Epigenetic targeting of the Nanog pathway and signaling networks during chemical carcinogenesis

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Chemical carcinogenesis has long been synonymous with genotoxicity, which entails DNA damage, genetic mutations and chromosomal abnormalities. The present study investigates a paradigm-shifting model in which epigenetic changes are key contributors to chemical carcinogenesis. Using genome-wide microarray-based analysis followed by conventional validation assays, we have progressively chronicled changes in the epigenetic landscape, as reflected in the patterns of DNA methylation, in the target organ of tumorigenesis in mice treated in vivo with a prototype chemical carcinogen (benzo[a]pyrene). Here, we demonstrate characteristic CpG island gain/loss of methylation and demethylation of repetitive DNA elements in carcinogen-treated mice, dependent on tumor progression. Alterations of the DNA methylome are accompanied by silencing of major DNA methyltransferases. Members of the Nanog pathway that establishes and maintains pluripotency in embryonic stem cells and possibly triggers uncontrolled proliferation of neoplastic cells are preferential targets of aberrant DNA methylation and concomitant gene dysregulation during chemical carcinogenesis. Several components of the MEK/ERK, JAK/STAT3, PI3K/AKT, WNT/β-catenin and Shh signaling cascades, which are known to modulate Nanog expression, also show concurrent changes in the patterns of DNA methylation and gene expression. Our data support an epigenetic model of chemical carcinogenesis and suggest that surveillance of the epigenetic landscape, particularly at the loci and in the pathways identified in this study, may have utility for early detection and monitoring of the progression of malignancy.

Introduction

Chemical carcinogenesis has long been ascribed to genotoxic events, which entail DNA damage, genetic mutations and chromosomal abnormalities (1–3). In addition to genotoxicity, chemical carcinogens may also possess a non-mutagenic mode of action, e.g. epigenetic effects, which are emerging as key contributors to carcinogenesis (4–6). Epigenetic effects include aberrant DNA methylation, histone modifications and variants, miRNAs dysregulation, chromatin remodeling and nucleosome positioning (7–9). Of these, aberrant DNA methylation is the most extensively studied epigenetic alteration in carcinogenesis (7,8). Gain of methylation (hypermethylation) at CpG islands, clustered at the promoter, 5′ untranslated region and exon 1 of known genes (promoter CpG islands) or localized within gene bodies (intragenic CpG islands) is a common event in cancer (9–11). Global loss of methylation (hypomethylation) at repetitive DNA elements, such as long and short interspersed nuclear elements (LINEs and SINEs, respectively), and long terminal repeat (LTR) retrotransposons is also a frequent occurrence in carcinogenesis (12–14).

Although human population studies and animal model experiments have established an association between exposure to chemical carcinogens and epigenetic effects, a direct cause and effect relationship has yet to be established (4,5). Suggestive evidence exists in support of a causal link between epigenetic effects and chemical carcinogenesis. For example, the reactive metabolite of a prototypical chemical carcinogen, benzo[a]pyrene (B[a]P) (15), binds preferentially to methylated CpG sites (16), thereby, possibly impeding the establishment and/or maintenance ofDNA methylation patterns by DNA methyltransferases (DNMTs) and methyl-CpG-binding domain (MBDs) proteins (17–19). It is also plausible that carcinogen-induced DNA damage may cause mutations in genes maintaining the epigenetic state, including those regulating the DNA methylation machinery and chromatin-modifying enzymes (20,21).

The objectives of the present study were 2-fold: (i) to investigate whether epigenetic effects occur in response to exposure to chemical carcinogens and, if so, (ii) to determine whether epigenetic effects are predictors of chemically induced carcinogenesis. To attain these objectives, we have performed genome-wide DNA methylation analysis in the target organ of tumorigenesis, i.e. seminal vesicles, in mice treated in vivo with B[a]P both before and after tumor development. For verification purposes, we have confirmed the data obtained by our genome-wide microarray-based analysis (22) using the conventional single-gene methylation detection assays (23,24). Furthermore, we have used a bisulfite sequencing-based assay (25) to determine the methylation status of major repetitive DNA elements, including LINE L1, intracisternal A particle (IAP) LTR and SINE B1 (26–28), in B[a]P-treated mice both before and after tumor development. Here, we demonstrate a relationship between carcinogen exposure, alterations of the epigenome and gene expression changes, particularly in the Nanog and its interconnected signaling pathways. Our data indicate that carcinogen exposure results in epigenetic modifications, specifically at loci that control key signaling pathways required for normal cell proliferation during development.

