Methylation Mediated Silencing of MicroRNA-1 Gene and Its Role in Hepatocellular Carcinogenesis

Jharna Datta,[1,5] Huban Kutay,[1,5] Mohd W. Nasser,[1,5] Gerard J. Nuovo,[2] Bo Wang,[1,5] Sarmila Majumder,[1,5] Chang-Gong Liu,[3,5] Stefano Volinia,[3,5] Carlo M. Croce,[3,5] Thomas D. Schmittgen,[4] Kalpana Ghoshal,[1,5] and Samson T. Jacob[1,5]

Departments of [1]Molecular and Cellular Biochemistry, [2]Pathology, and [3]Molecular Virology, Immunology and Medical Genetics, [4]College of Pharmacy, and [5]Comprehensive Cancer Center, Ohio State University, Columbus, Ohio

Abstract

MicroRNAs (miR) are a class of small (~21 nucleotide) noncoding RNAs that, in general, negatively regulate gene expression. Some miRs harboring CGIs undergo methylation-mediated silencing, a characteristic of many tumor suppressor genes. To identify such miRs in liver cancer, the miRNA expression profile was analyzed in hepatocellular carcinoma (HCC) cell lines treated with 5-azacytidine (DNA hypomethylating agent) and/or trichostatin A (histone deacetylase inhibitor). The results showed that these epigenetic drugs differentially regulate expression of a few miRs, particularly *miR-1-1*, in HCC cells. The CGI spanning exon 1 and intron 1 of *miR-1-1* was methylated in HCC cell lines and in primary human HCCs but not in matching liver tissues. The *miR-1-1* gene was hypomethylated and activated in *DNMT1*−/− HCT 116 cells but not in DNMT3B *null* cells, indicating a key role for DNMT1 in its methylation. miR-1 expression was also markedly reduced in primary human hepatocellular carcinomas compared with matching normal liver tissues. Ectopic expression of miR-1 in HCC cells inhibited cell growth and reduced replication potential and clonogenic survival. The expression of FoxP1 and MET harboring three and two miR-1 cognate sites, respectively, in their respective 3′-untranslated regions, was markedly reduced by ectopic miR-1. Up-regulation of several miR-1 targets including FoxP1, MET, and HDAC4 in primary human HCCs and down-regulation of their expression in 5-AzaC–treated HCC cells suggest their role in hepatocarcinogenesis. The inhibition of cell cycle progression and induction of apoptosis after re-expression of miR-1 are some of the mechanisms by which DNA hypomethylating agents suppress hepatocarcinoma cell growth. [Cancer Res 2008;68(13):5049–58]

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in the world and is the third leading cause of cancer-related death with annual death rate exceeding 500,000. The high mortality is due to late stage detection of this cancer when most of the therapies available are not effective (1). Primary HCC, the most common primary malignant tumor arising in the liver accounts for >90% of all primary liver cancer. In addition, metastatic liver tumor arises from colon, prostate, or breast carcinomas. The disease is progressive and death usually occurs within 10 months of initial diagnosis.

Recent demonstration of differential expression of microRNAs (miR) and their target mRNAs in cancer, and the function of some miRs as oncogenes or tumor suppressors has spurred considerable interest in elucidating their role in tumorigenesis (2, 3). MicroRNAs are highly conserved, small noncoding RNAs that play critical role in variety of biological processes including development, differentiation, apoptosis, cell proliferation, metabolism, and immunity (for review, see ref. 4). In general, miRNAs negatively regulate gene expression in vertebrates by multiple mechanisms such as complimentary base pairing with 3′ untranslated region (UTR) of their target mRNAs that results in translational repression, mRNA cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation (5). Under certain stressed conditions, miRs can also enhance expression of target mRNAs (5, 6). Primary miRNAs (pri-miRNA) are processed by RNases to generate the mature miRNAs that are recruited by RNA-induced silencing complex to exert their biological functions for review, see ref. 7.

