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Definition of the Landscape of Promoter DNA Hypomethylation in Liver Cancer

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Abstract

We use hepatic cellular carcinoma (HCC), one of the most common human cancers, as a model to delineate the landscape of promoter hypomethylation in cancer. Using a combination of methylated DNA immunoprecipitation and hybridization with comprehensive promoter arrays, we have identified approximately 3,700 promoters that are hypomethylated in tumor samples. The hypomethylated promoters appeared in clusters across the genome suggesting that a high-level organization underlies the epigenomic changes in cancer. In normal liver, most hypomethylated promoters showed an intermediate level of methylation and expression, however, high-CpG dense promoters showed the most profound increase in gene expression. The demethylated genes are mainly involved in cell growth, cell adhesion and communication, signal transduction, mobility, and invasion; functions that are essential for cancer progression and metastasis. The DNA methylation inhibitor, 5-aza-2′-deoxycytidine, activated several of the genes that are demethylated and induced in tumors, supporting a causal role for demethylation in activation of these genes. Previous studies suggested that MBD2 was involved in demethylation of specific human breast and prostate cancer genes. Whereas MBD2 depletion in normal liver cells had little or no effect, we found that its depletion in human HCC and adenocarcinoma cells resulted in suppression of cell growth, anchorage-independent growth and invasiveness as well as an increase in promoter methylation and silencing of several of the genes that are hypomethylated in tumors. Taken together, the findings define the potential functional role of hypomethylation in cancer. *Cancer Res; 71(17); 5891–903. ©2011 AACR.*

Introduction

DNA methylation is a chemical modification of DNA involved in gene expression programming (1). One of the hallmarks of cancer is aberrant DNA methylation. Three types of aberration in the DNA methylation machinery occur in cancer: hypermethylation of tumor suppressor genes, aberrant expression of *DNA methyltransferase 1* (*DNMT1*) and other *DNMTs*, as well as hypomethylation of unique genes and repetitive sequences (2–4). The role of increased DNMT1 activity and DNA hypermethylation in cancer has been well studied and has been the focus of anticancer therapeutics. Studies have shown that overexpression of *DNMT1* and deregulation of the proper cell-cycle–coordinated expression of *DNMT1* cause cellular transformation (5). On the other hand, knockdown of *dnmt1* by antisense oligonucleotide inhibitors blocks cancer growth (6), and knockout of *dnmt1* protects mice from colorectal cancer (7). The main mechanism of action of DNMT1 inhibitors was believed to be inhibition of DNA methylation and activation of tumor suppressor genes that were silenced by DNA methylation (8), although inhibition of DNMT1 could also induce tumor suppressor genes such as *p21* by a DNA methylation independent mechanism (9). The first DNA methylation inhibitor 5-azacytidine (AC) (Vidaza; ref. 10) was recently approved by the Food and Drug Administration (FDA) for treatment of myelodysplastic syndromes (11). Vidaza is now considered a new and promising approach to cancer therapy.

DNA demethylation in cancer is extensive and therefore was believed to mainly involve hypomethylation of repetitive sequences. Though its role in genes encoding proteins was unclear and the main focus in the field in the last 2 decades has been on the role of hypermethylation of tumor suppressor genes, a few screens for hypomethylated genes in different cancers have revealed several that were characteristically unmethylated in different types of cancer (12, 13). DNMT inhibitors cause DNA demethylation leading to induction of prometastatic genes such as *HEPARANASE* (14) and urokinase-type plasminogen activator (PLAU; ref. 15) and metastasis suggesting a possible role for hypomethylation in cancer metastasis. We previously proposed that the methylated

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DNA-binding domain protein 2 (MBD2) was required for expression and demethylation of *PLAU* and another prometastatic gene *MMP2* (15, 16). *MBD2* depletion by antisense oligonucleotides resulted in silencing of these genes and inhibition of invasiveness and metastasis of breast and prostate cancer cell lines, MDA-MB-231 and PC3, in nude mice (15).

These data suggest that demethylation of protein-coding genes might play an important role in cancer progression and metastasis. To understand the potential role of hypomethylation in cancer it is essential to delineate the landscape of hypomethylation in cancer samples. We therefore focused here on one of the most common cancers, hepatic cellular carcinoma (HCC) and examined the landscape of promoter demethylation in tumor samples. We mapped the hypomethylated sites to the genome to determine how they are distributed across the genome. We defined the functional pathways that are subject to hypomethylation in liver cancer using GO and KEGG databases. To determine how demethylation in liver cancer compares to demethylation in other cancers, we collected and analyzed published methylation profiles of other cancers.

To investigate the role of hypomethylation and its mechanisms in cancer, we used HCC cell line HepG2 and liver adenocarcinoma cell line SkHep1 as model systems. In particular, we knocked down *MBD2* in these cell lines and measured the resulting effect on cell growth, cell invasiveness, and expression of genes shown to be hypomethylated in liver cancer and in primary human liver cells overexpressing *MBD2*. At each step, we show that our results are consistent with the hypothesis that hypomethylation in liver cancer targets promoters of specific genes encoding functional pathways required for cell growth and invasion and that partial reversal of this process results in reversal of tumor growth and invasiveness in liver cancer cells but not in normal liver cells.

Materials and Methods

Patients and tissues

Cancerous and normal adjacent tissue samples were obtained from 11 patients with HCC in Chinese National Human Genome Center at Shanghai, China (Dr. Ze-Guang Han; Table 1). For 3 patients, the cancer samples were dissected using laser capture microdissection technique. All patients provided written informed consent, and the Ethics Committee from Chinese National Human Genome Center at Shanghai approved all aspects of this study.

Cell culture and transfection with siRNA directed to MBD2

Human HCC HepG2 cells and adenocarcinoma SkHep1 cells were authenticated by DNA profile using the short tandem repeat, cytogenetic analysis (G-banding, FISH), flow cytometry, and immunocytochemistry and obtained from the American Type Culture Collection (HB8065 and HTB52, respectively, ATCC) in December 2007 and December 2008, respectively. Human untransformed hepatocytes (normal hepatocytes, NorHep) derived from adult human liver were authenticated by tests for cytochrome P450 and albumin and obtained from Celprogen (33003-02) in September 2007, June 2009 and January 2011. After resuscitation of the received frozen cell ampule, the third, fourth, and fifth passage of cells was frozen, and only these frozen verified stocks were used for further experimental studies up to the twelfth passage (1–2 months). All cell lines were routinely verified by morphology and growth rate. HepG2 and SkHep1 cells were maintained in MEM medium (Gibco, Invitrogen, Life Technologies) supplemented with 2 mmol/L glutamine (Sigma-Aldrich), 10% FBS (Gibco), 1 U/mL penicillin and 1 μg/mL streptomycin (Gibco). NorHep cells were maintained in human hepatocyte cell culture complete medium (Celprogen). Cells were grown and transfected with siRNA as described in Supplementary Methods. The following siRNAs (50 nmol/L final concentration, Dharmacon, Thermo Fisher Scientific) were used in this study: control siRNA (siCtrl, antisense strand: 5′-UCGCCUAGG-CUGCCAAGGCUU-3′) and human MBD2 siRNA (siMBD2, antisense strand: 5′-UUACUAGGCAUCAUCUUUCUU-3′).