Materials and methods

B[a]P treatment of mice

Thirty adult male mice (6–8 weeks old) on a C57BL/6 genetic background (Stratagene, La Jolla, CA) were randomly divided into two groups: (i) experimental (B[a]P treatment; n = 15) and (ii) control (solvent treatment; n = 15), each subdivided into three categories, including (i) 6 week treatment (T0), (ii) 6 week treatment + 6 week latency (T1: early lesion formation) and (iii) 6 week treatment + 120 week latency (T2: tumor development). A flowchart of the study design is shown in Figure 1A. The mice assigned to each experimental or control group were kept in polypropylene cages in groups of two to three animals per cage and housed in an air-conditioned animal room with controlled ambient temperature, relative humidity and 12 h light/dark cycle. The mice had access to food (PicoLab Rodent Diet 20; PMI Nutrition International, LLC; Brentwood, MO) and water ad libitum at all times. All experiments were approved by the Institutional Animal Care and Use Committee in accordance with the recommendations of the National Institutes of Health provided in the Guide for the Care and Use of Laboratory Animals.

The experimental mice received intraperitoneal injections of B[a]P once per week for a duration of 6 weeks using the following protocol: first week: 25 mg/kg body weight (BW); second week: 50 mg/kg BW; third week: 75 mg/kg BW and fourth week to sixth week: 100 mg/kg BW of B[a]P. The specific doses of B[a]P were prepared fresh on the day of administration by dissolving the chemical in dimethyl sulfoxide (DMSO) (B[a]P) and DMSO (Sigma–Aldrich, St Louis, MO). The incremental doses of B[a]P were delivered to the mice by intraperitoneal injection (100 µl) on the lower right or left quadrant of the
abdomen in alternate weeks. Control mice received similar injections of solvent DMSO using the same dosing regimen, as described for B[a]P. All mice were monitored closely for development of any unusual symptoms. At the end of all experiments, the B[a]P-treated mice and controls were euthanized by CO₂ asphyxiation and subjected to necropsy and macroscopic examination. For biochemical assays, the accessory sex organs, including seminal vesicles...
and prostate glands or tumors, were harvested, snap frozen and preserved at −80°C until further analysis. Alternatively, the harvested tissues or tumors were fixed in formalin, embedded in paraffin and used for hematoxylin and eosin (H&E) slide preparation according to standard protocols.

**Genome-wide DNA methylation profiling**

We used the methylated CpG island recovery assay (MIRA) in combination with microarray analysis (22) to catalogue the DNA methylation profile, on a genome-wide scale, in the target organ of tumorigenesis, i.e. seminal vesicles, in mice treated in vivo with B[a]P both before and after tumor development. As a pull-down assay for enrichment of the methylated CpG content of DNA, the MIRA is based on the ability of the MBD2b protein to bind methylated CpG dinucleotides, while this reaction is enhanced in the presence of MBD3L1 protein (22). Briefly, genomic DNA was isolated from either seminal vesicles or tumors developed at the same organ site and subjected to MIRA enrichment, as described previously (22). Subsequently, the MIRA-enriched DNA and input DNA fractions were amplified by PCR and labeled and hybridized to the Roche NimbleGen Mouse DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Arrays (Roche NimbleGen, Indianapolis, IN). This set of microarrays covers 20 404 RefSeq gene promoters and 15 988 annotated CpG islands of the mouse genome. The raw microarray data were processed and analyzed using a standard bioinformatics approach, as described in ref. (22). The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (Accession No: GSE41422).

**Single-gene DNA methylation analysis by combined bisulfite restriction analysis and bisulfite sequencing**

We used the combined bisulfite restriction analysis (COBRA) (24) and sodium bisulfite sequencing (23) to verify the methylation status of individual target loci/gene identified by MIRA-microarray analysis in the target organ of tumorigenesis in B[a]P-treated mice both before and after tumor development. Briefly, 1 µg of genomic DNA was treated with sodium bisulfite using the Qiagen EpiTect kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). Subsequently, the bisulfite-treated DNA was assayed by standard COBRA (24) using sets of primers specifically designed for each target CpG island. The primer sequences used for PCR amplification of all the analyzed targets are available upon request. Mouse genomic DNA was methylated in vitro with M. Sssl CpG methyltransferase (New England Biolabs, Ipswich, MA) and served as positive control. For genomic sequencing, the PCR products obtained after bisulfite conversion of DNA were cloned into the TOPO-TA cloning vector according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Up to 10 randomly selected clones from each experimental and control group were sequenced using an ABI-3730 DNA Sequencer (ABI Prism; PE Applied Biosystems, Foster City, CA).