Mammalian DNA is predominantly methylated at C-5-position of complimentary CpG bp by concerted action of three DNA methyltransferases namely, Dnmt1, Dnmt3a, and Dnmt3b (for review, see ref. 8). This epigenetic modification is essential for mammalian development and its aberrations lead to a variety of diseases including cancer (9–11). Recent studies have established that such as mutation, methylation-mediated silencing of tumor suppressor genes play a causal role in tumorigenesis. Paradoxically, genome-wide DNA hypomethylation that induces chromosomal instability and spurious gene expression is also involved in carcinogenesis. Unlike mutation, methylation can be reversed by inhibitors of DNA methyltransferase, resulting in re-expression of silenced tumor suppressor genes. Approval of drugs, such as Vidaza (5-Azacytidine) and Dacogen (5-Aza-2′-deoxycytidine or decitabine), by the Food and Drug Administration as anticancer agents underscores the usefulness of epigenetic therapy. Recent studies showed that differential regulation of some miRs such as miR-127, miR-124a, and let-7a3 by differential methylation of associated CGIs occur in human cancers (12–14).

We have studied the efficacy of DNA methyltransferase inhibitors in a rat model of hepatocarcinogenesis and the molecular mechanisms of action of these drugs *in vivo* (15–23). This study showed that i.p. injection of 5-AzaC completely regressed growth of a transplanted tumor by demethylating and activating the

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J. Datta and K. Ghoshal contributed equally to this work.

Requests for reprints: Samson T. Jacob, The Ohio State University, 420 West 12th Avenue, 646B TMRF Building, Columbus, OH 43210. Phone: 614-688-5494; Fax: 614-688-5600; E-mail: Samson.Jacob@osumc.edu or Kalpana Ghoshal, The Ohio State University, 420 West 12th Avenue, 646C TMRF Building, Columbus, OH 43210. Phone: 614-292-8865; Fax: 614-688-5600; E-mail: kalpana.ghoshal@osumc.edu.

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antioxidant gene encoding metallothioneins (24) and a receptor-type tyrosine phosphatase PTPRO with tumor suppressor property (25, 26). We have also shown that these inhibitors induce degradation of DNMT1 in cancer cells and facilitate activation of silent genes (15, 27). To identify candidate tumor suppressor miRs that are silenced by epigenetic mechanism in human HCCs, we performed miRNA microarray analysis in HCC cells treated with 5-AzaC alone or in combination with trichostatin A (TSA), a histone deacetylase inhibitor. Deacetylation of histones promotes tumorigenesis by repressing genes that inhibit cell cycle progression, differentiation, apoptosis, cell adhesion, and inducing expression of genes involved in angiogenesis, cell migration, and invasion (28). HDAC inhibitors reverse cellular transformation by altering expression of the genes involved in these pathways. Inhibitors of DNMTs and HDACs are very promising anticancer agents, as many tumor suppressor genes are synergistically activated upon treatment with these two classes of inhibitors (29). The objective of the present study was to identify miRs activated by these chromatin-modifying agents. Here, we show that *miR-1-1* is one such gene that is methylated in human HCC cells and primary HCC, and its activation by the epigenetic drugs suppresses tumor cell growth by down-regulating its oncogenic targets MET, FoxP1, and HDAC4.

Materials and Methods

Cell culture, treatment with the drugs. Human HCC cell line (Hep3B, HepG2, Huh7, SK-HEP-1, and SNU449) were obtained from American Type Culture Collection (ATCC). Hep3B, HepG2, Huh7, and SK-HEP-1 cells were cultured in MEMα medium containing 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 1 mmol/L nonessential amino acids; SNU-449 cells were grown in RPMI in a 5% CO_2 incubator according to supplier's (ATCC) instructions. Exponentially growing cells were treated with 5-AzaC and/or TSA for different time periods. Cells were harvested for RNA/miRNA isolation, and whole cell extracts were subjected to Western blot analysis. DNMT1$^{+/+}$ and DNMT1$^{-/-}$, DNMT3B$^{-/-}$, and DKO (DNMT1$^{-/-}$ and DNMT3B$^{-/-}$) HCT116 cells were grown as described earlier (30).

Human primary HCC samples and matching controls were obtained from the cooperative human tissue network.

