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Deletion of the de novo DNA methyltransferase Dnmt3a promotes lung tumor progression

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Alterations in DNA methylation have been associated with genome-wide hypomethylation and regional de novo methylation in numerous cancers. De novo methylation is mediated by the de novo methyltransferases Dnmt3a and 3b, but only Dnmt3b has been implicated in promoting cancer by silencing of tumor-suppressor genes. In this study, we have analyzed the role of Dnmt3a in lung cancer by using a conditional mouse tumor model. We show that Dnmt3a deficiency significantly promotes tumor growth and progression but not initiation. Changes in gene expression show that Dnmt3a deficiency affects key steps in cancer progression, such as angiogenesis, cell adhesion, and cell motion, consistent with accelerated and more malignant growth. Our results suggest that Dnmt3a may act like a tumor-suppressor gene in lung tumor progression and may be a critical determinant of lung cancer malignancy.

Results

Dnmt3a Deletion Accelerates Tumor Growth. To test the effect of Dnmt3a deletion on lung cancer, we generated mice carrying a conditional K-ras<sup>L-SL-G12D</sup> allele (20) and 2Lox alleles of Dnmt3a (21) (K-ras<sup>L-SL-G12D/Dnmt3a<sup>2Lox/2Lox</sup></sup> (Fig. 1L). Oncogene activation and Dnmt3a deletion were induced by intratracheal infusion with adenoviral Cre recombinase (Ad-Cre) (22).

The lungs of infected animals were removed and prepared for histologic examination at weeks 8, 16, and 24 after Ad-Cre administration. No significant differences in tumor number and size were seen in lungs of animals at week 8. In contrast, Dnmt3a-deficient (KO) and WT mice showed a dramatic difference at weeks 16 and 24 after infection. Whereas most tumors in lungs of Dnmt3a WT animals were small (as large as 0.2 cm in diameter), lungs of Dnmt3a-deficient mice were characterized by a significant increase in the number of large tumors (Fig. 1B). This was confirmed on histological sections of the lungs (Fig. 1C). As summarized in Fig. 1D, the average size of Dnmt3a-deficient tumors was approximately four times larger at week 16 and six times larger at week 24 compared with WT tumors. Similarly, the fraction of the total lung area occupied by Dnmt3a-deficient tumors was approximately four times larger than that occupied by WT tumors at weeks 16 and 24 (Fig. 1E). However, the total number of tumors (adenoma and AD) and atypical adenomatous hyperplasia (a pretumor lesion) did not vary significantly between Dnmt3a-deficient and control animals at weeks 8 and 16 (Fig. 1F and Tables S1 and S2). These results suggest that Dnmt3a deficiency does not affect the initiation of K-ras-induced lung tumors but significantly promotes tumor growth.


The authors declare no conflict of interest.

Data deposition: The microarray and methylation data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE32487).

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Deletion of Dnmt3a in Tumors. To verify Dnmt3a protein expression, we performed immunohistochemical analyses on lung sections of Dnmt3a-deficient and WT mice by using an antibody that recognizes the aminoterminal region of the Dnmt3a protein. Fig. 2C shows strong nuclear as well as weak cytoplasmic staining in Dnmt3a WT tumors. In contrast, Dnmt3a-deficient tumors lacked nuclear staining and revealed only weak cytoplasmic immunoreactivity (Fig. 2D). This difference in staining pattern suggests that, although Dnmt3a mutant cells may produce low levels of truncated Dnmt3a protein, they do not express functional protein that can localize to the nucleus, and thus would be unable to methylate genomic DNA.

