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Targeted $p16^{ink4a}$ epimutation causes tumorigenesis and reduces survival in mice

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Cancer has long been viewed as a genetic disease; however, epigenetic silencing as the result of aberrant promoter DNA methylation is frequently associated with cancer development, suggesting an epigenetic component to the disease. Nonetheless, it has remained unclear whether an epimutation (an aberrant change in epigenetic regulation) can induce tumorigenesis. Here, we exploited a functionally validated cis-acting regulatory element and devised a strategy to induce developmentally regulated genomic targeting of DNA methylation. We used this system to target DNA methylation within the $p16^{ink4a}$ promoter in mice in vivo. Engineered $p16^{ink4a}$ promoter hypermethylation led to transcriptional suppression in somatic tissues during aging and increased the incidence of spontaneous cancers in these mice. Further, mice carrying a germine $p16^{ink4a}$ mutation in one allele and a somatic epimutation in the other had accelerated tumor onset and substantially shortened tumor-free survival. Taken together, these results provide direct functional evidence that $p16^{ink4a}$ epimutation drives tumor formation and malignant progression and validate a targeted methylation approach to epigenetic engineering.

Introduction
More than 25 years ago, it was proposed that epimutation — mitotically stable gene silencing associated with epigenetic alteration in DNA methylation — can act as 1 of Knudson’s 2 hits required for tumorigenesis (1). In subsequent decades, many promoter CpG island–associated (CGI-associated) genes have been shown to be aberrantly hypermethylated and silenced in various cancers (2). Indeed, recent epigenomic studies revealed that nearly all tumor types harbor hundreds of abnormally hypermethylated promoter CGIs (3), which indicates that epimutations are as common in tumors as genetic mutations. We and others have demonstrated that aberrant promoter CGI methylation is associated with distinct environmental exposures (4), gene mutation patterns (5), cancer prognosis (6), and response to therapy (7). Despite the undisputed importance of DNA methylation in cancer, however, its fundamental role in carcinogenesis remains unclear (8, 9). Most importantly, it remains unknown whether aberrant DNA methylation is a cause of tumorigenesis (8).

Cancer-related promoter CGI hypermethylation originates in normal tissues, which suggests that aberrant methylation could predispose to malignancy (10). $p16^{ink4a}$ (referred to hereafter as $p16$) is a tumor suppressor gene that regulates the ability of retinoblastoma protein to control exit from the G1 stage of the cell cycle (11). Inactivation of $p16$ by promoter CGI methylation is among the most common and earliest epigenetic events in human cancer (12) and is frequently detected in preneoplastic lesions (13–15). Mice provide an apt model in which to study epigenetic dysregulation of $p16$ in cancer; in a mouse model of chemically induced lung cancer, $p16$ methylation is a very early event (16). Moreover, $p16$ age-associated hypermethylation is observed in several normal human and mouse tissues (17–19). Together, these data suggest that epigenetic silencing of $p16$ in aging cells facilitates early abnormal clonal expansion, driving tumorigenesis (20). Directly testing this hypothesis, however, requires the ability to specifically target methylation to the $p16$ promoter CGI.

Results and Discussion
Because cis-acting DNA sequences are important for the establishment of genomic patterns of DNA methylation (21, 22), we set out to engineer a cis element to attract DNA methyltransferases during development to achieve targeted de novo DNA methylation in vivo. We built upon our recent demonstration that an exceptional class of promoter CGIs that are methylated and silenced in normal somatic tissues is associated with specific cis-acting DNA motifs (23). To test the function of the identified motifs in vitro, we constructed a 140-bp cis element containing the top 3 motifs in the order and orientation most frequently observed in the normally methylated CGIs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI76507DS1). We used a stable integrated system to test transgenes targeting 2 human promoter CGIs, one at INSL6 and another at $p16$, for 80 days in cell culture using both human (LNCaP) and mouse (NIH3T3) cell lines. The cis element induced extensive and progressive de novo methylation throughout the promoter CGIs of the juxtaposed transgenes (Supplemental Figures 2 and 3). These positive results prompted us to generate a mouse model with targeted knock-in of the promethylating cis element at the $p16$ locus.

