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Fundamental differences in promoter CpG island DNA hypermethylation between human cancer and genetically engineered mouse models of cancer

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Genetic and epigenetic alterations are essential for the initiation and progression of human cancer. We previously reported that primary human medulloblastomas showed extensive cancer-specific CpG island DNA hypermethylation in critical developmental pathways. To determine whether genetically engineered mouse models (GEMMs) of medulloblastoma have comparable epigenetic changes, we assessed genome-wide DNA methylation in three mouse models of medulloblastoma. In contrast to human samples, very few loci with cancer-specific DNA hypermethylation were detected, and in almost all cases the degree of methylation was relatively modest compared with the dense hypermethylation in the human cancers. To determine if this finding was common to other GEMMs, we examined a Burkitt lymphoma and breast cancer model and did not detect promoter CpG island DNA hypermethylation, suggesting that human cancers and at least some GEMMs are fundamentally different with respect to this epigenetic modification. These findings provide an opportunity to both better understand the mechanism of aberrant DNA methylation in human cancer and construct better GEMMs to serve as preclinical platforms for therapy development.

Introduction

Cancer initiation and progression is a multistep process that involves a complex interplay between genetic and epigenetic alterations in a cell.[1] Recent whole genome and exome sequencing studies have identified many genetic alterations in cancer cells, a small subset of which that is frequently mutated across many different types of cancer (such as *KRAS* and *TP53*, termed "mountains"), and a larger number of genetic perturbations that occur at low frequency (termed "hills").[2] Similarly, genome-wide studies of human tumors have demonstrated very large numbers of epigenetic changes, such as aberrant DNA methylation, that contribute to the cancer phenotype. Examples of epigenetic "mountains" that are frequently altered in many cancers include *CDKN2A* and *VHL* inactivation by promoter CpG island DNA hypermethylation.[3] Genes classified as "hills," regardless of whether their function is altered by genetic or epigenetic means, most likely define pathways that require multiple perturbations to drive cancer development.[4]

Genetically engineered mouse models (GEMMs) of cancer have been extensively used to study human cancer biology. Using sophisticated targeting and expression techniques, mouse models have been used as platforms to study drug resistance, early detection, metastasis, cancer prevention and for the preclinical development of novel targeted therapeutics.[5] While most cancer GEMMs have either overexpressed oncogenes or targeted deletions of tumor suppressor genes, very few of these models have examined how epigenetic changes contribute to the cancer phenotype.

We previously found common regions of cancer-specific DNA methylation changes in critical developmental regulatory pathways in primary medulloblastoma patient samples.[6] To determine whether GEMMs of medulloblastoma share a similar pattern of epigenetic dysregulation, we examined genome-wide DNA methylation in three different GEMMs of medulloblastoma.

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Results

Mouse models of medulloblastoma lack extensive promoter CpG island DNA hypermethylation

We initially chose a Shh-driven mouse model of medulloblastoma in which a constitutively activated form of the *Smoothened* gene (*SmoA1*) is expressed within cerebellar granule neuron precursors through the regulation of the NeuroD2 promoter. Medulloblastomas form in approximately 50% of SmoA1 mice by approximately 6 mo of age and show molecular profiles that resemble Shh-driven human medulloblastomas.[7] To detect differential DNA methylation, we performed the denaturation analysis of methylation differences (DAMD) assay, a technique that identifies DNA methylation differences in CpG islands based on the increased melting temperature of cytosine methylated DNA.[6,8] In contrast to the extensive promoter CpG island DNA hypermethylation in human medulloblastoma patient samples observed previously,[6] no DAMD-positive hypermethylated loci were observed in tumors from SmoA1 mice. Cerebella from wild-type mice served as the comparator. To determine if this finding was specific to the *SmoA1* allele, we analyzed a different allele of *Smoothened* (*SmoA2*) that drives medulloblastoma in ~75% of animals by 6 mo of age, and also displays aberrant cerebellar development.[9] Assaying tumors from SmoA2 mice, we again observed no DAMD-positive loci (DAMD data for SmoA1 and SmoA2 accessible via Gene Expression Omnibus database, accession number GSE45342). These results suggest that the extensive promoter CpG island DNA hypermethylation previously observed in patient samples is not present in these two Shh-driven mouse models.