Dnmt3a Deletion Facilitates Tumor Progression and Shortens Lifespan. To assess tumor progression and malignancy, we classified the tumors into three grades according to tumor differentiation (modified from ref. 23). Grade 1 tumors were well differentiated, and the tumor cells resembled type 2 pneumocytes with small round nuclei and granular cytoplasm. In contrast, grade 3 tumors represented poorly differentiated tumors with marked cellular and nuclear pleomorphism, high nucleus-to-cytoplasm ratio, prominent nucleoli, and numerous mitotic figures. Grade 2 tumors were moderately differentiated, with a degree of differentiation between those of grades 1 and 3. As summarized in Fig. 3A, a significantly higher fraction of tumors in Dnmt3a-deficient animals at weeks 16 and 24 after infection was of grades 2 and 3 compared with tumors in Dnmt3a WT mice (Tables S2 and S3).

We also evaluated the tumor growth pattern. Two types of tumor growth, a solid and a papillary growth pattern, can be distinguished in mouse lung tumor, with the papillary growth pattern being considered as characteristic for advanced tumors (24, 25). In solid tumors, tumor cells proliferate in a nest- or sheet-like fashion, whereas in papillary tumors, the cells grow...
around a fibrovascular core (Fig. 3B). Compared with the solid growth pattern, the papillary structure enables tumor cells to access blood circulation more efficiently, which is critical for tumor growth and progression. Fig. 3B shows that Dnmt3a-deficient mice had a significantly higher percentage of tumors with papillary structure (i.e., papillary tumors) at weeks 16 and 24 after infection than control mice (Tables S2 and S3). Finally, we observed tumor invasion in four Dnmt3a-deficient tumors (Fig. 3C), but no invasion was detected in WT tumors.

Consistent with the high tumor load and the more malignant tumor phenotype, the lifespan of Dnmt3a-deficient mice was significantly shorter than that of Dnmt3a heterozygous or WT mice (Fig. 3D). No significant difference was observed between Dnmt3a heterozygous and WT mice. Autopsies of approximately 70 animals revealed lung tumors as the most likely cause of death.

**Dnmt3a-Deficient Tumors Have a High Proliferation Index.** To investigate the possible basis of the accelerated growth of Dnmt3a-deficient tumors, we examined the cell proliferation and apoptosis indexes by immunostaining for proliferation marker Ki67 and apoptosis marker cleaved caspase-3. Fig. 4 A–C show that significantly more cells per unit area in Dnmt3a-deficient tumors were positive for Ki67 than in WT tumors, indicating that Dnmt3a mutant tumors were characterized by a higher proliferation index. In contrast, cells positive for cleaved caspase-3 were rare, and no significant difference was detected between Dnmt3a-deficient and WT tumors (Fig. 4D). These results suggest that the increased growth of Dnmt3a-deficient tumors is a result of increased proliferation rather than decreased apoptosis.

**Gene Expression and DNA Methylation in Dnmt3a-Deficient Tumors.** To gain insights into overall gene expression changes, we performed transcriptional profiling analyses by using whole mouse genome microarrays. A comparison between 12 pairs of Dnmt3a-deficient and WT tumors revealed that mRNA abundance of approximately 1,970 genes was significantly different between Dnmt3a-deficient and WT tumors [false discovery rate (FDR) < 0.05] with approximately 1,020 genes expressed at a higher and 940 genes expressed at a lower level in Dnmt3a-deficient tumors (Fig. S4 and Dataset S1). Gene Ontology enrichment analysis (26) revealed that the top three biological processes affected most significantly by these differentially expressed genes were blood vessel formation, cell adhesion, and regulation of cell motion (Fig. 5B and Dataset S1). These processes are key steps in cancer progression (27), consistent with the morphological findings that Dnmt3a-deficient tumors appear to be larger and more advanced. Also, no significant difference (using a raw P value threshold of 0.05) of the mRNA abundance was detected in Dnmt1 (P = 0.46) or Dnmt3b (P = 0.34) between Dnmt3a-deficient and WT tumors.