Plasmid construction. 3′-UTR of FoxP1 and MET were amplified from human lymphocyte DNA using Accuprime Taq polymerase (Invitrogen) and cloned into PCR2 cloning vector (Qiagen). Inserts were retrieved with Mlu I and cloned into the same sites of a luciferase reporter vector, pIS0, obtained from Addgene (31). Deletion of miR-1 complimentary site from 3′-UTR of FOXP1 and MET were performed by PCR. The following primers were used to amplify 3′-UTRs: FoxP1-3′UTR-F, CCCCGAGAATGAAGATTGG and FoxP1-3′UTR-R, CAGTGGTAGGATAAA CACAAGGG; FOXP1-Δmir1-1F, TCTTCCTTCAGACATCACCACG and FOXP1-Δmir1-2R, CGTGGTGATGTCTGAAGGAAGA; MET-3′UTR-F, CAATGGTTTT TTCACTGCCTGAC and MET-3′UTR-R, AGCCAGGTGAAATCCATCTTAGG; MET-Δmir1-#1-F, AACCTCCACCTCCCAGGC TC and MET-δmir1#2-R, TTGAG CCTGGGAGGTG GAGGTTGC.

RNA isolation and miRNA microarray analysis. Total RNA was isolated from HCC cells by Trizol (Invitrogen), according to the manufacturer's protocol. RNA was labeled and hybridized on miRNA microarray chips as previously described (32). Briefly, 5 μg of RNA from each sample was biotin labeled during reverse transcription using random hexamers. Hybridization was carried out on version 4 of our miRNA chip (ArrayExpress accession number AMEXP-258), which contains 381 probes for mature and precursor human miRNAs and 393 probes for mouse miRNAs, together with controls. There are four spot replicates for each probe on the chip. Hybridization signals were detected by biotin binding of a Streptavidin-Alexa 647 conjugate using a GenePix 4000B scanner (Axon Instruments). Images were quantified using the GenePix Pro 6.0 apparatus (Axon Instruments). We used the Eisen CLUSTER and TREEVIEW programs for hierarchical clustering and visualization of the data. Before applying the clustering algorithm, we log transformed the fluorescence ratio for each expression and then average centered the data for all samples.

Isolation of miRNA. Total RNA was extracted from cell lines and frozen tissue samples using Trizol reagent (Invitrogen) as described (16). Total RNAs isolated from HCC and matching normal tissues were further enriched using mirVana miRNA isolation kit (Applied Biosystems).

Taq Man reverse transcription-PCR for quantification of miR-1. For mature miR-1 detection, reverse transcription was performed following Applied Biosystems Taq Man MicroRNA Assay protocol. PCR reaction mixtures contained Taq Man human miR-1 and Universal PCR Master Mix in a total volume of 20 μL. Cycling variables were as follows: 95°C for 10 min followed by 40 cycles at 95°C (15 s) and annealing/extension at 60°C (1 min). All reactions were performed in triplicate. MiR-1 expression was normalized using 18S rRNA. The expression of miR-1 relative to 18S rRNA was determined using $2^{-\Delta CT}$ method (33). An aliquot of cDNA (1.33 ng for miR-1 and 5 pg 18S rRNA) were used for each assay.

Transfection of cells and reporter assay. Luciferase reporter plasmid (pIS0) containing 3′-UTR of FoxP1-or FoxP1-ΔmiR-1-3′-UTR were transiently transfected with SV40-βgal plasmid into Hep3B cells. Briefly, the cells were seeded into 24-well plates and, 24 h later, were cotransfected with 100 ng of pIS0 and 10 ng of pSV40-βgal along with 60 nmol/L hsa-pre-miR-1 or control RNA #1 or #2 using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. After 48 h, cells were washed with PBS and resuspended in the lysis buffer [100 mmol/L potassium phosphate (pH 7.8), 0.2% Triton X-100, and 0.5 mmol/L DTT] and followed by assay of luciferase activity in a luminometer using the Dual-Light System (Applied Biosystems). All experiments were performed in quadruplicate, and the results are mean of three separate experiments.