The DAMD assay detects differential DNA methylation based on the increased melting temperature (T_m) of methylated DNA.[6] Because DNA sequence composition also strongly affects the T_m of DNA, the denaturation conditions used in the DAMD assay do not detect differential DNA methylation in low-density CpG regions of the genome. Thus, the lack of DAMD-positive loci in tumor specimens from mice could formally be due to differences in sequence composition between the mouse and human genomes.

To address this possibility, we performed Reduced Representation Bisulfite Sequencing (RRBS), a high throughput sequencing-based method for quantitative, single-nucleotide resolution of cytosine methylation that provides good coverage of CpG islands and promoter regions across the genome.[10] RRBS was performed on a representative human medulloblastoma patient sample, and tumors from three different mouse models of medulloblastoma: the two Shh-driven medulloblastoma mouse models (SmoA1 and SmoA2) previously examined using the DAMD assay, and a MYCN-driven mouse model of medulloblastoma (Glt1-tTA:TRE-MYCN/Luc) that recapitulates the histology of human classic and large cell anaplastic medulloblastoma.[11] For the identification of differentially methylated regions in tumors, human and mouse strain-specific normal cerebella were used as controls. RRBS analysis of the human medulloblastoma patient sample revealed strong promoter CpG island DNA hypermethylation (>60% more methylated than normal cerebellum) of 121 loci (**Table 1**; **Table S1**), while the mouse models of medulloblastoma displayed far fewer loci, ranging from 0 to 16 loci (**Table 1**; **Table S2**). If the stringency for methylation in the tumors was reduced to identify loci with >33% more methylation than the control, the patient sample had 315 additional loci, while tumors from the mouse models of medulloblastoma still demonstrated fewer loci, ranging from 7 to 70 (**Table 1**; **Tables S1 and S2**).

RRBS library characteristics between the human and mouse samples were similar, with the mouse samples having greater CpG coverage (**Table S3**). Metaplots of the RRBS data from both human and mouse promoters containing a CpG island demonstrate the expected pattern of relative protection from CpG DNA methylation of the transcriptional start site (TSS) in both tumor and normal cerebellum (**Fig. S1**). When the subset of promoters displaying CpG island DNA hypermethylation or hypomethylation are similarly analyzed, the TSS continues to show relative hypomethylation (**Figs. S1B and C**).

Traditional bisulfite sequencing of representative loci confirmed the results obtained by RRBS. In the human sample, loci that had >60% methylation in the tumor displayed extensive CpG methylation, while normal cerebellum was largely unmethylated at those loci (*GPR75*, *MIR155HG*, and *TMEM176B_A*; **Fig. 1A**). Good agreement between RRBS and traditional bisulfite sequencing was also obtained for loci that displayed 33–59% methylation in the human samples (*SALL4*; **Fig. 1A**), as well as the SmoA2 tumor and normal cerebellum (**Fig. 1B**). However, the mouse samples did not show the same degree of dense methylation as was present in the human samples with the exception of one locus (*1810013D10Rik*) from SmoA2 that demonstrated a pattern consistent with one allele of the gene being densely methylated, while the other allele was completely unmethylated (**Fig. 1B**).

Although the RRBS identified loci in the mouse models of medulloblastoma showed only modest changes in CpG island methylation, a few common methylated loci (>33% hypermethylation) were identified among the different mouse tumor samples. One region showed increased CpG island methylation in all of the mouse tumor samples, and this locus encompasses two genes (*Cbln1* and *Gm2694*) that are divergently transcribed and share a common promoter CpG island (**Fig. S2**). *Cbln1* encodes for Cerebellin precursor protein 1, a secreted glycoprotein essential for normal synaptic structure and function in the cerebellum,[12] while *Gm2694* encodes a protein of unknown function. As shown in **Fig. S3A**, the RRBS data demonstrated minor differences in DNA methylation between the tumors and normal cerebellum control at this locus. Common to SmoA1, SmoA2, and one of the MYCN derived tumors was *Grm4*, encoding a type 4 metabotropic glutamate (mGlu4) receptor. mGlu4 receptors are expressed by cerebellar granule precursor cells (CGPs), and activation of these receptors reduces proliferation and enhances differentiation of CGPs.[13] A previous study also demonstrated that activation of mGlu4 receptors reduces medulloblastoma cell proliferation by inhibiting the PI-3-K pathway.[14] Lastly, common to SmoA2 and one of the MYCN tumors was *Map3k6*, a proapoptotic gene that has been shown to be a tumor suppressor in a keratinocyte tumorigenesis model.[15] There was only one methylated