**DNA methylation analysis in repetitive DNA elements**

We used a bisulfite sequencing-based assay (25) to analyze the methylation status of major repetitive DNA elements, including LINE L1, IAP-LTR and SINE B1, in the target organ of tumorigenesis in B[a]P-treated mice both before and after tumor development. The LINE L1, IAP-LTR and SINE B1 comprise 18.78, 3.13 and 2.66%, respectively, of the mouse genome (26–28). The assay is based on sodium bisulfite treatment of the genomic DNA, followed by primer amplification of the consensus sequences of the respective elements and direct sequencing, thereafter (25). Detailed information on the genomic sequence and primer design of the repetitive DNA elements analyzed in the present study is available in ref. (22).

**Motif discovery and canonical pathway analysis**

Differentially methylated CpG islands were analyzed for shared de novo motifs and known transcription factor recognition sites, using Partek® Genomics Suite™ software (Partek, St. Louis, MO). Novel motifs of length 6–16 bases were identified based on their highest score (score = log ratio of the probability that the sequence was generated by the motif versus that by background distribution). Binding sites for known transcription factors were selected from the JASPAR database in Partek based on their high probability of occurrence (P value). Functional identification of gene networks and canonical pathways analysis were performed using the Ingenuity Pathway Analysis® program (IPA®: v 9.0) and the GO Enrichment Analysis in Partek® Genomics Suite™.

**Quantitative real-time PCR**

Standard quantitative real-time reverse transcription–PCR (qRT–PCR) was used to determine the level of transcription of individual target genes identified by MIRA-microarray analysis, as described previously (22). Briefly, total RNA was extracted from the seminal vesicles of mice treated in vivo with B[a]P both before and after tumor development, using the RNeasy kit (Qiagen). DNase-treated RNA (0.5 µg) was reverse transcribed into cDNA using SuperScript® VILO™ cDNA Synthesis kit (Invitrogen). The mRNA expression level of target genes was determined by qRT–PCR using the EXPRESS SYBR® GreenER™ qPCR SuperMix (Invitrogen) and the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad). All reactions were performed in triplicate and fold changes were determined using the 2−ΔΔCt method (22). The primer sets used for qRT–PCR are listed in Supplementary Table S1, available at Carcinogenesis Online.

**Results**

**Mice survival and tumorigenicity**

We have treated adult male mice with progressively increasing doses of B[a]P weekly for a duration of 6 weeks, as described in Materials and methods. For control purposes, we have treated counterpart mice with solvent DMSO using the same protocol as described for B[a]P. Subgroups of animals were euthanized immediately after treatment (T0), 6 week posttreatment (T1) or after tumor development (T2), as outlined in Figure 1A. The mice generally well tolerated B[a]P treatment and had a survival rate of >75%, as determined in two independent sets of experiments. All surviving B[a]P-treated mice developed large aggressive tumors in the seminal vesicles as early as 10 week posttreatment. Small lesions and other abnormalities were also detectable in the secondary sex organs of B[a]P-treated mice at T1. Control mice treated with DMSO showed 100% survival at the end of all experiments and were lesion free at all organ sites.

**Histological analysis of B[a]P-induced tumors**

The accessory sex organs, including the target organ of tumorigenesis, i.e. seminal vesicles, were collected from all B[a]P-treated and control mice at the time of necropsy, fixed in formalin and subjected to H&E staining. Consistent with landmark studies (29), all surviving B[a]P-treated mice developed large solid masses (Δ: 2.5 × 2 × 2 cm) in the seminal vesicles and prostate glands. Histopathological examination identified the tumors as high-grade sarcomas, mostly leiomyosarcoma. Prominent features of the tumors included characteristic spindle-like cells, abundance of apoptotic cells and mitotic figures, as well as areas of pleomorphism and infiltration (Figure 1B, panels a–f). One mouse from group T0 and all mice from group T1 had macroscopic abnormalities in the genitourinary tract. These aberrations often consisted of (uni-)testicular atrophy and/or hyperplasia of seminal vesicles/prostate (mostly on the left side), which are likely to have preceded the tumors developed in older mice. Hyperplasia and other abnormalities were also observed sporadically in the digestive tract of several mice at the end of treatment (T0), and thereafter (T1 and T2). One mouse from group T2 (#313) also developed an aggressive sarcoma on the intestinal wall. Representative H&E sections of tissues/tumors collected from B[a]P-treated mice are shown in Figure 1B, panels a–f.