Quantitative real-time PCR and Western blot

Total RNA was isolated using TRIzol (Invitrogen, Life Technologies) according to the manufacturer's protocol. One microgram of total RNA served as template for cDNA synthesis using AMV reverse transcriptase (Roche Diagnostics), as recommended by the manufacturer. Quantitative

Table 1. Clinicopathologic characteristics of 11 HCC patients

Patient ID	Gender	Age	Cellular type	Differentiation stage	Size (cm)	Portal vein infiltration
1	Male	48	HCC	Middle	10 × 8	Yes
4	Male	31	HCC	Low	>20	Yes
5	Female	69	HCC	Middle	11 × 9	Yes
6	Male	51	HCC	Middle	12 × 10	Yes
8	Male	43	HCC	High	6 × 5	No
9	Male	50	HCC	Middle	4 × 4	No
10	Male	44	HCC	High	3 × 3	No
11	Male	73	HCC	Middle to low	8 × 8	No
12	Female	51	HCC	Middle to high	6 × 6	No
14	Male	52	HCC	Middle	3 × 3	No
15	Male	31	HCC	Middle	3 × 3	No

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real-time PCR (qPCR) reactions were done as described in Supplementary Methods using primers listed in Supplementary Table S1. The proteins were immunoblotted with an anti-MBD2 antibody at 1:1,000 dilution, followed by a secondary anti-rabbit IgG antibody at 1:4,000 dilution. The anti-MBD2 antibody was developed in our laboratory and is specific for 2 MBD2 isoforms, A and B. The membranes were blotted with an anti-β–actin antibody as loading control (Sigma–Aldrich). See Supplementary Methods for further details.

Pyrosequencing

Bisulfite conversion was done as previously described (17). Specific bisulfite converted promoter sequences were amplified with HotStar Taq DNA polymerase (Qiagen) using biotinylated primers listed in Supplementary Table S2. The biotinylated DNA strands were pyrosequenced in the PyroMark Q24 instrument (Biotage, Qiagen) as previously described (18). Data were analyzed using PyroMark Q24 software.

Methylated DNA immunoprecipitation and promoter methylation microarrays

Purified DNA from cancerous samples and normal adjacent samples as well as HepG2 and NorHep cells was enriched for methylated DNA using the methylated DNA immunoprecipitation (MeDIP) protocol developed by Cedar's group (19). The labeled input and bound DNA samples were hybridized to a custom designed 244 K promoter tiling array (Agilent Technologies) that contained probes covering all transcription start sites at intervals from 800 bp upstream to 200 bp downstream of all genes described in Ensembl (version 44) and within 250 bp of approximately 400 microRNAs from miRBase, all at 100 bp-spacing. The array covered 36,957 transcription start sites corresponding to 18,468 genes. All the steps of hybridization, washing, and scanning were done following the Agilent protocol for ChIP-on-chip analysis. The methods for analysis of promoter methylation microarray are included in Supplementary Methods.

Quantitative chromatin immunoprecipitation

Quantitative chromatin immunoprecipitation (qChIP) was done as previously described (ref. 20; see Supplementary Methods for further details). ChIP DNA was used as a template for qPCR. A total of 25 ng of bound and input DNA was used as starting material in all conditions. Level of MBD2 binding was expressed as (Bound-IgG)/Input. Primers used for ChIP validation are depicted in Supplementary Table S3.

Statistical analysis

Statistical analysis for pyrosequencing and qPCR data was done using unpaired and paired *t* tests. Each value represents the mean ± SD of 2 or 3 independent experiments. The results were considered statistically significant when $P < 0.05$.

Results

Promoter DNA hypomethylation is widespread in HCC

It has been well established that global demethylation of repetitive sequences such as Line1 repeats is a hallmark of several cancers including HCC (21). It was generally assumed that DNA demethylation in cancer occurs mainly in repetitive, CpG-sparse regions of the genome (22) in contrast to DNA methylation that targets CpG-rich islands in promoters and first exons (23). To delineate the scope and landscape of differential DNA methylation between cancer and adjacent normal tissue in the promoters of genes, we subjected DNA prepared from 11 patients with HCC at low to high differentiation stage and a HCC cell line HepG2 and a nontransformed primary human liver cell line (NorHep) to MeDIP and hybridization to a custom-designed 244 K promoter tiling array. Hierarchical clustering of the 500 of the most variable promoters in this data set separates cancer from adjacent normal samples (Fig. 1A). Contrary to the usual emphasis on gene promoter hypermethylation in cancer, there are approximately an equal number of promoters that are hypermethylated (3,517 corresponding to 1,894 genes) and hypomethylated (3,689 corresponding to 1,974 genes). Surprisingly, the NorHep cell line is included in the clustering with the tumor samples and the HepG2 HCC cell line (Fig. 1A). This suggests that a significant fraction of the gene promoters whose DNA methylation levels classifies tumors also undergo methylation changes when nontransformed cells are placed in culture media. Nevertheless, a careful examination of the differences in DNA methylation between normal cultured hepatocytes and HepG2 HCC cells identifies approximately 3,800 differentially methylated promoters that distinguish NorHep from HepG2 cells. Because many of these gene promoters also differentiate liver tumor tissue from adjacent normal tissue, methylation changes in these promoters are likely critical for the state of cellular transformation and cell invasiveness as will be elaborated below. The array data was validated by pyrosequencing (methylation arrays) and qPCR (expression arrays) of several genes as described in Supplementary Methods (Supplementary Fig. S1). We ruled out the possibility that the demethylation observed in our pyrosequencing assays reflects either mutations of C to T or deletions (see Supplementary Results for further details, Supplementary Fig. S2 and Supplementary Table S4).

Methylation profiles were compared with gene expression profiles for the same patients. Overall, genes that are highly expressed in tumors have lower promoter methylation in both normal liver tissue (Fig. 1C) and HCC (Fig. 1D) suggesting that there is an inverse correlation between promoter DNA methylation and gene expression in both normal and cancer tissues. Opposed to this overall correlation is a significant fraction of lowly expressed genes with lower promoter methylation levels and a few highly expressed genes with high promoter methylation.

Hypomethylation targets functional pathways critical for cancer growth and metastasis

From our gene expression and promoter methylation profiles, we identified 230 genes whose promoters contain reduced methylation levels and whose expression levels are elevated in cancer and 322 genes whose promoters contain increased methylation levels and whose expression levels are reduced in cancer (Fig. 1B, Supplementary Table S5). For

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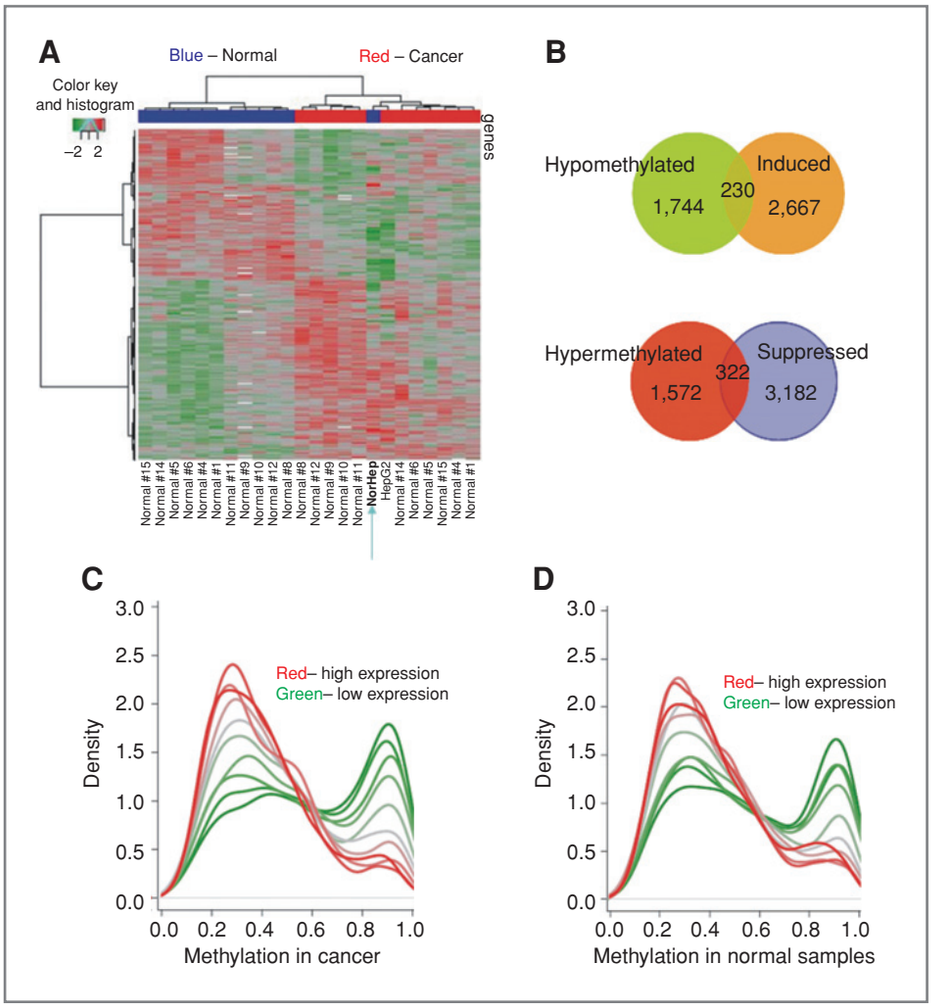