Table 1. DNA methylation of promoter CpG islands in human and mouse medulloblastoma

CpG methylation tumor vs. normal	Genes with promoter CpG islands				
	Human	Mouse			
	MB	SmoA1	SmoA2	MYCN[1]	MYCN[2]
>60% hyper	121	0	5	15	16
33–59% hyper	315	7	53	70	67
1–32% hyper	313	34	120	85	154
1–32% hypo	8	140	21	6	5
33–59% hypo	15	36	29	10	12
>60% hypo	10	3	2	6	2

Promoter CpG islands are defined as ±2 kb of a gene's transcriptional start site. Analysis of regions of the autosomal genome with a statistically significant difference in DNA methylation of P value < 1 x 10^{-9}. Methylation percentages are reported as tumor compared with normal cerebellum. MYCN[1] and MYCN[2] refer to tumors from two independent animals.

(>33%) locus shared between any of the mouse medulloblastoma tumors and the patient sample. Common to SmoA2 and the patient sample was *FAM83F* (family with sequence similarity 83, member F), a gene of unknown function. This locus showed a modest increase in DNA methylation in the mouse tumors compared with the DNA hypermethylation observed in the patient sample (**Fig. S3B**). Therefore, although minor methylation differences can be found at a small number of mostly non-overlapping loci in mouse models of medulloblastomas, the mouse models do not display the strong promoter CpG island hypermethylation of a large set of shared loci present in human tumors.[6] However, the limited number of affected genes in the mouse models may be of interest for future studies in medulloblastoma biology.

Mouse models of Burkitt lymphoma and breast cancer do not display extensive promoter CpG island DNA hypermethylation

Given our findings examining promoter CpG island DNA hypermethylation in mouse models of medulloblastoma, we wanted to determine whether this phenomenon was common to other GEMMs. We performed the DAMD assay on tumors from the Eμ-Myc mouse model of Burkitt lymphoma[16] and the MMTV-rtTA/TetO-NeuNT transgenic mammary carcinoma model.[17] Similar to our findings examining mouse models of medulloblastoma, we did not detect any DAMD-positive loci (DAMD data available via accession number GSE45342), suggesting that the extensive promoter CpG island DNA hypermethylation observed in human cancers is not a common feature of mouse models of cancer.

Previously published studies of breast cancer patient samples have demonstrated extensive promoter CpG island DNA hypermethylation,[18-21] For Burkitt lymphoma, however, data are more limited. Candidate gene studies[22,23] and an array-based examination of a small subset of the genome[24] suggested that promoter CpG island DNA hypermethylation can occur in Burkitt lymphoma. Since we did not have access to Burkitt lymphoma patient samples, we performed the DAMD assay on two human Burkitt lymphoma cell lines, Daudi and Raji. While the long-term propagation of cancer cell lines in culture can lead to increased promoter CpG island DNA hypermethylation, genome-wide DNA methylation studies in cell lines can still inform on cancer biology.[25] In contrast to Eμ-Myc mice, extensive promoter CpG island DNA hypermethylation was observed for Daudi (1148 loci) and Raji (1075) with a high degree of overlap (945 loci; **Fig. 2A**; **Table S4**). Traditional bisulfite sequence analysis confirmed that the DAMD-positive loci represented regions of DNA hypermethylation (**Fig. 2B and C**). Taken together with the previously published reports of Burkitt lymphoma and breast cancer patient samples, these results suggest that human medulloblastomas, Burkitt lymphomas, and breast cancers display aberrant promoter CpG island DNA hypermethylation, whereas this epigenetic change is greatly diminished, or absent, in their corresponding genetically engineered mouse models, indicating a fundamental epigenetic difference between the mouse models and human disease.