We introduced the cis element approximately 1 kb upstream of the $p16$ transcription start site (TSS) in mouse ES cells (mESCs)
To initially determine whether the knockin cis element specifically induces DNA methylation at the endogenous p16 promoter, we studied mESCs in vitro. Differentiating ESCs recapitulate the earliest stages of embryonic lineage development. We therefore analyzed the progression of DNA methylation before and after induced differentiation (25, 26) in mESCs carrying either knockin allele. To characterize the dynamics of methylation establishment, we performed detailed methylation profiling by quantitative bisulfite pyrosequencing at CpG sites spanning the knockin sequence and the endogenous p16 promoter (–906 to –313 bp relative to TSS).

Prior to differentiation, this region was essentially unmethylated in both control (p16+/ctr-neo) and cis-knockin (p16+/cis-neo) mESCs (Figure 1, A–C). Upon differentiation of p16+/ctr-neo mESCs, a few CpG sites through homologous recombination (Supplemental Figure 4). The insertion leaves the p16 core promoter intact and avoids affecting the p15 promoter of targeted alleles, including CpG maps, are shown above. Ex1a, exon 1a. (C) Examples of bisulfite pyrograms of 3 CpG sites between –615 to –589 bp relative to TSS in p16+/ctr-neo and p16+/cis-neo mESCs before and after differentiation. The y axis represents the signal intensity of luminessence as a measure of nucleotide incorporation, and the x axis shows the dispensation order of nucleotide. CpG sites are shaded yellow, and the percentage methylation (blue boxes) was measured based on the signal intensities of C and T (representing methylated and unmethylated cytosines, respectively) in each C of a CpG site. (D) Clonal bisulfite sequencing analysis of 11 CpG sites (white, unmethylated; black, methylated) between –814 to –589 bp relative to TSS. Each row represents an individual clone. Whereas essentially no promoter methylation was observed in undifferentiated p16+/cis-neo mESCs, extensive methylation was found in differentiated p16+/cis-neo mESCs. (E) Quantitative p16 gene expression analysis showed strong transcriptional suppression in differentiated p16+/cis-neo cells compared with controls. Values are mean ± SD. **P < 0.01, Student’s t test.
Interestingly, methylation at the cis-targeted alleles exhibited clonal heterogeneity. Since our assay was designed to detect methylation at the targeted allele using a forward primer specific for the knockin sequence, our data suggest that the increased methylation occurs in a cell type–specific fashion. Finally, to monitor the functional effects of induced methylation, we assessed p16 expression using quantitative TaqMan real-time RT-PCR. In p16<sup>cis/cis</sup>-neo cells, p16 expression was upregulated during differentiation (Figure 1E), consistent with its functional role in limiting the replicative capacity of stem cells (27). In p16<sup>cis/cis</sup>-neo cells, however, induced promoter methylation during differentiation led to strong transcriptional repression (Figure 1E). p16 expression was restored by treatment with the DNA hypomethylating agent 5-aza-2′-deoxycytidine (DAC) (Supplemental Figure 5A), providing further validation that p16 silencing is not due to unforeseen side effects of the cis element knockin. Importantly, consistent with previous studies (28, 29), we observed concomitant increases in repressive histone markers (H3K9me2 and H3K27me3), with no changes of active markers (H3K9Ac and H3K4me3), across the p16 promoter (Supplemental Figure 5B), which suggests that cis-mediated epigenetic silencing could be mechanistically reinforced by changes in chromatin configuration. Having verified that our engineered cis element specifically induced developmentally regulated promoter methylation and transcriptional silencing, we analyzed p16 methylation in multiple tissues from p16<sup>cis/cis</sup>-neo mice after excision of the Frt-flanked selection marker (Figure 2A). p16<sup>cis/cis</sup>-neo mice were viable and fertile and did not display any developmental abnormalities (Supplemental Figure 6). In agreement with our data in differentiating mESCs, we observed initial methylation seeding within the cis element in almost all mouse tissues at birth (P0) (Figure 2B). The notable exception was testis, in which we detected low methylation at all CpG sites. This was remarkably consistent with our previous observations, since the promoter CGIIs with which the sequence motifs were originally associated are also highly methylated in most tissues, except testis and sperm (23). With aging, cis element–mediated methylation spread toward the endogenous p16 promoter (Figure 2C). In spleen, liver, and colon, normal age-related increases in DNA methylation at the p16 promoter were significantly accelerated by the cis element, with commensurate reductions in gene expression (Figure 2, D and E, and Supplemental Figure 7). Collectively, these results demonstrated that our approach successfully induced developmentally regulated somatic p16 epimutation, leading to transcriptional repression in vivo.