**DNA methylation profiling during B[a]P-induced tumorigenesis**

We have used a genome-wide microarray-based approach (22) to detect aberrant DNA methylation in the target organ of tumorigenesis, i.e. seminal vesicles, in mice treated in vivo with B[a]P immediately after treatment (T0) and following tumor development (T2). For control purposes, we performed similar analysis on counterpart organs from solvent-treated mice. Due to small size of lesions in mice at T1, the seminal vesicles of these animals were excluded from the analysis. Briefly, genomic DNA was isolated from either seminal vesicles or tumors developed at the same organ site and subjected to MIRA enrichment, as described previously (22). Following pull-down of the methylated CpG islands by MIRA, the enriched- and input DNA fractions were labeled, mixed and hybridized to the mouse CpG island plus promoter tiling arrays (Roche NimbleGen). The microarray data were normalized and analyzed using rigorous algorithms for peak calling, as described in ref. (22). Two independent lists of differentially methylated targets were generated based on the level of stringency used for data analysis, as follows: (i) stringent: methylation differences detected in four out of four biological replicates within a treatment group and absent in all respective controls and (ii) relaxed:
methylation differences detected in three out of four biological replicates within a treatment group and absent in all respective controls. When the stringent criteria were used for analysis, we identified 372 CpG targets that displayed aberrant methylation in tumors from B[a]P-treated animals relative to controls (251 hypermethylated and 121 hypomethylated CpG islands) (Figure 2A). A lower yet significant number of differentially methylated CpG islands was detected in the seminal vesicles of apparently asymptomatic mice treated with B[a]P at T0 (immediately after treatment), i.e. 123 aberrantly methylated targets, of which 60 were hypermethylated and 63 were hypomethylated (Figure 2A). Seventy-two percent of these 123 CpG islands coincide with those identified in tumors (T2), suggesting that aberrant DNA methylation in these targets occurs early in the process of tumorigenesis. Principal component analysis of the differentially methylated CpG islands confirmed that all samples within a treatment group clustered together, with a significant overlap between the T0 and T2 groups (Figure 2B and C). In tumors (T2), hypermethylated CpG islands map mostly within annotated genes (41%), whereas loss of methylation occurs predominantly at upstream regulatory regions (77%) (Figure 2D). Likewise, a characteristic pattern for genomic distribution of the aberrantly methylated targets was found in seminal vesicles of mice at T0, with 46% of hypermethylated CpG islands being intragenic and 76% of hypomethylated CpG islands mapping to the 5'-end of annotated genes (Figure 2D). The latter reaffirms that aberrant DNA methylation patterns are established immediately after carcinogen exposure, at a time preceding lesion formation (Figure 2D). We also observed that aberrant DNA methylation preferentially targets genes that are normally associated with either active histone mark (H3K4me3) or bivalent marks (H3K4me3 and H3K27me3) in murine embryonic stem cells (ESCs), as established by comparison of our data to published databases (30) (Figure 2E).

Validation of differentially methylated targets by COBRA and bisulfite sequencing
To validate the DNA methylation microarray results, we have randomly selected and analyzed several targets identified by MIRA-microarray analysis using the conventional COBRA (24) and bisulfite DNA sequencing (23). Representative COBRA results for hyper- and hypomethylated targets are shown in Figure 3A and B (upper panels) and Supplementary Figure S1, available at Carcinogenesis Online. In all cases, we confirmed DNA methylation differences between control and experimental samples collected at different time points posttreatment, which mirrored the differences observed in the array analysis. These confirmatory results indicate that epigenetic marks, i.e. CpG methylation, are established or lost during B[a]P-induced...
Fig. 3. Representative gene targets identified by MIRA-microarray analysis. (A) Top: genomic DNA from B[a]P-treated and control mice was treated with sodium bisulfite, and the hypermethylated CpG island downstream of the Cacng7 gene was amplified with gene-specific primers and subjected to COBRA. Digested fragments on the gel are indicative of methylated restriction sites within the CpG island. In vitro methylated mouse genomic DNA served as positive control (Pos). The symbols (+) and (−) show the presence and absence, respectively, of the restriction enzyme in reaction mix. M = 100bp ladder DNA marker. Bottom: the extent of CpG methylation in the Cacng7-associated CpG island was determined by sodium bisulfite sequencing in B[a]P-treated mice at T0 and T2 and control (C). The methylation status of precursor lesions (a) and adjacent normal tissues (b) in B[a]P-treated mice at T1 was also compared. The sequencing results of up to 10 independent clones and the respective percentage of methylation per sample are shown. Open and closed circles represent unmethylated and methylated CpG dinucleotides, respectively. MI = methylation index. (B) Top: COBRA analysis of the hypomethylated CpG island located downstream of the Pax2 gene in B[a]P-treated mice. Digested fragments on the gel are indicative of methylated restriction sites within the CpG island. Bottom: the extent of CpG methylation in the Pax2 CpG island was determined by sodium bisulfite sequencing in B[a]P-treated mice at T0 and T2 and control (C). The methylation status of precursor lesions (a) and adjacent normal tissues (b) in B[a]P-treated mice at T1 was also compared. The sequencing results of several independent clones and the respective percentage of methylation per sample are shown.
tumorigenesis. One of such targets is the \textit{Cacng7} gene, whose product plays a role in the synaptic expression of cerebellar alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (31). A normally unmethylated CpG island located downstream of the murine \textit{Cacng7} gene becomes increasingly methylated immediately after treatment with B[a]P (T0), and thereafter (T1 and T2) (Figure 3A, top). A detailed methylation map for each CpG within the \textit{Cacng7} CpG island was also constructed using the bisulfite sequencing technique (Figure 3A, bottom). A steady increase in the median percentage of CpG methylation at this locus was detectable in B[a]P-treated mice, as reflected in the methylation indices of 18, 26, 52 and 60\% for samples of control, T0, T1 and T2 mice, respectively. Despite the substantial methylation increase in the \textit{Cacng7} gene in precursor lesions of B[a]P-treated mice at T1, the methylation indices of this gene in the adjacent normal appearing tissues were comparable with that in solvent-treated controls. We have also verified B[a]P treatment-associated loss of methylation at several loci using both the COBRA and bisulfite sequencing. Figure 3B combines the COBRA and bisulfite sequencing results for a CpG island located 3’-end to the murine \textit{Pax2}, a key gene involved in normal prostate development (32). DNA methylation at this downstream CpG island decreases immediately after the cessation of B[a]P treatment (from 62\% in control animals to 48\% in T0 mice to 19\% in T1 mice) and is virtually lost in tumor-bearing animals at T2 (5\%) (Figure 3B, bottom). Notwithstanding the loss of methylation in \textit{Pax2} in early lesions of B[a]P-treated mice at T1, the surrounding apparently normal tissues had comparable methylation indices to that of DMSO-treated controls (Figure 3B, bottom). We have also detected agglomerate loss of methylation within gene families, e.g. the \textit{Hoxa} and \textit{Hoxb} homeobox gene clusters, a phenomenon commonly found in various types of human cancer (Supplementary Figure S2, available at Carcinogenesis Online) (33). Supplementary Table S2, available at Carcinogenesis Online, lists the hypermethylated and hypomethylated CpG islands identified in samples of mice at T0 and T2.