Mouse fibroblasts passaged through crisis obtain extensive genome-wide promoter CpG island DNA hypermethylation

Previous work over 20 y ago demonstrated that immortalizing mouse fibroblasts could lead to an increase in DNA methylation of the CpG island encompassing the *MYOD1* gene.[26] Experiments using human cell lines have shown that genome-wide DNA methylation of promoter CpG islands increases during the process of immortalization,[27,28] and most recently, this result was confirmed in mouse embryonic fibroblasts.[29]

To determine whether the genome-wide DNA hypermethylation of promoter CpG islands that occurs in mouse cells during the process of immortalization is more similar to human cancers than the modest methylation changes observed in GEMMs, we created newly immortalized mouse fibroblasts by serially passaging primary cells in vitro and selecting clones that spontaneously acquired the ability to bypass replicative senescence.[30] The DAMD assay was performed on two independent newly immortalized mouse fibroblast cell lines (iMF1 and iMF2), and two well-established mouse fibroblast immortalized cell lines (3T3 and 10T1/2); an early passage culture of non-immortalized mouse primary fibroblasts served as a control. When compared with the early passage mouse primary fibroblast culture, all of the immortalized mouse fibroblasts displayed extensive promoter CpG island DNA hypermethylation of a large number of loci (3T3, 2071 loci; 10T1/2, 1196; iMF1, 1158; iMF2, 915), similar