To test whether p16 promoter methylation predisposes animals to tumor development, we used heterozygous intercrossing to generate a cohort of p16<sup>cis/cis</sup>, p16<sup>cis/cis</sup>, and p16<sup>cis/cis</sup>-neo mice (on a mixed 129/C57 genetic background) and monitored tumor development and survival. In mice aged 35–100 weeks, spontaneous tumors developed in 0 of 18 p16<sup>cis/cis</sup> (0%), 3 of 51 p16<sup>cis/cis</sup> (5%), and 6 of 22 p16<sup>cis/cis</sup>-neo mice (27%); *P = 0.02 vs. p16<sup>cis/cis</sup> mice. Of the 3 p16<sup>cis/cis</sup> mice with spontaneous tumor development, 1 developed lymphoma and 2 developed sarcoma (Supplemental Figure 8, A–C). The malignancies in the 6 p16<sup>cis/cis</sup>-neo mice were sarcoma (n = 3), lymphoma (n = 2), and lung adenocarcinoma (n = 1) (Figure 3A and Supplemental Figure 8, D–I). Given that p16 deficiency in many human cancers involves mutation of one allele and promoter hypermethylation of the other (30), we next assessed the cooperative tumorigenic effects of combined...
p16 mutation and epimutation. We bred the p16<sup>−/−</sup> allele into mice on a p16 exon 1a deletion background (31) to generate p16<sup>cis/Δexon1</sup> mice (mixed FVB/129/C57 background; referred to herein as p16<sup>−/−</sup>) mice. In 20 p16<sup>−/−</sup> mice aged 40–91 weeks, 6 (30%) tumors were found; the malignancies were sarcoma (n = 3), lung carcinoma (n = 2), and lymphoma (n = 1) (Figure 3B and Supplemental Figure 9). Moreover, consistent with the notion of p16 epimutation serving as 1 of Knudson’s 2 hits, p16<sup>−/−</sup> mice had accelerated tumor onset and shortened survival (Figure 3C). Our results compare favorably with previous studies that characterized the effects of p16-specific knockout on tumorigenesis (31, 32). Although rigorous comparisons in congenic strains have not yet been performed, the cancer-prone conditions in p16<sup>−/−</sup> and p16<sup>−/−</sup> mice were strikingly similar: tumor spectra were predominantly sarcoma in both, tumor incidence was 30% and 35%, respectively, and tumor latency was 80 and 76 weeks, respectively. In contrast, p16<sup>−/−</sup> mice developed significantly fewer spontaneous tumors and exhibited significantly longer tumor-free survival. To ascertain the role of p16 promoter methylation in the oncogenic pathway in vivo, we measured p16 promoter methylation and gene expression in the tumor tissues. Hypermethylation of the p16 promoter in tumors was associated with essentially complete loss of protein expression (Figure 3, D and E, and Supplemental Figures 10 and 11). Taken together, our results provide direct evidence for a driving role of p16 epigenetic silencing by promoter hypermethylation in tumor formation and progression (Figure 3F).

In conclusion, our present study provides the first clear demonstration that p16 epimutation causes tumorigenesis. Potential applications of this novel mouse model include the testing of targeted epigenetic therapies for prevention and treatment of human cancer. Our straightforward approach to epigenetic engineering should be useful in testing the causal role of other epigenetic alterations implicated in carcinogenesis, and broadly applicable toward elucidating epigenetic etiology in a wide range of diseases.

Methods

Further information can be found in Supplemental Methods and Supplemental Tables 1–3.

Statistics. 2-tailed Student’s t test and Fisher exact test were used to determine the significance of differences. Survival analysis was conducted with Kaplan-Meier analysis and log-rank test. A P value less than 0.05 was considered significant.

Study approval. All animals were treated in accordance with NIH guidelines as approved by the Baylor College of Medicine Animal Care committee.

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