Western blot analysis. HCC cells (3×10^5) were plated per 60-mm dish 24 h before transfection. Cells were transiently transfected with 100 nmol/L pre-miR-1 or negative control RNA as described above. HepG2 cells were transfected with Mirus TransIT-siQuest (Mirus Bio Corporation) transfection reagent following the supplier's protocol. After 24 h, cells were allowed to grow in regular culture medium for an additional 24 h before further studies. The whole cell extracts prepared after 48 h of transfection or from 5-AzaC–treated cells were immunoblotted with anti-MET, anti-HDAC4 (Santa Cruz Biotech), anti-FOXP1 (Abcam), and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Molecular Probes) antibodies following published protocol (34). The signal was developed with ECL (GE Healthcare) after incubation with appropriate secondary antibodies.

Immunohistochemistry. Immunohistochemical testing was performed using the Ventana Benchmark System (Ventana Medical Systems) according to the manufacturer's recommendations. Optimal detection of FoxP1, c-MET, and HDAC4 required the antigen retrieval CC1 for 30 min and dilutions of antibodies 1:1,000-, 1:500-, and 1:500-fold, respectively.

***In situ* hybridization.** Our protocol for detection of RNAs by *in situ* hybridization has been previously published (35). In brief, the tissue was deparaffinized, proteased (30 min in 2 mg/mL of pepsin in RNase free water), washed in sterile water, then 100% ethanol, and air dried. For each miR studied, LNA modified cDNA probes were used. The probes were labeled with the 3′ oligonucleotide tailing kit using biotin as the reporter nucleotide (Enzo Diagnostics). Hybridization was done at 37°C overnight and followed by a wash in 0.2XSSC and 2% bovine serum albumin. The probe-target complex was seen due to the action of alkaline phosphatase (as part of the streptavidin complex) on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP; Enzo Diagnostics). Nuclear fast red served as the counterstain. The negative controls were the omission of the probe and the use of a scrambled probe (the same sequence as the miR cDNA but where the nucleotides have been "scrambled" at random so that is very low homology with the target sequence).

Cell proliferation assay. Cell proliferation was monitored using Cell Proliferation reagent kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Roche Molecular Biochemicals]. HepG2 or Hep3B cells (3,000 per well) transfected with pre-miR-1 or control pre-miR were allowed to grow in 96-well plates. Cell proliferation was documented every

24 h following the manufacturer's protocol. To measure cell proliferation, 10 μL of MTT labeling reagent I was added to each well and incubated at 37°C for 4 h followed by the addition of 100 μL solubilization reagent in each well. Absorbance was measured at 570 nm in the ELISA reader (Tristar; Berthold Technology) after overnight incubation.

DNA replication assay. Cells (10,000 per well) transfected with hsa-pre-miR-1 or control RNA were plated in 24-well plates in 1 mL culture medium and incubated at 37°C in 5% CO_2 atmosphere in a humidified incubator. After 48 h, the cells were incubated with serum-free medium for an additional 16 h. Serum-free medium was replaced with complete MEMα medium, and 30 min later, 1 μCi of $^3H_1$-thymidine were added to each well. Six hours later, cells were washed twice with ice-cold PBS followed by estimation of $^3H_1$ incorporation into DNA in a Hitachi scintillation counter (27).

Fluorescence-activated cell sorting analysis. To analyze DNA content, HepG2 cells (5×10^5) transfected with hsa-pre-miR-1 or control RNA were plated in 10-cm tissue culture plates and incubated at 37°C in 5% CO_2 atmosphere in a humidified incubator. Cells were allowed to grow before they were fixed and stained with propidium iodide solution (20 μg/mL propidium iodide, 200 μg/mL RNase A) for 15 min at 37°C in the dark. Cell were analyzed in a FACSCalibur flow cytometer (BD Biosciences). Flow cytometric data were analyzed using Cell Quest Pro software (BD Biosciences).