\textbf{Methylation profiling of major repetitive DNA elements during B[a]P-induced tumorigenesis}

To determine whether in vivo treatment of mice with B[a]P can induce global hypomethylation events, we have analyzed the methylation status of major repetitive DNA elements in the target organ of tumorigenesis in B[a]P-treated mice both before and after tumor development. Using a bisulfite sequencing-based approach (25), we determined the status of CpG methylation in LINE L1, IAP-LTR and SINE B1, which are routinely used as surrogate markers to estimate the overall DNA methylation level in the mouse genome (26–28). As shown in Supplementary Figure S3, available at Carcinogenesis Online, there was a significant reduction in CpG methylation in the IAP-LTR between experimental mice and controls ($P = 0.02$). However, no appreciable differences in CpG methylation were observed in the SINE B1 and LINE L1 between experimental and control mice.

\textbf{Motif discovery}

We next used the Partek\textsuperscript{®} Genomics Suite\textsuperscript{™} Software (v 6.6) to search for common non-redundant sequence instances across the differentially methylated CpG islands in samples of B[a]P-treated mice at T0 and T2. The top-scored \textit{de novo} motifs, identified by Partek analysis, are illustrated in Figure 4A and show an overall prevalence of purine residues (mostly guanines, followed by adenines), often in the context of CpG dinucleotides. Of note, B[a]P epoxy diols (B[a]PDE) are known to react with DNA to form covalent adducts preferentially at the N2 position of guanines, and to a lesser extent, at N6 position of adenines. It is well established that B[a]P-N2-dG adducts form more efficiently at methylated CpGs than non-methylated CpGs (18,19). We have also explored the occurrence of known transcription factor recognition sites within genomic loci targeted by aberrant DNA methylation during chemical carcinogenesis. A list of potential transcription factors with their respective consensus binding sites is shown in Figure 4B. Of interest, the non-histone \textit{HMG-I/Y} transcription factor binds to A-T rich DNA sequences and participates in enhanceosome formation, chromatin remodeling and regulation of transcription, with a crucial role in many cellular processes, including cell growth and differentiation (34). Other potentially relevant transcription factors include the zinc finger protein \textit{Mzf1}, which plays an important role in cell proliferation and tumorigenesis (35), and the forkhead box protein \textit{Foxd3}, a novel epigenetically regulated tumor suppressor gene that controls ESC self-renewal and pluripotency as well as cell growth (Figure 4B) (36). Enrichment of consensus binding sites within the differentially methylated CpG islands indicates that aberrant DNA methylation can also interfere with \textit{in vivo} binding of key transcription factors and/or recruitment of methyl-binding proteins (37).