To examine whether Dnmt3a deficiency resulted in genomic methylation changes, we performed an unbiased methylome analysis. Methylated DNA isolated from three Dnmt3a-deficient and two WT tumors was immunoprecipitated and subjected to high-throughput sequencing [methylated DNA immunoprecipitation (MeDIP)-Seq], producing a total of 101 million reads. Because of the CpG density dependency of MeDIP, we only analyzed relative methylation levels comparing the two exact same regions in Dnmt3a-deficient and WT tumors. To identify differentially methylated regions (DMRs), we mapped the reads and scanned the genome in 1-kb windows with 100 bp overlap (Materials and Methods). We observed that 99.6% of the DMRs were less methylated in the Dnmt3a-deficient compared with the WT tumors (Table S4 and Dataset S2). Based on their overall distribution on the genome, a higher than expected percentage of less methylated regions overlapped gene bodies, particularly exons (Table S4). We further tested all genes and promoter regions for differential methylation (Materials and Methods and Dataset S2) and observed that genes that were less expressed in Dnmt3a-deficient tumors tended to have lower methylation levels in gene bodies (Fig. S1A). In contrast, gene expression changes did not correlate with promoter methylation (Fig. S1B). These results are consistent with a previous report showing a positive correlation between gene body methylation and gene expression in Dnmt3a-deficient neural cultures, suggesting that Dnmt3a-mediated nonpromoter methylation facilitates gene expression (28).

A previous study found methylation changes of repetitive sequences in established Dnmt3a mutant ES cells (29). We therefore performed Southern blot-based methylation assays to compare the DNA methylation status of minor satellite repeats and detected no obvious differences between Dnmt3a-deficient and WT tumors (Fig. S2). This suggests that Dnmt3a-dependent methylation changes of these sequences become manifest only after extensive cell passage and cell proliferation.

**Discussion**

The role of DNA methylation in the APCMin model of intestinal tumors has been well established, with Dnmt3b deletion protecting against tumorigenesis and overexpression increasing tumor load by de novo methylation and silencing of tumor-suppressor genes (9, 10). In contrast, no role of Dnmt3a in
tumor formation has been established. Here we present data supporting this view. Deletion of Dnmt3a in the mutant K-ras lung cancer model affects tumor growth and progression, consistent with an effect on tumor growth and progression.

The methylation status of DNA may affect cancer by several mechanisms: (i) global hypomethylation may increase genomic instability (3, 4), and (ii) hypermethylation of promoters can mediate tumor suppressor gene silencing (5, 9). However, the mechanism of how Dnmt3a affects gene expression and tumor formation is unclear. The genome of embryonic stem cells, in contrast to that of somatic cells, has methylated cytosine residues at non-CpG contexts, which has been suggested to result from the activity of Dnmt3a (30). More recently, whole-genome profiles of DNA methylation at single-base pair resolution of ES cells detected non-CpG methylation in gene bodies, which was positively correlated with gene expression (31). Another study demonstrated that Dnmt3a-dependent gene body methylation correlated with expression of genes involved in neural differentiation (28). Consistent with this observation, we found that gene bodies were less methylated in Dnmt3a-deficient tumors than in WT tumors, which also correlated with lower gene expression. Given that tumors in the mutant K-ras lung cancer model may arise from stem cells giving rise to more differentiated cells in the tumor (32), it is possible that Dnmt3a-dependent gene body methylation may be important for expression of genes that promote differentiation in a fraction of the tumor cells. Although mechanistic insights are lacking, our observation that Dnmt3a-deficient mice harbored more poorly differentiated and more advanced tumors is consistent with the notion that Dnmt3a deficiency interferes with the differentiation process in tumor cells, promoting the formation of less mature and more malignant tumors.

The majority of DNM73a mutations found in myeloid neoplasm patients are missense mutations, and some of the mutations have been shown or predicted to result in reduced translation (14–16). However, because nearly all patients are heterozygous for the mutant allele it is not clear whether the DNM73a mutations have dominant-negative effects or cause hemizygous insufficiency. In this context, our data using a Dnmt3a-null allele are of interest, as they argue against the possibility that hemizygous insufficiency affects lung tumors.