Combined bisulfite restriction analysis and bisulfite sequencing. University of California Santa Cruz (UCSC) database was used to identify CpG islands (CGI) spanning *miR-1-1* gene. The primers for Combined Bisulfite Restriction Analysis (COBRA) for all four CGIs were designed using Methprimer software.[6] Genomic DNA isolation and bisulfite conversion were performed as described (36). Bisulfite-converted genomic DNA, which converts only unmethylated cytosines to uracils, was amplified with strand-specific primers followed by digestion with methylation-sensitive enzymes. The primers used for amplification of different CGI on miR-1-1 gene are described below.

miR1-CGI81-BSF1: TGGGGTTAAATTTATTTTGAATTTG and miR1-CGI81-BSR1: ACTCCC CAACAAAAACCTACAC; miR1-CGI81-BSF2: TTTGGAAAATTGTAGAGTTAGTAGT and miR1-CGI2-BSR2: AAATT-CAAAATAAATTTAACCCCAC; miR-1-CGIdown-BSF: TGTTTGTGTTA GTAGGTGGAAGTGT and miR-1-CGIdown-BSR: TTCCAAAATAACCAT-CAATAAAACC; miR1-PRO-BSF: GAGGGGTAGGATAGTAGTTTAGTT and miR1-PRO-BSR: ACCATATTTAATCAA ACAAAAAAAA.

Statistical analyses were performed using Student's *t* test. Box and whisker plots were generated using QI Macros[7] software.

Results

miR-1 is activated in HCC cells after treatment with epigenetic drugs. Treatment of cancer cells with inhibitors of DNMTs or HDACs inhibits cell growth by activating genes encoding tumor suppressors, including some miRNAs (15, 29, 37). To identify potential growth regulatory miRNAs silenced by epigenetic mechanisms in HCC cells, we compared the miRNA expression profiles of HepG2 and Hep3B cells treated with 5-AzaC (DNA hypomethylating agent), TSA (HDAC inhibitor), or both to those of untreated cells using miRNA microarray analysis. Cluster analysis showed that treatment with these drugs deregulated expression of 23 miRs in both cell lines (Fig. 1*A*). Among these miRs, miR-1-1 was significantly up-regulated ($P \leq 0.0001$) in both cell lines upon treatment with 5-AzaC alone or in combination with TSA.

Next, we validated up-regulation of miR-1 in different HCC cells treated with 5-AzaC by real-time reverse transcription-PCR (RT-PCR). miR-1 expression was almost undetectable in all six HCC cell lines tested, which increased after treatment with 5-AzaC for 36 h (Fig. 1*B, top*). In HepG2, Hep3B, SK-Hep1, and SNU-449, miR-1 level increased with increasing concentration of the drug from 1 to 5 μmol/L, whereas its maximal induction was observed in Huh-7 and peritoneal lymphocytes/PRF5 cells treated with 1 μmol/L 5-AzaC. The differential activation of miR-1 in different HCC cell lines is likely due to differential availability of transcription factors and chromatin structure. To confirm that the RT-PCR product is indeed miR-1, we separated it on a denaturing polyacrylamide gel and subjected to Northern blot analysis with antisense miR-1 as probe. The results showed that miR-1 was barely detectable in control HCC cells, which increased with increasing concentrations of 5-AzaC (Fig. 1*B, bottom*). Overall, the real-time RT-PCR and Northern blot data corroborated well with the microarray data confirming that miR-1 is indeed silenced by epigenetic mechanism in HCC.

We next measured the mature miR-1 levels in human primary HCCs and matching liver tissues by real-time RT-PCR analysis that showed significant reduction in miR-1 level in five HCCs relative to the matched controls among six samples analyzed (Fig. 1*C*). *In situ* hybridization of tissue sections with locked nucleic acid (LNA)-modified antisense miR-1 oligo showed miR-1 expression in many of the benign hepatocytes in the tissue adjacent to the HCC (Fig. 1*D*). Hybridization to scrambled oligo did not generate any signal (data not shown). The miR-1 expression was not evident in the fibrotic foci of the cirrhotic livers. In comparison, miR-1 expression was much reduced in the HCCs being evident in rare cancer cells. These results indicate that down-regulation of miR-1 occurs specifically in primary HCCs.

