoters etc. Relative methylation of the clusters is plotted for A549 cells that were exposed to 250 nM 5-Azacytidine.

D) Chromosomal distribution of clusters and DMRs. The curves show the density distribution of hypomethylated DMRs and CpG clusters in relation to their chromosomal position: 0 indicates chromosomal end and 1 the centromeric region. Hypomethylated DMRs (green) and CpG clusters (black) are shown. Loss of DNA methylation at day 6 of 5-Azacytidine preferentially occurred near chromosome ends. Depicted here as an example is chromosome 10 in A549 cells exposed to 250 nM 5-Azacytidine.

Figure 5: Polycomb binding sites are preferentially demethylated by 5-Azacytidine and methylation remains decreased after the end of 5-Azacytidine-exposure.

A) Left: The plot depicts the representation of Transcription Factor Binding sites (TFBS) in DNA hypomethylated regions after a 6 days exposure to 5-Azacytidine. Only Polycomb binding sites (Suz12) in ES cells as represented by two independent ChIP-Seq binding profiles were more frequently DNA hypomethylated than expected.
Right: DNA hypomethylation of genomic regions and TFBS correlated closely between cell lines. Plotted are the ratios of observed/expected for the hypomethylated regions of 5-Azacytidine exposed HTB56 cells (x-axis) and A549 cells (y-axis).

B) The bar graph depicts the percentage of DMRs, which remain hypomethylated after release from 5-Azacytidine-exposure. After the release of cells from 5-Azacytidine-exposure, DNA methylation of initially hypermethylated regions remained low.

C) Smoothed scatterplot of DNA methylation levels for initial metastasis-prone A549 cells versus A549 cells on day 13 (7 days after release from 5-Azacytidine treatment).

D) Smoothed scatterplot of DNA methylation levels for 5-Azacytidine treated A549 and HTB56 on day 6 versus cells after release from 5-Azacytidine on day 13. HTB56 cells regained DNA methylation faster than A549 cells.
Figure 1

A)...
B)...
C) Methylation in high metastatic cells vs. Methylation in low metastatic cells for A549 and HTB56 cell lines.
Table 1: Enrichment of transcription factor binding sites in genomic region with decreased methylation levels upon 5-Azacytidine exposure in lung cancer cell lines.

<table>
<thead>
<tr>
<th>Factor</th>
<th>HTB56–250nM</th>
<th>A549–250nM</th>
<th>A549–1µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio p-value</td>
<td>Ratio p-value</td>
<td>Ratio p-value</td>
</tr>
<tr>
<td><strong>Enrichment</strong></td>
<td></td>
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</tr>
<tr>
<td>SUZ12_a</td>
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<td>2.10 &lt;1 X 10^{-5}</td>
<td>2.14 &lt;1 X 10^{-5}</td>
</tr>
<tr>
<td>SUZ12_b</td>
<td>1.72 &lt;1 X 10^{-5}</td>
<td>1.57 &lt;1 X 10^{-5}</td>
<td>1.46 &lt;1 X 10^{-5}</td>
</tr>
</tbody>
</table>

| **Depletion** | | | |
| CtBP2      | 0.79 <1 X 10^{-5} | 0.53 <1 X 10^{-5} | 0.54 <1 X 10^{-5} |
| Nrsf       | 0.74 4.5 X10^{-3} | 0.56 1.12 X10^{-3} | 0.59 2.20 X10^{-3} |
| CTCF       | 0.64 <1 X 10^{-5} | 0.45 <1 X 10^{-5} | 0.46 <1 X 10^{-5} |
| Max        | 0.61 <1 X 10^{-5} | 0.55 <1 X 10^{-5} | 0.55 <1 X 10^{-5} |
| TCF12      | 0.48 <1 X 10^{-5} | 0.40 <1 X 10^{-5} | 0.39 <1 X 10^{-5} |
| JunD       | 0.45 <1 X 10^{-5} | 0.35 <1 X 10^{-5} | 0.36 <1 X 10^{-5} |
| NRF1       | 0.41 <1 X 10^{-5} | 0.24 <1 X 10^{-5} | 0.25 <1 X 10^{-5} |
| Jun        | 0.35 <1 X 10^{-5} | 0.27 <1 X 10^{-5} | 0.26 <1 X 10^{-5} |
| RFX5       | 0.28 <1 X 10^{-5} | 0.19 <1 X 10^{-5} | 0.22 <1 X 10^{-5} |
| SP1        | 0.20 <1 X 10^{-5} | 0.15 4.78 X10^{-114} | 0.16 <1 X 10^{-5} |

Lung cancer cell lines were exposed to 5-Azacytidine at either 250 nM (HTB56 and A549) or at 1 µM (A549) for six days. To determine whether transcription factor binding sites, were over- or underrepresented in hypomethylated regions we supposed that the number k 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 two-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. Transcription factor binding sites were obtained from a public ChIP-Seq data set (23). In addition a ChIP-CHIP dataset for Suz12 binding sites was used to confirm the results (24).
DNA Methyltransferase inhibition reverses epigenetically embedded phenotypes in lung cancer preferentially affecting Polycomb target genes


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