Based on the well established role of de novo DNA methylation-mediated gene silencing in cancer, inhibitors of methyltransferases are being actively investigated (33) and two drugs, azacitidine and decitabine, have been approved by the Food and Drug Administration for treatment of myelodysplastic syndrome (34, 35). Our data raise the possibility that such treatments, in addition to activating silenced tumor-suppressor genes, may have the unintended consequence of inhibiting DNM73a, thereby affecting its proposed tumor-suppressor function. Therefore, it will be of great importance to elucidate the molecular mechanisms of how this gene affects cancer progression. The availability of mouse models of Dnmt3a deficiency will be of great importance to elucidate the molecular mechanisms of how this gene affects cancer progression.
ability of a genetically defined experimental system will greatly facilitate these efforts.

Materials and Methods

Animal Models. K-ras<sub>SL-G12D</sub> mice, which were provided by T. Jacks (Massachusetts Institute of Technology, Cambridge, MA) (20), were crossed with the Dnmt3a conditional KO mice (21) to generate mice with the following genotypes: K-ras<sub>SL-G12D WT</sub>/Dnmt3a<sub>Dnmt3a<sub>fl/fl</sub>/2lox/WT</sub>, K-ras<sub>SL-G12D WT</sub>/Dnmt3a<sub>Dnmt3a<sub>fl/fl</sub>/2lox/2lox</sub>, and K-ras<sub>SL-G12D WT</sub>/Dnmt3a<sub>Dnmt3a<sub>fl/fl</sub>/2lox/2lox</sub>. After Ad-Cre-mediated recombination, mice were generated with lung cells that carried the K-ras mutation and were homozygous (i.e., KO), heterozygous, and WT at the Dnmt3a locus.

Ad-Cre (Gene Transfer Vector Core, University of Iowa) was delivered via an intratracheal approach (22). Briefly, 8- to 12-wk-old mice were anesthetized by i.p. injection of tribromoethanol (0.4 mg/kg body weight) and suspended on a bar of a mouse platform via their front teeth. A Fiber-lite Illuminator was used to illuminate the opening of the trachea, an i.v. catheter (22 gauge) was inserted into the trachea until the top of the catheter was near the mouse’s front teeth, and 60 μL Ad-Cre (5 X 10<sup>9</sup> PFU in MEM containing 10 mM CaCl<sub>2</sub>) was directly delivered into the catheter. In some of the microarray studies, we also used an intranasal infection approach. The procedure was similar to the intratracheal approach except that the virus was directly pipetted over the opening of one nostril of the mouse instead of delivering Ad-Cre via a catheter into the trachea.

Animal care was in accordance with institutional guidance and all animal studies were reviewed and approved by the Committee on Animal Care, Department of Comparative Medicine, Massachusetts Institute of Technology.

Mouse Examination, Tissue Preparation, and Histological Examination. For histological comparison, the mice were euthanized at weeks 8, 16, and 24 after Ad-Cre infection (no. of mice analyzed: week 8, Dnmt3a WT, n = 4; KO, n = 4; three pairs were littermates; week 16, WT, n = 5; KO, n = 5; four littermate pairs; week 24, WT, n = 4; KO, n = 4; three littermate pairs). During dissection, each organ was carefully examined with particular attention to mediastinal structures, especially lymph nodes. Any abnormal tissue was sampled for histological examination. The lungs were fixed by infusion of 10% formalin. After infiltrated by paraffin, the left lobe was trisected and the other lobes were bisected; they were then embedded in paraffin, sectioned (4 μm), and stained by H&E. Thus, each mouse’s lungs were represented by 11 sections that were used for histopathological comparisons. In the survival study, mice were euthanized at end stage (mouse numbers: Dnmt3a WT, n = 29; homozygous, n = 23; heterozygous, n = 24). Before tissue fixation, representative lung tumors and normal tissue were flash-frozen in liquid nitrogen and stored at −80 °C for genomic DNA and RNA extraction. Tumor images were captured and analyzed by Pixel Link Capture SE (Pixel Link).