Figure 1. DNA methylation signature of HCC and its association with gene expression. A, a heatmap showing relative methylation levels for the 500 most variably methylated promoters. Hierarchical clustering separates tumor from normal liver (2-tailed *t* test, $P < 0.05$). The heatmap represents raw data in Supplementary Table S10. The white-line gaps in the heatmap indicate that data for the specific probes was unavailable. B, the number of differences in methylation and expression levels between HCC samples and matched adjacent normal tissues. C and D, relationship between promoter methylation and gene expression in tumor samples (C) and normal tissues (D) in 11 HCC patients using promoter methylation and expression microarray data. The *x*-axis is the methylation level estimated from MeDIP microarray data by a Bayesian deconvolution method (see Supplementary Methods) where 0 indicates no methylation and 1 indicates a complete methylation, and the *y*-axis shows the relative number of gene promoters at each methylation level. The promoters were grouped by the expression level of their corresponding genes, and densities are shown for each level with the displayed density colored according to expression level, from dark red indicating the highest expression level to dark green indicating the lowest expressed.

convenience, we call the first set of genes "epigenetically induced" and the latter set "epigenetically suppressed." These results support the hypothesis that promoter hypomethylation might play as important role in modulating gene expression levels in cancer as promoter hypermethylation. Functional analysis reveals that the epigenetically induced genes are enriched in pathways which were reported before to drive cellular transformation, cancer growth, angiogenesis, and cancer metastasis (Supplementary Fig. S3A). It may suggest that these genes are important for liver cancer development and progression, however, this needs to be confirmed in future molecular studies. Further details about these pathways are included in Supplementary Results. In contrast, epigenetically suppressed genes are enriched in signaling cascades that are well known to be suppressed/attenuated in cancer. Most of the identified pathways are responsible for regulation of proliferation, cell-cycle progression, apoptosis and adhesion, all which are essential for normal cell function and integration (Supplementary Fig. S3B).

In general, the epigenetically induced genes are enriched in biological processes that are known to be critical for tumor progression, survival and motility, differentiation, transcription regulation, and signal transduction (Table 2). Many of the epigenetically induced genes in these critical processes are known to play a role in cancer while at least 20 of these genes, to our knowledge, play unknown roles (Tables 2 and 3,

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Table 2. Functional analysis of 111 genes with HCP promoters that are epigenetically induced in HCC patients[a]

Biological processes	Genes
Cell cycle	ARL2, CENPH, CKS2, CSPP1, FAM83D, MAPRE1, PLK1, FOXQ1
Cell differentiation	IFT81, NENF, PPARG, RELB
Cell proliferation and survival	ARF1, ASRGL1, CENPH, CKS2, EXOSC4, FAM83D, IPO7, MAPRE1, NEIL3, NENF, PLK1, POLD3, RAD50, RELB, RRM2
Cell adhesion and cell communication	CASD1, CRELD2, DPP3, DYNLL1, JPH3, MAPRE1, MYO6, PSEN1, RALA, CABYR
Transcription regulation and chromatin remodeling	ASF1B, EXOSC4, MED30, PRPF6, PSEN1, SOX9, TBP, SENP6
Signal transduction pathways	ARF1, ARL2, DPP3, MAP3K4, MATN3, MED30, MYO6, NENF, PLK1, PPARG, PSEN1, RALA, RASAL2, RIOK1, RRM2, SENP6, SLC39A13, FOXQ1, CABYR
Apoptosis	ATAD5, BAG2, DYNLL1, FAIM, MAPRE1, PFKFB2, PPARG, PSEN1, RELB, TBP
Transport	ARF1, ATP1B3, COPG, IPO7, JPH3, MYO6, SLC39A13
Embryonic development	IFT81, NEIL3, PPARG
Immune and inflammatory response	RELB
Lipid metabolic process	GDPD1, PPARG
Transcription factors	MED30, PPARG, RELB, SOX9, TBP, FOXQ1
Oncogenes and cancer antigens	HEATR6, PLK1, RELB, MAGEC1
Metastasis	ENPP4, PPARG, PRPF6, RALA, CKS2, IPO7, MMP14
Invasion	RALA, CSK2, IPO7, TMX2
Migration	ARF1, MMP14
Known to be involved in cancer	ARF1, ASRGL1, CENPH, CKS2, COPG, CRELD2, DPP3, DYNLL1, ENPP4, FAIM, HEATR6, IPO7, MAP3K4, MAPRE1, MATN3, MED30, MMP14, MYO6, ORC6L, PLK1, POLD3, PPARG, PRPF6, PSEN1, RAD50, RALA, RELB, RIOK1, RRM2, SERF2, TRIM8, WHSC1
New candidates	CASD1, CCDC138, CSPP1, FAM83D, EXOSC4, GDPD1, IFT81, JPH3, KCTD2, NEIL3, NENF, PAQR4, PFKFB2, RASAL2, RNMT, SENP6, SMYD5, SRRT, TMX2, WBSCR22

[a]Functional analysis of 111 genes with HCP promoters that were significantly hypomethylated and induced in liver cancer samples compared with matched adjacent normal tissue.

Table 3. Comparative functional analysis of hypomethylated genes in liver cancer that are new cancer associated gene candidates[a]

New candidate pathways	Known pathways in cancer development and progression
Positive regulation of MAPKKK cascade	Phosphorylase kinase and metallopeptidase activity
Histone binding	Histone binding and transcription cofactor activity
Methyltransferase activity	Receptor signaling protein activity
RNA methyltransferase activity	Cell division and cell-cycle progression
Spermatogenesis	Immune response
Base-excision repair	Nucleotide-excision repair and mismatch repair
Monosaccharide, glycerol, and alcohol metabolic processes	Glycogen, energy reserve, polysaccharide, and nucleoside diphosphate metabolic processes
3′-5′-exonuclease activity	Protein *C*-terminus and vitamin D receptor binding

[a]Functional analysis of genes hypomethylated and induced in liver cancer that were not reported previously to be involved in cancer. The genes fall into 2 different functional pathways. The left column lists pathways that were not known to be involved in cancer but are plausible candidates. The right column lists pathways that are known to play a role in human cancer development and progression. Functional analyses were done using GO database.

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Supplementary Results). These 20 genes are enriched in biological processes such as histone binding, positively regulating MAPK pathway, methyltransferase activity, and involvement in base-excision repair, suggesting that these new candidates play a role in liver cancer and are regulated by epigenetic mechanisms.

To determine causal relationship between promoter hypomethylation and activation of these genes in HCC, we determined whether the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-azaCdR) would induce these genes in a primary hepatocyte (NorHep) cell culture. We examined by qPCR the expression of 84 genes following 5-azaCdR treatment of NorHep including 65 with high CpG-dense promoters (see Supplementary Results for a rationale for choosing these genes). Seventy-one of the 84 examined genes were induced upon treatment with 5-azaCdR at 1.0 μmol/L concentration for either 5 or 20 days (Supplementary Table S6, Supplementary Fig. S4B). As expected, 5-azaCdR treatment of NorHep cells led to global hypomethylation (Supplementary Fig. S4A) and a decrease in promoter methylation of *MMP2*, *NUPR1*, *PLAU*, and *S100A5* genes (Supplementary Fig. S4C). Using microarray data for each patient, we also found a correlation between promoter hypomethylation and gene induction for 230 genes epigenetically induced in HCC samples (Wilcoxon rank-sum test, $P \leq 0.005$) indicating that promoter hypomethylation does have impact on the increase in gene expression (see Supplementary Results for details and Supplementary Fig. S4D).