\textbf{Functional pathway analysis of differentially methylated genes}

Using a combination of the Ingenuity Pathway Analysis\textsuperscript{®} (IPA\textsuperscript{®}: v 9.0) and the GO Enrichment Analysis in Partek\textsuperscript{®} Genomics Suite\textsuperscript{™} (v 6.6), we obtained gene ontology information for the annotated genes identified as aberrantly methylated by MIRA-microarray analysis. Functional annotation analysis revealed that gene targets involved in connective tissue development and function, embryonic development, cell-to-cell signaling and interaction and nervous system development and function were particularly enriched (Figure 2F and IPA results). These target genes are members of the frequently disrupted signaling networks in cancer, including the MEK/ERK, JAK/STAT3, PI3K/AKT, WNT/β-catenin and Shh cascades (38–40) (Figure 5B and Supplementary Figure S4, available at Carcinogenesis Online). These signaling networks cross-talk to and modulate the Nanog pathway, which is overall the most represented canonical pathway, according to IPA (Figure 5A). The significance of the Nanog pathway in the establishment and maintenance of the pluripotent state and carcinogenesis is increasingly appreciated (see Discussion).

\textbf{Correlation between DNA methylation and gene expression during B[a]P-induced carcinogenesis}

To shed light into the underlying mechanisms of aberrant DNA methylation, we next measured the mRNA levels of major murine methyltransferases including the ‘maintenance’ \textit{Dnmt1} and the ‘\textit{de novo}’ \textit{Dnmt3a} and \textit{Dnmt3b} methyltransferases. As shown in Figure 6A, we detected cumulative loss of expression of \textit{Dnmt3a} and \textit{Dnmt3b} in samples of B[a]P-treated mice both before (T0) and after tumor development (T2), whereas no significant changes were detectable in the expression level of \textit{Dnmt1}. Misregulation of \textit{Dnmt3a} and \textit{Dnmt3b} can interfere with the establishment and maintenance of normal patterns of DNA methylation and, in turn, lead to tumorigenesis (41).

To investigate the impact of DNA methylation on gene expression during chemical carcinogenesis, we have also quantified the transcription level of several functionally important genes that were aberrantly methylated, as identified by our MIRA-microarray analysis. The examined genes are known components of the signaling cascades linked to the Nanog pathway and are potentially relevant for the establishment of a malignant phenotype (Figure 5B). Figure 6 shows the mean normalized expression levels of the \textit{Wnt4} and \textit{Fzd3} (Figure 6B), \textit{Mmp9} and \textit{Erk1} (Figure 6C), \textit{Foxd3}, \textit{Nanog} and \textit{Gata6} genes (Figure 6D) in samples of B[a]P-treated mice at T0 and T2 relative to control. With the exception of the \textit{Gata6} gene, which is upregulated, most of the above genes show overall reduction in transcription levels during B[a]P-induced carcinogenesis.

\textbf{Discussion}

Chemical carcinogenesis has historically been accounted for by genotoxicity, which entails DNA damage, genetic mutations and chromosomal abnormalities (1–3). However, an emerging model of carcinogenicity recognizes that epigenetic alterations can also play an important role in the initiation and progression of cancer, although the underlying molecular mechanisms remain to be elucidated (4–6). To investigate whether epigenetic alterations are a determinant of...
motif discovery analysis of the aberrantly methylated CpG islands. This sequence specificity of the aberrantly methylated targets is consistent with preferential binding of the reactive metabolite of B[a]P, B[a]PDE, to guanines in the DNA (15, 18). The enrichment of guanine residues, often in the context of CpG dinucleotides, in samples of B[a]P-treated mice indicates a high enrichment of guanines, often in the context of CpGs. The 'Sequence Logo' windows graphically display the best motifs found in the hyper- and hypomethylated CpG islands in samples of B[a]P-treated mice at T0 and T2. The height of each position is the relative entropy (in bits) and indicates the importance of a base at a particular location in the binding site. (B) Known motifs were identified by using the JASPAR database in Partek®, based on the number of occurrences above the threshold.