Immunohistochemistry. Immunohistochemistry was performed by using Vectastain Elite ABC kit (Vector) following the manufacturer’s instructions, with the following primary antibodies: anti-Dnmt3a (sc-20703, 1:200 dil-
lution; Santa Cruz), anti–K–67 (clone TEC-3; 1:20 dilution; DakoCytomation), and anti–cleaved caspase-3 (no. 9661, 1:1,000 dilution; Cell Signaling). Dnmt3a-deficient tumors were performed on 160 tumors in six Dnmt3a-deficient mice and 170 tumors in six WT mice. K–67 and cleaved caspase-3 staining were performed on 57 Dnmt3a-deficient tumors from two mice and 69 WT tumors from two mice.

PCR and RT-PCR. We used regular PCR to detect Cre-mediated recombination in tumors of Dnmt3a KO mice. The sequences of the PCR primers (Fig. 1A) were as follows: sense, 5′-gggctttccctgacagttg3′; anti-sense, 5′-taaattcatacgagggatag3′. PCR program was 95 °C for 2 min, 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 45 sec, 30 cycles; 72 °C for 6 min. Forty tumors with diameters from 0.1 cm to 0.4 cm were tested from nine Dnmt3a-KO mice. Genomic DNA (for large tumors) or tumor lysis (for small tumors) was used as template. Genomic DNA was isolated with AllPrep DNA/RNA mini kit (Qiagen). We used Prism 5 (GraphPad Software) to perform statistical analysis, with two-tailed Student t test for the comparison of tumor number, size, grade, growth pattern, invasion, and fraction of tumor area; Kaplan–Meier survival analysis for comparison of lifespan; and two-way ANOVA for comparison of proliferation and apoptosis indexes. Unless indicated otherwise, 0.05 was used as the P value threshold for statistical significance.

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Statistical Analysis. We used Prism 5 (GraphPad Software) to perform statistical analysis, with two-tailed Student t test for the comparison of tumor number, size, grade, growth pattern, invasion, and fraction of tumor area; Kaplan–Meier survival analysis for comparison of lifespan; and two-way ANOVA for comparison of proliferation and apoptosis indexes. Unless indicated otherwise, 0.05 was used as the P value threshold for statistical significance.

Microarray. Total RNA was isolated from 12 matched Dnmt3a KO – WT tumor pairs from eight pairs of Dnmt3a KO – WT mice. Each pair of mice shared at least one parent. These 12 pairs of tumors included six pairs with tumors (~0.4 cm in diameter) and six pairs of small tumors (<~0.5 cm in diameter). The hybridization was performed on Agilent Whole Mouse Genome 4 × 44K microarrays (two-channel) by Whitehead Genome Technology Core.

DNA Methylation Assays. MeDIP-seq was performed on three Dnmt3a-deficient and two WT tumors by using the Magmedip kit (Diagenode) according to the manufacturer’s protocol. Libraries were sequenced on the genome analyzer II (Illumina). Data analysis was similar to that described by Bock et al. (36).

Details of MeDIP-Seq and Southern blot analysis are provided in SI Materials and Methods.

The two-color microarray raw data were normalized across channels by loess (locally weighted scatter-plot smoothing, using spot quality weights) and then quantile normalized by quantile normalization of average intensities (“Aguantile”) using Bioconductor. Following summarization of replicate probes by median, differential expression was assayed by moderated t test and corrected for FDR, as implemented by the limma package in Bioconductor.

For gene enrichment analysis, we used the Database for Annotation, Visualization, and Integrated Discovery (28) to analyze the differentially expressed genes list between Dnmt3a-deficient and WT tumors (FDR < 0.05).