Properties of genes demethylated in liver cancer

We first identified promoters that are hypomethylated in HCC compared with normal liver cells. We then compared the state of methylation of all genes that are targeted for hypomethylation in HCC with the state of methylation of these genes in several normal tissues (24). The heatmap in Figure 2A reveals that the promoters that are hypomethylated in HCC exhibit tissue-specific methylation patterns in normal tissues that is consistent with a role for these genes in cellular differentiation.

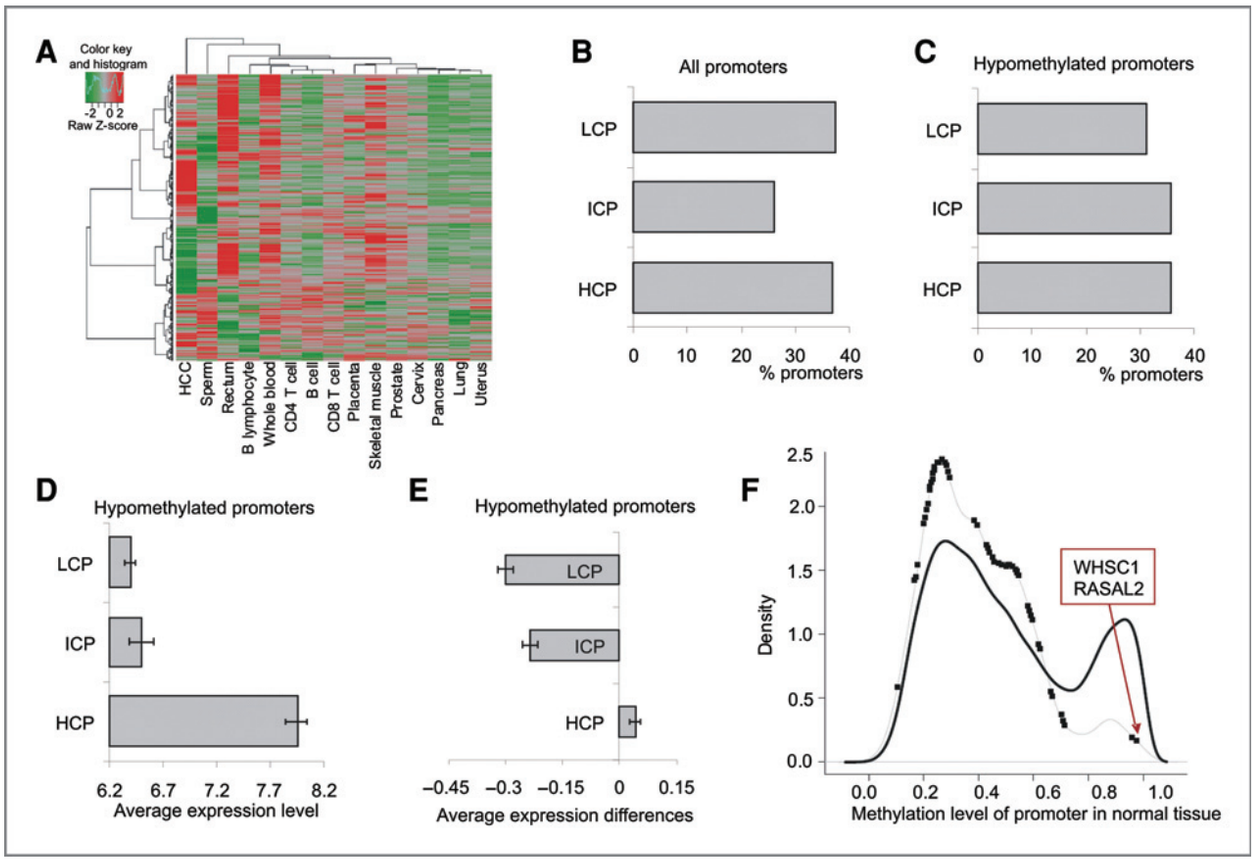

Figure 2. Properties of gene promoters demethylated in HCC. A, heatmap showing promoter methylation levels estimated from microarray data in different tissues corresponding to genes that are demethylated in HCC. The order of the genes in the heatmap is given in Supplementary Table S11. B, percentage of promoters in 3 different CpG density classes, low (LCP), intermediate (ICP), and high (HCP) for all known gene promoters. C, percentage of promoters hypomethylated in HCC in the 3 different CpG density classes. D, expression levels in HCC of genes controlled by hypomethylated promoters in each of the 3 CpG density classes. E, differential expression between cancer and normal tissue of genes [log(cancer)–log(normal)] whose promoters are hypomethylated in HCC in each of the 3 CpG density classes. F, distribution of methylation levels in normal liver estimated from microarray data of all promoters (black line) and HCP promoters that become hypomethylated in HCC (grey line). Black dots on the graph identify the methylation levels of the 100 most significantly demethylated genes with HCP promoters.

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It has been previously suggested that while hypermethylation in cancer targets CpG island-containing promoters, hypomethylation occurs mainly in sparsely distributed CpGs (22, 23). We therefore tested whether there was any discrimination for CpG sparse sequences among the hypomethylated promoters. All promoters were divided into 3 CpG density groups, high (HCP, 37%), intermediate (ICP, 25%), and low (LCP, 38%; Fig. 2B; ref. 25). Our analysis indicates that hypomethylation targets promoters with a wide range of CpG densities, which is consistent with the broad scope of DNA hypomethylation seen in liver cancer (Fig. 2C). As expected the hypomethylated promoters with high CpG density are also highly expressed in tumors (Wilcoxon rank-sum test, $P = 1.05 \times 10^{-9}$; Fig. 2D), and hypomethylation of these promoters in tumors is correlated with the most profound increase in gene expression in HCC as compared with normal liver (average expression differences are > 0, Wilcoxon rank-sum test, $P = 1.37 \times 10^{-5}$; Fig. 2E). We therefore focused on this gene set for further analysis. Our data suggest that CpG rich promoters that are hypomethylated in HCC are methylated at an intermediate level in normal liver (Wilcoxon rank-sum test, $P = 1.32 \times 10^{-38}$) (Fig. 2F, Supplementary Fig. S5), although there are exceptions (see Supplementary Table S7 for a list of genes that are heavily methylated in normal liver and hypomethylated in HCC).

Early reports of changes in DNA methylation in cancer focused on extreme on-off changes. Indeed, there is a small fraction of gene promoters demethylated in HCC that have little or no expression in normal liver and are highly expressed having their promoters hypomethylated in tumors (Supplementary Fig. S5, right panel). This group includes genes that are overexpressed in many types of human malignancies such as members of *MAGE* and *GAGE* families, metalloproteinases and amino acid transporters (26). However, the global picture emerging from our study is that hypomethylation in HCC takes place in promoters that, in normal tissue, are already partially hypomethylated ($P \leq 5.8 \times 10^{-6}$; Wilcoxon rank-sum test that these promoters have lower than average methylation levels) and whose corresponding genes are quite active ($P \leq 4.2 \times 10^{-5}$; Wilcoxon rank-sum test that these genes have higher than average expression). This suggests that overall induction of gene expression and hypomethylation in liver cancer modulates an existing program rather than switching on genes by promoter demethylation. On the other hand, we cannot rule out the possibility that "normal" liver tissue from HCC patients consists of heterogeneous cell populations comprising normal and cancer cells which may affect the results.