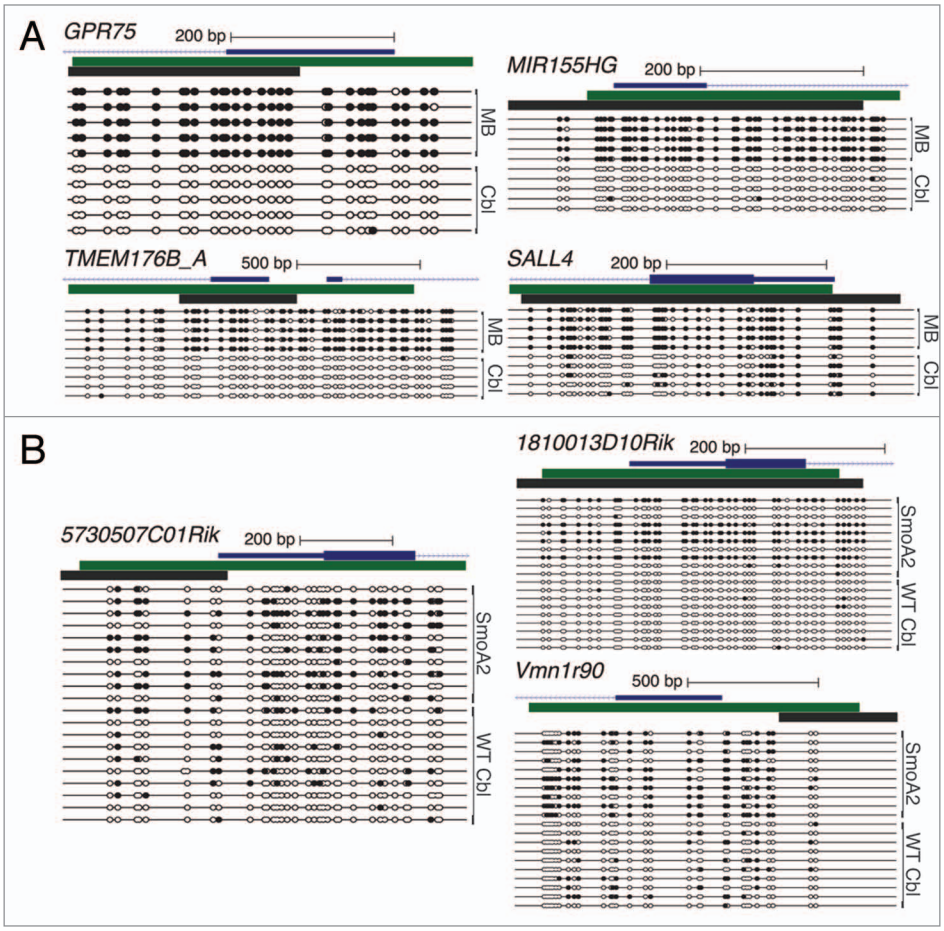

Figure 1. Bisulfite sequence analysis of loci identified by Reduced Representation Bisulfite Sequencing from human and SmoA2 medulloblastoma. (**A**) Bisulfite sequence analysis of *GPR75*, *MIR155HG*, *TMEM176B_A*, and *SALL4* from human medulloblastoma (MB) and normal cerebellum (Cbl). The black rectangle shows the genomic region subjected to bisulfite sequence analysis; the mRNA structure (exon, large rectangle; intron, thin line; untranslated region (UTR), small rectangle; arrow, direction of transcription) is shown in blue; any associated CpG island is shown using a green rectangle. Solid circles represent CpG methylation, and open circles depict unmodified CpG dinucleotides. (**B**) Bisulfite sequence analysis of *5730507C01Rik*, *1810013D10Rik*, and *Vmn1r90* from SmoA2 and wild-type cerebellum (WT Cbl).

to the dense hypermethylation in human cancers. Many of these loci were common to all four of the immortalized mouse cell lines (770 loci; **Fig. 3A**; **Table S4**), and bisulfite sequence analysis confirmed that these loci displayed DNA hypermethylation (**Fig. 3B and C**). These results demonstrate that mouse cells are capable of undergoing extensive promoter CpG island DNA hypermethylation, similar to what is observed in human cancers, when they are spontaneously immortalized. Furthermore, this finding implies that GEMMs of cancer do not activate a similar process of immortalization-associated CpG island hypermethylation.

Discussion

We examined genome-wide DNA methylation in GEMMs, with a primary focus on examining promoter CpG island DNA hypermethylation in primary tumor samples from mouse models of medulloblastoma. In contrast to the extensive DNA hypermethylation of these regions of the genome observed in patient samples,[6] relatively few promoter CpG islands in three different mouse models of medulloblastoma displayed this epigenetic modification, and those loci did not show the same degree of hypermethylation seen in human cancers.

A very limited number of genome-wide DNA methylation studies of GEMMs of cancer have been reported. One of these examined genome-wide DNA methylation in the same genetically driven Eμ-Myc mouse model of Burkitt lymphoma used in our current study.[31] Robust promoter CpG island DNA hypermethylation of individual genes was not described, but rather only a trend toward increasing promoter CpG island DNA hypermethylation could be discerned when data for all genes were analyzed in aggregate. A separate study in Eμ-Myc mice demonstrated a modest increase in DNA methylation by transgenic expression of *DNMT3B7*, a truncated isoform of DNA methyltransferase 3B that has been found in human cancer cells.[32] This finding is not cancer-specific, however, since an increase in promoter CpG island DNA methylation was observed by ectopic expression of mouse *Dnmt3b* in normal mouse colon cells.[33]

Mouse cells are capable of obtaining extensive promoter CpG island DNA hypermethylation when they are immortalized, as recently shown[29] and in this study. It is possible that this