Fig. 4. Summary of motif occurrences across differentially methylated CpG islands in B[a]P-treated mice at T0 and T2. (A) The top de novo motifs were selected using the Partek® Genomics Suite™ software and show high enrichment of guanines, often in the context of CpGs. The 'Sequence Logo' windows graphically display the best motifs found in the hyper- and hypomethylated CpG islands in samples of B[a]P-treated mice at T0 and T2. The height of each position is the relative entropy (in bits) and indicates the importance of a base at a particular location in the binding site. (B) Known motifs were identified by using the JASPAR database in Partek®, based on the number of occurrences above the threshold.

chemical carcinogenesis, we have chronicized changes in the epigenetic landscape during chemically induced carcinogenesis. Accordingly, we have assessed the alterations of the epigenome, as reflected in the patterns of DNA methylation, in mice treated in vivo with a representative chemical carcinogen, B[a]P (15), before and after tumor development. It is well established that rodents exposed to B[a]P develop large aggressive tumors (including sarcoma) at the accessory sex organs (29). Thus, we constructed the whole DNA methylome in the seminal vesicles or tumors formed at this organ site in B[a]P-treated mice (Figure 2A). Genome scale analysis, supported by standard validation assays, showed locus-specific hyper- and hypomethylation in B[a]P-treated mice, even at a time preceding detectable lesion formation. Of the 123 aberrantly methylated loci identified in the normal appearing seminal vesicles in B[a]P-treated mice at T0, a significant portion (72%) overlapped with those identified in tumor-specific differentially methylated targets in T2 mice (Figure 2A–C).

De novo motif discovery analysis of the aberrantly methylated CpG islands, in samples of B[a]P-treated mice at T0 and T2, indicates a high enrichment of guanine residues, often in the context of CpG dinucleotides (Figure 4A). This sequence specificity of the aberrantly methylated targets is consistent with preferential binding of the reactive metabolite of B[a]P, B[a]PDE, to guanines in the DNA (19). Given the fact that these epigenetic changes manifest early and in the absence of evident morphological abnormalities, it is tempting to speculate that these alterations are the initiating oncogenic events, directly related to the effect of carcinogen exposure, although a causal relationship remains to be established. At this time, we cannot exclude that other carcinogen-induced epigenetic effects, such as histone modifications, chromatin remodeling and/or microRNA gene modulation, may have triggered the aberration of DNA methylation patterns observed in the present study. Alternatively, it is plausible that the detected epigenetic changes are the result of B[a]P genotoxicity, e.g. mutations in crucial genes that can directly or indirectly influence key pathways involved in the establishment and maintenance of DNA methylation patterns. Of relevance, we observed downregulation of the 'de novo' Dnmt3a and Dnmt3b methyltransferases (Figure 6A), which can lead to global loss of DNA methylation. In confirmation, we have found demethylation of the IAP-LTR repetitive elements in tumors of B[a]P-treated mice (Supplementary Figure S3, available at Carcinogenesis Online) (41). The IAP retrotransposons are often associated with 'metastable epialleles' in mouse, with varying methylation at specific CpG sites that can be influenced by in utero and/or early life exposure to environmental toxicants (6). Furthermore, we have detected agglomerates of differentially methylated CpG islands along extended chromosomal regions containing groups of consecutive genes, such as the Hoxa and Hoxb gene clusters, in B[a]P-treated mice both before
Fig. 5. Functional pathway analysis of aberrantly methylated genes in B[a]P-treated mice prior to (T0) and after (T2) tumor development. (A) The Canonical Pathway Heat Map was generated using the Comparison Analysis in IPA®. The heat map visualizes the enriched canonical pathways simultaneously in T0 and T2, allowing a direct comparison between the two datasets. The pathway scores are displayed using a blue color gradient, where darker blue corresponds to higher scores. The score represents the negative log of the $P$ value derived from the Fisher’s exact test. (B) The top canonical pathway (Nanog pathway) is crucial in mammalian ESC pluripotency and appears to play a role in chemical carcinogenesis. Aberrant DNA methylation targets several components of the signaling cascades that modulate Nanog expression and increases progressively from T0 to T2. Red and green nodes represent hyper- and hypomethylated gene targets identified by MIRA-microarray analysis, respectively. Adapted from IPA with some modifications.
and after tumor development (Supplementary Figure S2, available at Carcinogenesis Online). Agglomerative aberrant DNA methylation is an epigenetic signature commonly found in several types of human malignancy (33), cancer cell lines and other toxicant-induced models of cell transformation (42). Our overall data confirm that known hallmarks of human cancer, including locus-specific gain/loss of CpG methylation and global loss of methylation in repetitive DNA elements, are key components of chemically induced carcinogenesis.