A common demethylation signature in liver, breast, and ovarian cancer

As the hypomethylation events in liver cancer affect basic pathways in cancer growth (Supplementary Fig. S3A), we reasoned that several of these events occur in other cancer types as well. To test this, we obtained published tumor and normal methylation profiles for breast, ovarian, and colorectal cancers (27, 28) and computed the methylation differences (tumor over normal) in the 500 most demethylated gene promoters in liver cancer. As expected, although these differences are mainly tumor type specific, several genes are hypomethylated in all selected cancers (Fig. 3A and B). In further analysis, we omitted colorectal cancer since its methylation profiles covered fewer promoters, and we identified 42 gene promoters that are hypomethylated in liver, ovarian, and breast cancers (Supplementary Table S8). For further details about genes that are hypomethylated in different types of cancer see Supplementary Results. Interestingly, although the identity of a specific member of a gene family is cell-type specific, we found that the involvement of a few gene families such as *MAGE*, *SNORA*, *KIAA*, is common to several cancers. We suspect that this might be a general rule for other gene families, pathways, and processes.

Clustering of DNA hypomethylation in liver cancer

Having shown that hypomethylated promoters in liver cancer share several common functions relevant to cancer, we then asked whether the distribution of these hypomethylated promoters in the genome exhibited evidence of some higher-level organization. First, we examined the distribution of hypomethylated promoters at the chromosomal level. We noticed overrepresentation of hypomethylated promoters in 7 chromosomes as shown in Figure 3C (Wilcoxon rank-sum test, $P = 8.25 \times 10^{-5}$). Second, we asked whether the hypomethylated promoters are randomly distributed or whether they form clusters. Several such clusters are identified in Figure 3C. Clustering of differentially hypermethylated promoters in cancer was previously reported in a study comparing breast cancer to normal samples; these clusters were frequently found at gene family clusters (29). Several of our clusters are similarly found at gene family clusters that were previously shown to be important in cancer. Further details about identified hypomethylated clusters are included in Supplementary Results. Beyond local clusters of genes, differential promoter methylation shows evidence of long-range dependencies; in fact, these dependencies are statistically significant ($P < 0.05$) up to a distance of 15 Mbp (Fig. 3D). This indicates a highly large-scale organization of DNA hypomethylation in HCC.

Invasiveness of liver cancer is dependent on *MBD2* expression

To test whether epigenetic regulators could play a causal role in HCC through induction of the genes delineated in this study, we first examined whether the expression of any of the known epigenetic proteins is altered in the tumor samples. Among genes implicated in regulation of DNA methylation, *MBD2* showed the highest induction of expression in the HCC samples (Fig. 4A). MBD2 was previously shown to be involved in the activation and demethylation of *PLAU* and *MMP2* in breast and prostate cancer cell lines (15, 16). Interestingly, our results indicate that *MBD2* expression correlates with the extent of hypomethylation in the individual HCC samples (Fig. 4A, right panel). Although we cannot determine causality in human clinical samples, we can determine whether or not MBD2 plays a causal role in cellular transformation and invasiveness using a human liver cell culture model. We

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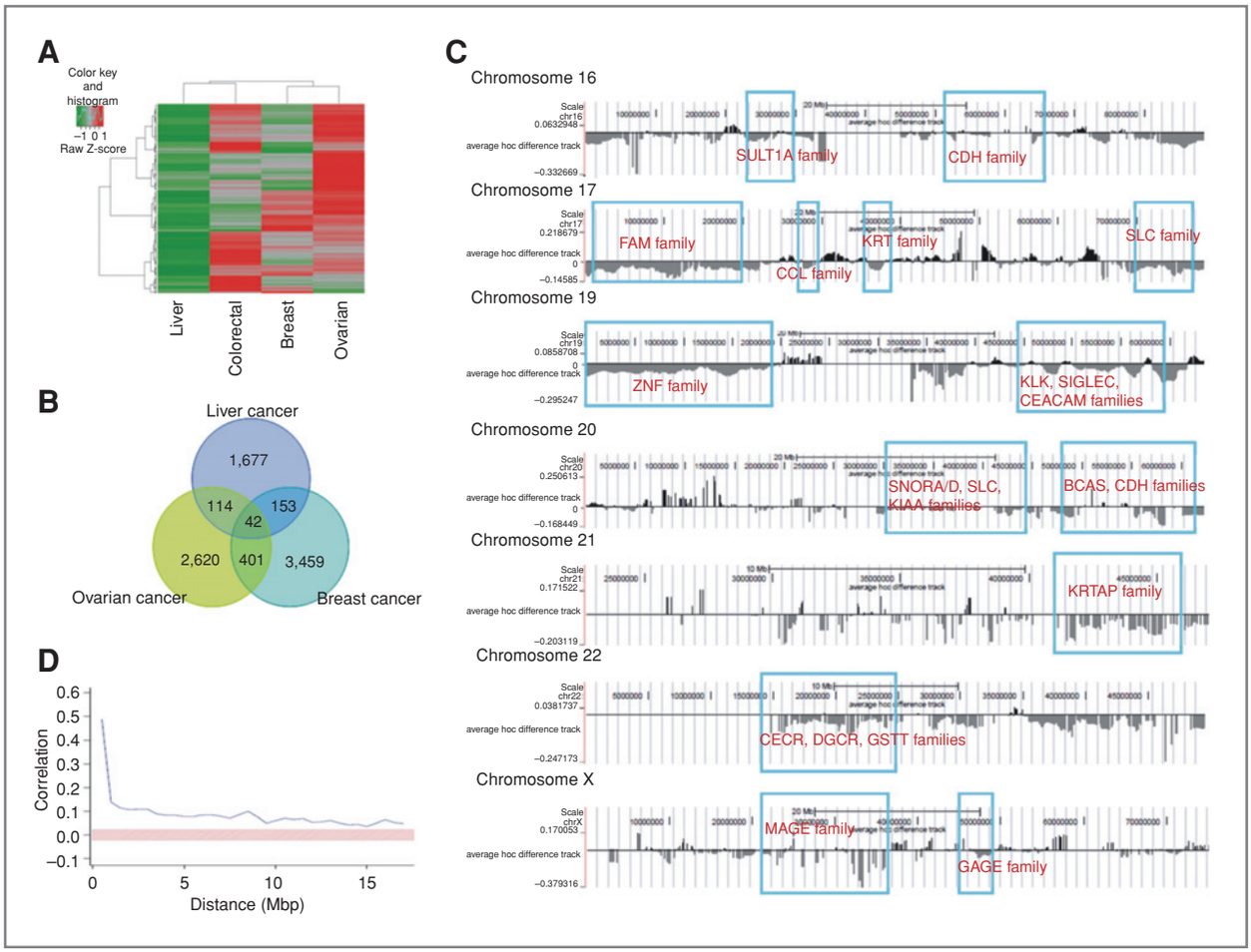

Figure 3. Common hypomethylation signature in human cancers and large-scale genomic organization of hypomethylated clusters in HCC. A, heatmap showing the differential state of methylation (cancer-normal) in liver, ovarian, breast, and colorectal cancers of a list of promoters that are hypomethylated in HCC. The order of genes in the heatmap is shown in Supplementary Table S12. B, Venn diagram showing overlap between the hypomethylated gene promoters in 3 human cancers listed in Supplementary Table S9. C, chromosomal views of the methylation difference between HCC and normal liver. Chromosomes with extensive hypomethylation in HCC are shown. Microarray data were visualized in the UCSC genome browser (44). The tracks show differences in mean methylation between 11 HCC samples and normal liver samples. Downward peaks (below the 0 line) represent lower mean methylation in HCC versus normal liver. The horizontal line represents the 0 point where there is no difference in methylation between the groups. Clusters of gene families that are hypomethylated in cancer are indicated for chromosomes 16, 17, 19, 20, 21, 22, X. Other chromosomes did not exhibit long range DNA hypomethylation. D, differential methylation in cancer correlates up to megabase distances along the genome. By examining methylation differences in all 500 kbp regions a given length apart, we showed that adjacent differential methylation is correlated up to distances of several mega-base pairs. The pink rectangle represents the 95% CI of the background distribution created by randomly permuted probes.

therefore depleted *MBD2* in the liver cancer cell lines, HepG2 and SkHep1, as well as nontransformed normal liver cells (NorHep) to compare the effects of *MBD2* depletion in cancer cells to that in normal cells. Depletion of *MBD2* expression in the liver cancer cell lines (Fig. 4B and C, mRNA; protein) was followed by inhibition of the rate of cancer cell growth (Fig. 4D), anchorage-independent growth and cell invasiveness (Fig. 4E and F).