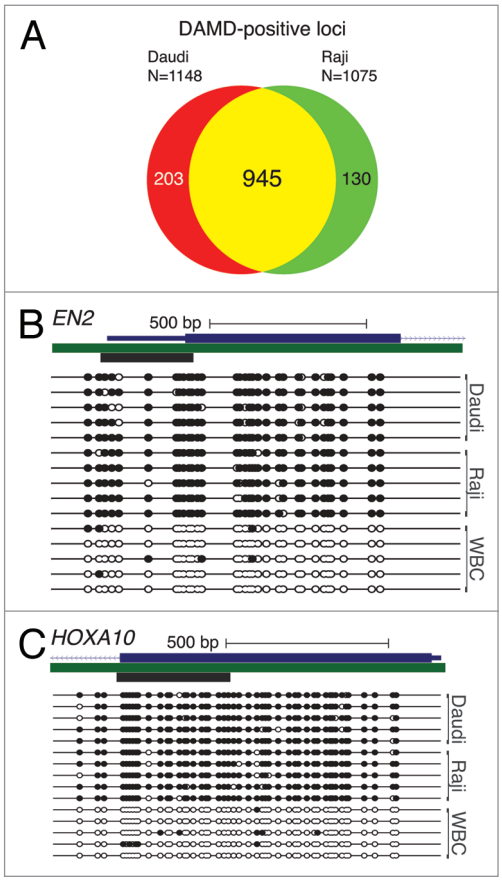

Figure 2. Human Burkitt lymphoma cell lines demonstrate extensive promoter CpG island DNA hypermethylation. (**A**) DAMD-positive loci from the human Burkitt lymphoma cell lines Daudi and Raji are depicted, and common loci are indicated with a Venn diagram. (**B and C**) Bisulfite sequence analysis of *engrailed homeobox 2* (*EN2*) and *homeobox A10* (*HOXA10*) from Daudi, Raji, and peripheral white blood cells (WBC). See **Figure 1** legend for labeling schematic.

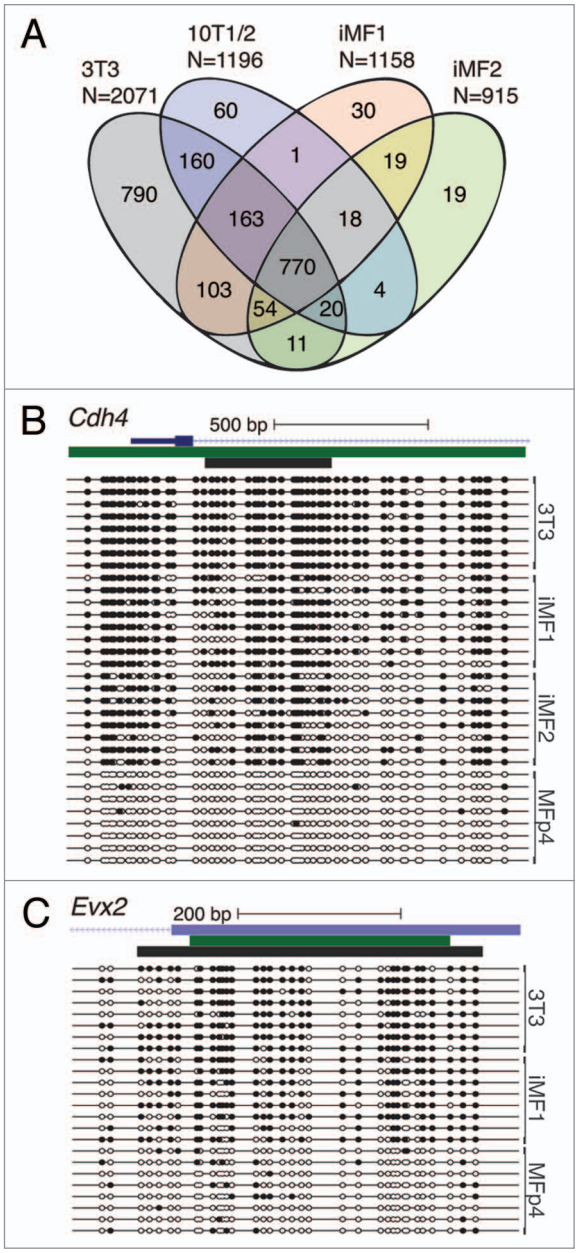

Figure 3. Mouse fibroblast cells obtain global promoter CpG island DNA hypermethylation during the process of spontaneous immortalization. (**A**) DAMD-positive loci from the established mouse fibroblast cell lines 3T3 and 10T1/2, and two independent newly immortalized mouse fibroblast cell lines (iMF1 and iMF2), are depicted. Common loci are indicated on a Venn diagram. (**B and C**) Bisulfite sequence analysis of *Cdh4* and *Evx2* from 3T3, iMF1, iMF2, and an early passage primary mouse fibroblast culture (MFp4). See **Figure 1** legend for labeling schematic.

phenomenon is a result of culturing cells, as it has been observed that progressive promoter CpG island DNA hypermethylation occurs after extensive passaging of cancer cells in vitro. However, while differential CpG methylation has also been observed upon culturing non-transformed cells, these methylation differences occur predominately as unmethylated CpGs in the bodies of genes involved in regulating cellular proliferation, rather then promoter CpG island DNA hypermethylation.[25] Given that extensive promoter CpG island DNA hypermethylation occurs as an early step in the process of immortalization in both mouse and human fibroblasts, these findings suggest that this phenomenon may contribute to the bypass of senescence, an important barrier to cancer development.