In the present study, the identified tumor-specific aberrantly methylated genes colocalize mostly with the murine stem cell active histone marker (H3K4me3), and to a lesser extent, with the bivalent histone marker (H3K4me3 and H3K27me3), as deduced by comparing our methylation data with the published mouse databases (30) (Figure 2E). Nearly half of the annotated targets associated with the H3K4me3 active histone mark are phosphoproteins, mostly involved in intracellular signaling cascades. This is consistent with the functional pathway analysis that shows the convergence of top aberrantly methylated targets at the MEK/ERK, JAK/STAT3, PI3K/AKT, WNT/β-catenin and Shh signaling cascades. The annotated targets associated with bivalent marks are mainly involved in the control of transcription and pattern formation and include several developmental regulators. In agreement with findings by others (43,44), we have also found that 10% of the tumor-associated hypermethylated CpG islands overlap with loci that are bound by components of the polycomb repressive complex 1 (PRC1) (Rnf2 and Phc1) and PRC2 (Eed and Suz12) that are associated with transcriptionally silent chromatin in ESCs (45). Together, these data indicate an interplay between aberrant DNA methylation and histone modifications that shapes the epigenetic landscape during chemical carcinogenesis.

Gene ontology analysis of tumor-specific differentially methylated targets shows enrichment of genes involved in connective tissue development and function, embryonic development and organ development (Figure 2F and IPA results). The vast majority of these genes are normally expressed in the brain, a trend described in several human malignancies. The top canonical pathway whose members are preferential targets of aberrant DNA methylation is the Nanog pathway (Figure 5A). Nanog is a well-studied transcription factor that plays a crucial role in the maintenance and self-renewal of undifferentiated ESCs (46). Nanog expression is normally restricted to pluripotent cells and is downregulated upon differentiation; however, Nanog has also been found to be dysregulated in cancer cell lines and tumors (47). Accumulating evidence supports that Nanog and other pluripotency genes can function as neoplastic engines to drive tumorigenesis, probably promoting infinite self-renewal of a distinct subset of stem-like cells within tumors (46,48). Interestingly, we have observed methylation defects in genes upstream of Nanog, particularly in

Fig. 6. Relative quantification of gene expression by standard qRT–PCR. The expression status of major methyltransferases and that of individual target genes identified by MIRA-microarray analysis was analyzed by standard qRT–PCR using the 2^ΔΔCt method. Bars represent the mean normalized expression (±SD) of three replicates in samples of B[a]P-treated mice at T0 (gray) and T2 (black) relative to control. Data were normalized using an endogenous housekeeping gene as the reference (Gapdh) and untreated control as the calibrator (with expression equal to 1). Relative transcription levels of Dnmt1, Dnmt3a and Dnmt3b (A), Wnt4 and Fzd3 (B), Mapk3 (Erk1) and Mapk11 (C) and Fodd3, Nanog and Gata6 genes (D) are shown.
components of the oncogenic signaling cascades, including the MEK/ERK, JAK/STAT3, and WNT/beta-catenin, etc., which modulate Nanog expression.

Consistent with DNA methylation data, standard qRT–PCR analysis confirmed abnormal and deregulated expression of several components of the signaling cascades interconnected with the Nanog pathway, which may altogether cause perturbations in cell fate decision and differentiation and, ultimately, lead to cancer (Figure 6B–D). Apart from altering the expression of key genes (i.e., transcription factors), aberrant DNA methylation can also disrupt their respective genomic binding sites, thus, interfering with regulation of downstream effectors. For instance, epigenetic downregulation of the Foxd3 gene (Supplementary Table S2, available at Carcinogenesis Online and Figure 6D) and functional inactivation of Foxd3 recognition sites by aberrant DNA methylation (Figure 4B) can both contribute to loss of Foxd3 tumor suppressive function. Of note, ~10% of the cancer-related hypermethylated genes identified in this study are potential binding targets of Nanog in murine ESCs (49). Our findings accord with a recent report by Varley et al. (50) who demonstrated that cancer-specific hypermethylation is enriched at sites bound by Nanog in human ESCs.

In summary, our study indicates that in vivo exposure of mice to a prototype chemical carcinogen can alter the epigenetic landscape in a similar fashion to that found in human cancer. The alterations of the epigenome, as reflected in the patterns of DNA methylation, progressively increase during chemical carcinogenesis. Members of the Nanog pathway, which establishes and maintains pluripotency in the mammalian ESCs and possibly triggers uncontrolled proliferation of neoplastic cells (46), are preferential targets of aberrant DNA methylation during chemical carcinogenesis. Moreover, top gene networks targeted by aberrant DNA methylation are components of the signaling cascades that cross-talk to Nanog and are frequently disrupted in human cancer. Altogether, our data show the predictive value of aberrant DNA methylation in carcinogenesis and the potential utility of this epigenetic marker for early detection and monitoring of the progression of malignancy. Given the reversibility of this epigenetic change, e.g., through pharmacologic interventions, mitigating aberrant DNA methylation, particularly at the loci and in the pathways identified in the present study, may serve as a therapeutic approach for cancer.

Supplementary material
Supplementary Tables S1 and S2 and Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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References

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