Reduction of *MBD2* in SkHep1 cells dramatically reduced cell viability prompting us to examine whether *MBD2* depletion triggered apoptosis. We observed a 3-fold increase in apoptosis rate in HepG2 and SkHep1 cells on day 9, and when SkHep1 cells were split during the experiment the rate of apoptosis increased 8-fold on day 6 (Fig. 4G). A possible explanation for this heightened rate of apoptosis is the fact that *MBD2* depletion resulted in downregulation of an apoptosis inhibitor, *BIRC3*, by 80% and 65% in HepG2 and SkHep1, respectively (Fig. 4G). In untreated SkHep1 cells compared with NorHep, *BIRC3* is 10-fold more expressed (Fig. 4G), a fact that might be related to the higher sensitivity of this cell line to *MBD2* depletion.

Contrary to cancer cells, NorHep cells that were treated with siMBD2 proliferated at almost the same rate as control cells and there was no significant effect on invasiveness or anchorage-independent growth (Fig. 4D, E, and F). The basis for this difference is probably a consequence of the fact that there are 2 known isoforms of *MBD2*, *MBD2A* initiating at an upstream ATG and *MBD2B* initiating at a downstream ATG.

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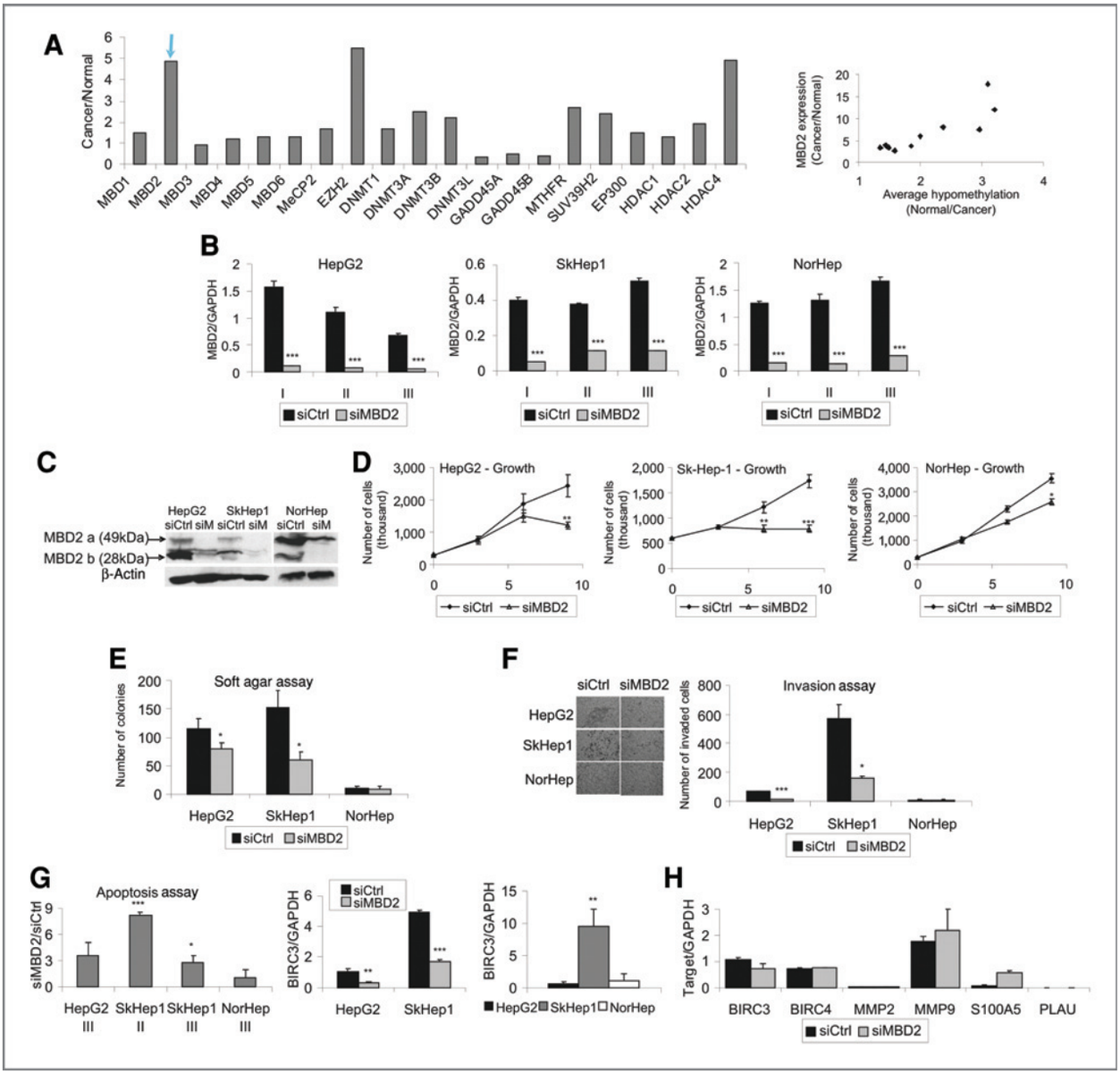

Figure 4. Regulation of cancer growth and invasion by MBD2 in HCC cell lines; selectivity for cancer versus nontransformed cells. A, expression of genes that are implicated in regulation of epigenetic modifications in liver cancer samples compared with matched adjacent normal tissues. A chart in the right panel shows a correlation between *MBD2* upregulation and average level of hypomethylation in HCC patients. *MBD2* expression is higher in HCC samples that show more profound DNA hypomethylation. The *x*-axis shows the average level of hypomethylation in every patient that represents the mean hypomethylation of 9 genes: *AKR1B10*, *CENPH*, *MMP2*, *MMP9*, *MMP12*, *NUPR1*, *PAGE4*, *PLAU*, and *S100A5* as determined by pyrosequencing. The mean hypomethylation in each gene was calculated by dividing the percentage of methylation in normal adjacent tissue by the percentage of methylation in matched cancer sample. The *y*-axis shows a fold change in *MBD2* expression in every patient that was calculated by dividing *MBD2* expression in cancer sample by *MBD2* expression in normal adjacent tissue based on array data. B and C, *MBD2* expression quantified by qPCR (B) after first (I), second (II), and third (III) transfection and by Western blot (C) after third transfection with siCtrl or siMBD2. D, effect on cell growth after first (day 3), second (day 6), and third (day 9) transfection with siCtrl or siMBD2 estimated as described in Supplementary Methods. E and F, effect on anchorage-independent growth and cell invasion as measured by soft agar and Boyden chamber invasion assays, respectively, as described in Supplementary Methods after triple transfection with siRNA. G, effect of *MBD2* depletion on apoptosis in HepG2, SkHep1, and NorHep cells and on *BIRC3* expression in HepG2 and SkHep1 cancer cells. The last chart displays *BIRC3* relative expression in all tested cell lines. Apoptotic assay was done as described in Supplementary Methods after third transfection with siRNA (on day 9). For SkHep1 cells, the number of apoptotic cells was estimated after second transfection (on day 6) when cells were split and after third transfection (on day 9) when cells were cultured without passaging. H, expression of a set of genes in NorHep after *MBD2* knockdown. Contrary to cancer cells, *MBD2* depletion in NorHep does not suppress the selected genes. All results represent mean ± SD of 2 or 3 independent experiments, measured in triplicate ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

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As shown in Figure 4C, the cancer cell lines selectively express higher levels of the B isoform while the normal liver cell expresses the A isoform predominantly. Our data suggest that a similar difference exists at the mRNA level, that the mRNA encoding isoform B is elevated in the cancer cell lines (Supplementary Fig. S6A). In accordance with this hypothesis, specific knockdown of the *MBD2A* isoform in HepG2 cells did not affect cell growth and invasive capacities (Supplementary Fig. S6B–E). Furthermore, because the *MBD2B* isoform was previously reported by us to act as a cytosine DNA demethylase (30), the *MBD2B* isoform may be responsible for the cancer-specific effects of *MBD2* depletion. However, further experiments are required to investigate this hypothesis.