While many GEMMs use an oncogenic driver to initiate cancer, chronic inflammatory states have been shown to lead to tumor formation in mice. Patients with inflammatory bowel disease (IBD) are at an increased risk of developing colorectal cancer,[34] and mouse models of this disease recapitulate this phenomenon. Using glutathione peroxidase *Gpx1* and *Gpx2* double-knockout (Gpx1/2-KO) mice, genome-wide DNA methylation studies of tumors and pre-cancerous lesions demonstrated a limited number of loci with moderate degree of promoter CpG DNA methylation.[35] This result, along with our findings in spontaneously immortalized mouse fibroblasts, suggests that the molecular machinery responsible for promoter CpG island DNA hypermethylation is present in mouse cells, but this global epigenetic event is not initiated nor required for tumorigenesis in GEMMs driven by an oncogene, at least not in the five GEMMs

we examined. Cancer arising from inflammatory states, however, may require the initiation of global epigenetic dysregulation in pre-neoplastic tissues to allow for the selection of clones that harbor the prerequisite epigenetic and genetic changes found in a malignant cell. In support of the hypothesis that a global change in DNA methylation may be an early event in inflammatory cancer, it was demonstrated that oxidative damage could cause rapid redistribution of a silencing complex containing DNA methyltransferases and Polycomb members to promoter CpG islands.[36] Future studies to elucidate the differences between GEMMs and human cancers will hopefully give insight into what changes are necessary for human carcinogenesis as well as aid in developing more faithful GEMMs as platforms for preclinical drug testing, especially for those drugs that target the epigenome.

Materials and Methods

Detailed Materials and Methods may be found in **Supplemental Materials and Methods**.

Ethics statement

Pre-existing patient de-identified samples were obtained in accordance with IRB protocol.

Cell culture, mouse strains, and genomic DNA isolation

The human Burkitt lymphoma cell lines Daudi[37] and Raji[38] were maintained in RPMI-1640; mouse fibroblast cells were all propagated in DMEM. Media in all cases was supplemented with 10% FBS and 1% Pen/Strep. Newly immortalized mouse fibroblast cell lines were obtained by serially passaging cells until cell division ceased (approximately at passage 16), and then spontaneously immortalized clones were subsequently isolated.[30] Genomic DNA for RRBS analysis was obtained by growing an isolated clone immediately after immortalization. Mouse medulloblastoma tumors were obtained from SmoA1,[7] SmoA2,[9] and Glt1-tTA:TRE-MYCN/Luc mice.[11] All mice were maintained in accordance with the NIH Guide for the Care and Use of Experimental Animals with approval from the Institutional Animal Care and Use Committee from each institution. Mouse tumor or normal tissue was dissected and snap frozen prior to genomic DNA isolation. Genomic DNA from all sources was isolated using the DNeasy Blood and Tissue Kit (Qiagen, cat# 69504). Genomic DNA from human peripheral white blood cells was obtained from Promega (cat# G3041).

DNA methylation and bioinformatics analysis

The Denaturation Analysis of Methylation Differences (DAMD) assay was performed and analyzed as previously described.[8] Sample library preparation for Reduced Representation Bisulfite Sequencing (RRBS) was performed by service provider (Zymo Research). Briefly, mapping of RRBS reads was performed using BSMAP (v2.74).[39] Methylation analysis was performed using R. See **Supplemental Materials and Methods** for detailed description of the data analysis. Bisulfite DNA conversion, PCR, and sequence analysis was performed as described.[8] Primer sequences can be found in **Table S5**. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession number GSE45342).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/26486

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