CGI of *miR-1-1* is methylated in human HCC cells and primary HCCs. Because *miR-1-1* gene was activated in HCC cell lines in response to 5-AzaC, we next explored its methylation status in cell lines and primary liver tumors. Searching the UCSC database revealed that miR-1 coded by an intron 1 of the putative ORF166 is embedded in CGIs (Fig. 2*A*). We used COBRA to assess methylation status of the largest CGI (CGI-81) spanning exon 1 and intron 1 in HCC cells. The amplicon harbors one *Taq* I site that is retained after bisulfite conversion provided genomic DNA is methylated. PCR product from HepG2 cells was almost completely digested with *Taq* I, whereas ~50% of the amplicon from Huh7 cells was cleaved with *Taq* I (Fig. 2*B, left*). Complete digestion of the PCR products with *Tsp509* I confirmed complete conversion of unmethylated cytosines to uracils. These results show that the CGI located upstream of miR-1 is methylated in both HCC cell lines. To determine whether demethylation of CGI-81 indeed resulted in *miR-1-1* activation, we performed COBRA of genomic DNA obtained from Huh7 cells treated with 5-AzaC. As expected, the amplicon from 5-AzaC–treated cells was refractory to *Aci* I or *Taq* I digestion compared with that from the control cells (Supplementary Fig. S1). These data show that the activation of *miR-1-1* correlates with hypomethylation of its CGI upon 5-AzaC treatment.

Next, we examined the methylation status of CGI-81 in human primary HCCs and matching liver tissues by COBRA. The amplicon also harbors 4 *Aci* I sites. Digestion of the amplicon with methylation-sensitive enzymes such as *Taq* I or *Aci* I indicates methylation of genomic DNA. Analysis of four pairs of human primary HCCs showed significant methylation at both *Aci* I and *Taq* I sites in tumor T_2 and at the *Taq* I site in T_4 (Fig. 2*B, right*). A low-level methylation was observed at both restriction enzyme sites in T_1. In contrast, CGI-81 was

[6] http://www.urogene.org/methprimer/index1.html
[7] http://www.qimacros.com/free-spc-software.html