Given that *MBD2* depletion in cancer cells reduces cell growth and invasiveness, we then asked whether or not MBD2 could play a role in inducing the epigenetically induced genes in HCC. Among the 230 genes epigenetically induced in HCC and another 188 genes that were either hypomethylated or induced in both HCC and HepG2, we found 82 genes that were implicated in pathways and biological processes important for regulation of cellular transformation and migration (Supplementary Table S9). Moreover, an experimental upregulation of many of these genes has reportedly led to increased growth and aggressiveness. Of these selected genes, we identified 15 genes that were suppressed after *MBD2* knockdown in HepG2 and/or SkHep1 cells (Fig. 5, Supplementary Fig. S7B). Most of these 15 genes are overexpressed in HepG2 and/or SkHep1 untreated cells compared with NorHep (Fig. 5, Supplementary Fig. S7A). Consistent with the observation that *MBD2* depletion had little effect on NorHep cell growth and invasion, depletion of *MBD2* in NorHep cells did not silence (or induction in the case of *S100A5*) the genes that were suppressed in the cancer cell lines by siMBD2 (Fig. 4H).

MBD2 was previously shown to act as both a transcriptional repressor and an activator of gene expression, and several studies from our lab suggested that it promoted demethylation of its target-activated genes (31). MBD2 might therefore regulate these hypomethylated genes in liver cancer either by interacting with the chromatin at the gene regulatory sequences to activate them or indirectly by suppressing genes that in turn negatively regulate the genes that we found epigenetically induced in liver cancer. We therefore asked if MBD2 binds the promoter regions of 4 of the genes (*MMP2*, *PLAU*, *S100A5*, and *NUPR1*), whether depletion of *MBD2* reduces any such binding, and whether *MBD2* depletion changes their methylation status as tested by pyrosequencing (Fig. 5). Our data show that MBD2 binds these promoters, that *MBD2* depletion results in reduced MBD2 binding (Fig. 5), and that *MBD2* depletion results in 2 to 5-fold increase in methylation at certain CpG sites (see Figure 5B–E for the exact position of the CpGs that change). We further showed that MBD2 upregulation in NorHep cells leads to induction and hypomethylation of several of these genes (see Supplementary Methods and Supplementary Results for details, Supplementary Fig. S8). These studies support the hypothesis that MBD2 regulates several of the genes, that are activated and hypomethylated in HCC and liver cancer cell lines, and therefore likely conducts a similar role in liver cancer.

Discussion

The hypermethylation of CpG islands in promoters of tumor suppressor genes has been the focus of attention in the study of the role of DNA methylation in cancer. This focus has guided as well therapeutic approaches to DNA methylation in cancer. Unsurprisingly, the first DNA methylation drug that the FDA approved for cancer therapy was 5-aza-cytidine, a DNA methylation inhibitor (11). Although it has been known for more than 2 decades that cancer cell DNA is globally hypomethylated when compared with normal DNA, DNA hypomethylation of promoters in cancer has not attracted much attention. It was generally believed that areas with sparsely distributed CpGs such as repetitive sequences and satellite DNA are hypomethylated (22). It was therefore suspected that global hypomethylation is mainly involved in structural-nuclear functions such as chromosomal stability but not in promoter function in contrast to hypermethylation. Indeed there is pharmacological and genetic evidence that chromosomal and genomic stability might be compromised by global hypomethylation (32, 33). The immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) is a genetic disease caused by *DNMT3B* mutation resulting in hypomethylation of pericentromeric DNA and chromosomal instability (34).

There have been periodic reports of promoters that were hypomethylated in cancer (35, 36); however, it is generally accepted that the weight of the balance is tilting toward hypermethylation of tumor controlling genes. Our study shows that promoter hypomethylation is as broadly distributed as promoter hypermethylation suggesting a central role for promoter hypomethylation in cancer growth and metastasis. A bioinformatic analysis of promoter hypomethylation suggests that it targets some of the cardinal pathways involved in cancer growth, metastasis, and angiogenesis (Supplementary Fig. S3A, Table 2). The functional pathways affected by hypomethylation are fundamentally different from those affected by hypermethylation (Supplementary Fig. S3).

Owing to the fact that DNA methylation is known almost exclusively as a repressive regulatory mark, it is perhaps unexpected to find that only a fraction of the genes whose promoters were hypomethylated show significant increase in gene expression. Hypomethylation might be conditioning these genes to be expressed only in response to specific triggers. A good example of conditioning of genes by epigenetic programming is the programming of responsivity to glucocorticoid hormones by DNA demethylation in glucocorticoid responsive genes (37).

Changes in DNA methylation of CpG rich promoters in cancer involve both hypermethylation as is widely accepted and hypomethylation (Fig. 2C). Interestingly, in contrast to the common "stereotype," the genes that are hypomethylated in HCC exhibit a broad range of methylation levels in normal liver. The global picture emerging from our study is that hypomethylation in HCC occurs in genes that are characterized by an intermediate level of methylation and expression in

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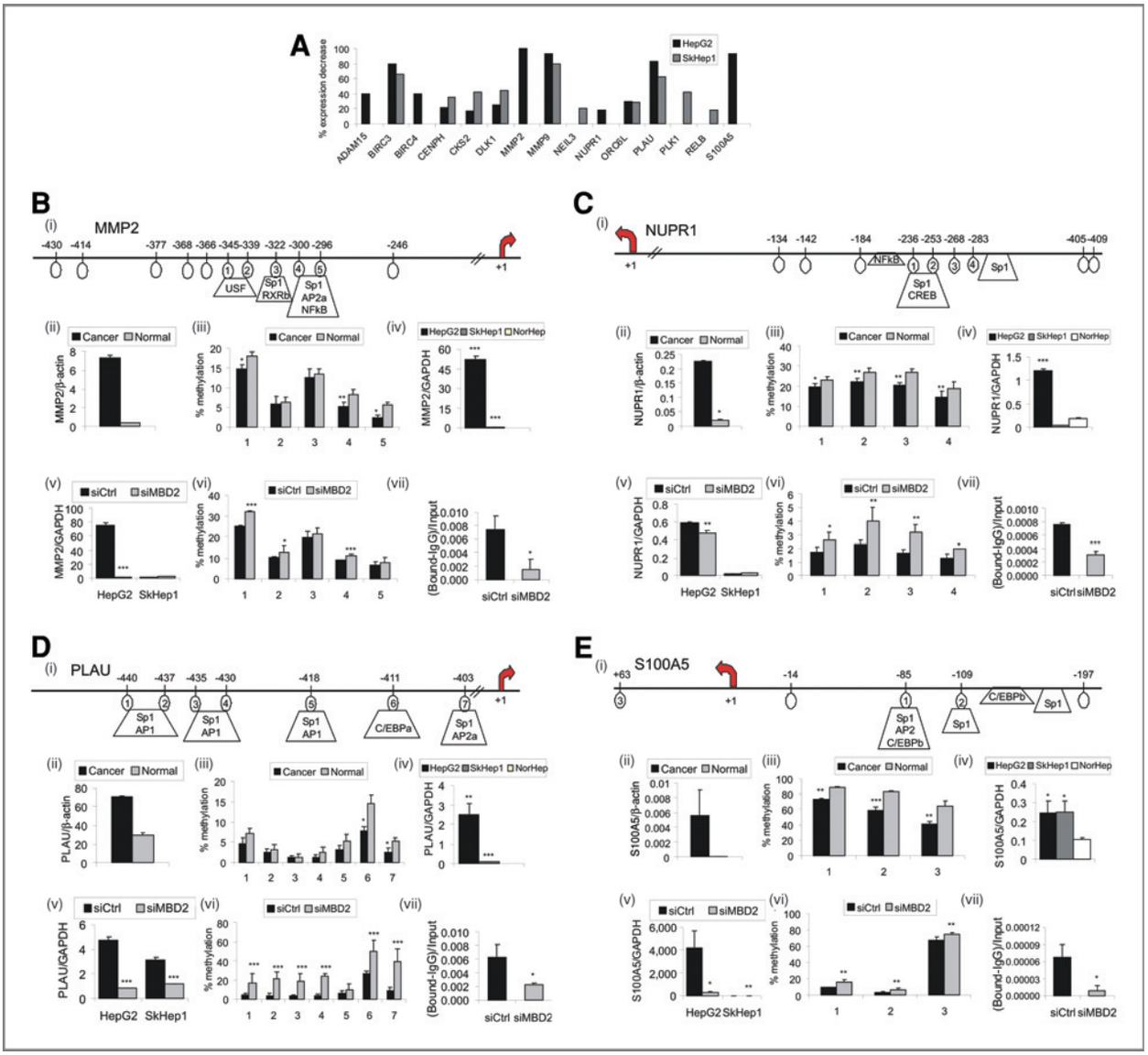

Figure 5. Silencing of genes that are hypomethylated in liver cancer by *MBD2* depletion in HCC cell lines; selectivity for cancer versus nontransformed cells. A, level of expression of 15 genes in siMBD2 and siCtrl treated HepG2 and SkHep1 cells. The data are presented as percentage inhibition in siMBD2 as compared with siCtrl treatment. B–E, state of methylation as determined by pyrosequencing and MBD2 binding as determined by qChIP to the promoter region of *MMP2* (B), *NUPR1* (C), *PLAU* (D), and *S100A5* (E), in HepG2 cells treated with either siMBD2 or siCtrl: (i) a map of the tested promoter fragment flanking the transcription start site. The CpG sites that were chosen for pyrosequencing are circled and numbered; (ii) average gene expression in HCC patients versus normal liver; (iii) average methylation state of CpG sites in the tested promoter fragment between HCC patients and normal liver; (iv) expression of these genes in HepG2, SkHep1, and nontransformed NorHep cells; (v) expression of these genes in HepG2 and SkHep1 cells treated with either siCtrl or siMBD2; (vi) methylation state of CpG sites in the tested promoter fragment in HepG2 cells treated with either siCtrl or siMBD2; (vii) MBD2 binding to the tested promoter region in HepG2 cells treated with either siCtrl or siMBD2 as assessed by qChIP. All results represent mean ± SD of 3 determinations in either 2 or 3 independent experiments; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

normal liver (Supplementary Fig. S5). Nevertheless, as previously shown there is a small subset of genes that undergo dramatic off-on changes in methylation in HCC (see Supplementary Fig. S5, right panel).

Hypomethylation is organized not only at the functional level through hypomethylation of several members of specific functional gene pathways but also at the structural level by clustering of hypomethylated gene promoters in regions and chromosomes (Fig. 3C). The clustering of hypomethylated promoters in gene families is consistent with the hypothesis that there is a yet unknown common mechanism that targets members of the same family to become hypomethylated in liver cancer perhaps through a common cis-acting element. It is unclear what mechanism is responsible for coordinated hypomethylation of multiple promoters from different gene families residing in regions that span up to 15 MB of sequence

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(Fig. 3D). Large-scale functional relationships in gene clusters such as the beta globin gene cluster were studied for decades (38) and it is known that large-scale regions such as imprinted genes clusters are regulated by long-acting cis-acting elements such as CTCF (39, 40). Although the mechanisms are yet to be elucidated, the genome-scale response seen here suggests an organized response rather than multistep selection of random events. Understanding the fundamental players coordinating such responses is obviously critical for identifying targets for cancer therapeutics.

The apparent functional and spatial organization of the demethylation events suggests a common factor responsible for hypomethylation and activation of these genes. We have previously shown that MBD2 plays a causal role in the activity and state of methylation of *PLAU* and *MMP2* in breast and prostate cancer cell lines (15, 16). We took advantage of human liver cancer cell lines as experimental models for testing the hypothesis that several of the genes that are activated and hypomethylated in HCC in the patients are regulated by MBD2. Although MBD2 has been shown to serve as a repressor of methylated genes in several instances (41), our data (Fig. 5, Supplementary Fig. S7B, Supplementary Fig. S8) suggest that it acts as an activator of critical genes that are hypomethylated in liver cancer. MBD2 was previously shown to associate with transcriptional activation complexes. It is interesting to note that MeCP2, the prototype of methylated DNA binding proteins (42) and the prime repressor of methylated promoters, was recently shown to also act as an activator of gene expression and the switch between repressor and activator was found to be a serine phosphorylation event (43). We previously suggested that MBD2B is associated with DNA demethylase activity, which could explain the involvement of MBD2 in demethylation and activation of these genes. Our pyrosequencing data (Fig. 5, Supplementary Fig. S8) suggest indeed that several CpG sites in several gene promoters have their methylation levels changed upon *MBD2* depletion and upon *MBD2* overexpression. However, we cannot rule out that observed CpG demethylation may be an indirect consequence of the transcriptional activation of promoters by MBD2. Importantly, depletion of *MBD2* not only silences the expression of at least 15 of the genes induced in liver cancer (Supplementary Fig. S7A and B), but it also blocks the transformation and invasive properties of the cell (Fig. 4D, E, and F). It also increases apoptosis in the cancer cells but not in nontransformed liver cells (Fig. 4G). The effects of *MBD2* depletion in liver cancer cells could be explained by the fact that depletion of *MBD2* silences genes known to be involved in metastasis and apoptosis (Fig. 4G and Fig. 5A). Taken together, the data provide support not only for the involvement of MBD2 as a master regulator of some of the genes activated in liver cancer but also for the hypothesis that hypomethylation and activation of this set of genes play a causal role in cancer, particularly in cancer metastasis.

A remarkable observation is the exquisite specificity of the effect of *MBD2* depletion on cancer cells. One possible explanation is that *MBD2* depletion results in silencing of genes that are activated in cancer. Because these genes are silenced already in normal cells, *MBD2* depletion would have no impact on these genes. A possible explanation for the fact that this set of genes is hypomethylated in HCC but not in normal liver cells is that cancer cells express a specific isoform *MBD2B* which is abundant in HCC. It is clear from our results that the putative targets of *MBD2A* are not involved in driving cancer cell growth and invasive capacities since *MBD2A* depletion does not result in significant changes in growth rate, anchorage-independent growth and invasiveness in cancer cells (Supplementary Fig. S6).

There are several implications of these studies for cancer therapeutics. First, our data raise the concern that demethylating drugs that are currently being used in cancer therapy might have adverse effects due to activation of oncogenes and prometastatic genes. Second, our data suggest that targeting the DNA demethylation machinery might serve as a new approach to liver cancer and cancer therapy. Third, *MBD2* inhibition has potential as an anticancer therapeutic strategy targeting some of the cardinal processes that lead to activation and demethylation of these genes in liver cancer. The exquisite specificity of *MBD2* depletion to cancer cells as far as both gene expression and cell biology effects are concerned supports the idea that *MBD2* depletion could serve as a strategy for specifically silencing critical genes in liver cancer and stopping cancer growth and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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