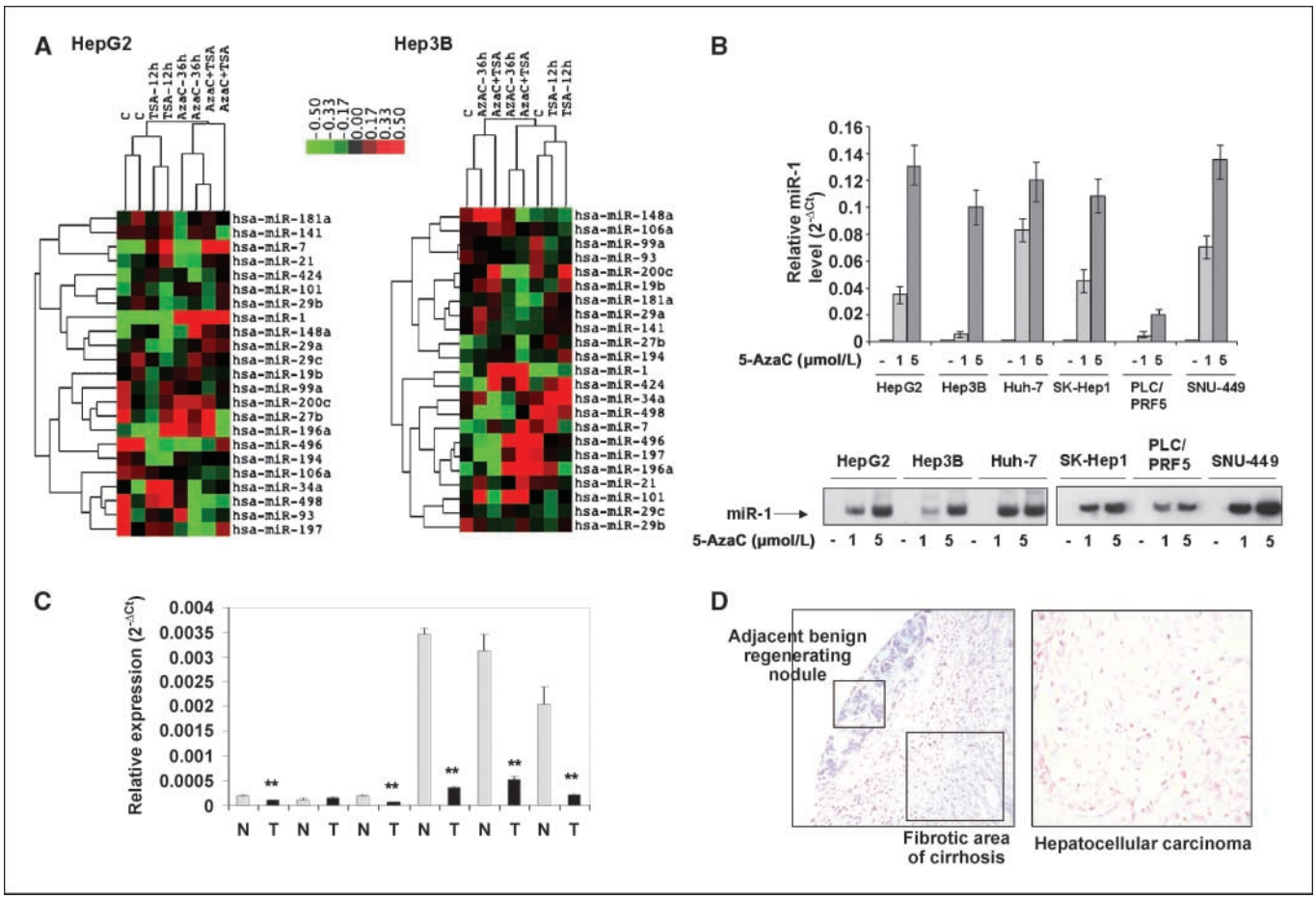

Figure 1. *A,* epigenetic drugs deregulate miR expression in HCC cells. Cluster analysis of differentially regulated miRNA in HCC cells after treatment with inhibitors of DNA methyltransferase (5-azacytidine), HDAC (TSA), or both. Cells in log phase were treated with 5-AzaC (1 μmol/L) for 24 h followed by treatment with TSA (0.3 μmol/L) for an additional 12 h. Cells treated with the vehicle (DMSO) or the drugs alone were used as the control. Total RNA isolated was subjected to miRNA microarray analysis, and data were normalized to the average median of all the genes in the array. Hierarchical clustering was performed with the normalized, log-transformed data using Cluster 3.0 and TREEVIEW software. The results are mean of two different batches of cells treated under identical conditions. Only 23 miRs were used for clustering, whose expression was >1.5 or <0.5 compared with the control cells. *B,* miR-1 expression is up-regulated in HCC cell lines upon treatment with 5-AzaC. *Top,* cells in log phase were either untreated or treated with 1 μmol/L of 5-AzaC for 36 h. Real-time RT-PCR analysis of mature miR-1 was performed using Taqman primers and probe. The data were normalized to 18S rRNA. *Columns,* mean of three replicates; *bars,* SD. *Bottom,* Northern blot analysis of the PCR product generated in *B* using ^{32}P-labeled antisense miR-1 deoxyolgonucleotides. *C,* miR-1 level is reduced in human primary HCCs. Real-time RT-PCR analysis of mature miR-1 in human primary HCCs and matching liver tissues as described in *B*. The data were normalized to 18S rRNA. Each sample was analyzed in triplicate. *Columns,* mean of three values; *bars,* SD. *D,* detection of miR-1 in HCC tissue sections and adjacent nonmalignant tissue with LNA-modified antisense miR-1 probe. miR-1 was detected in the hepatocytes of a regenerating nodule in this section of cirrhotic liver adjacent to a HCC (positive is blue, due to NBT/BCIP; negative cells pink from nuclear fast red counterstain); note the cytoplasmic localization. In comparison, miR-1 was not detected in the malignant hepatocytes of the adjacent HCC.

methylation free at these restriction enzyme sites in all four matching control livers because respective amplicon could not be cleaved by *Aci* I or *Taq* I. Thus, miR-1 CGI-81 is specifically methylated in human HCCs. We also measured methylation status of other CGIs located upstream or downstream of *miR-1-1* by COBRA, none of which was methylated in human livers, primary HCCs, or HCC cell lines (data not shown).

To determine the methylation status of each CpGs within the amplicon, we sequenced 12 randomly selected TA clones of PCR products obtained from the liver and tumor of sample #2. The results showed dense methylation of certain CpGs located in this region of the tumor, whereas only a few scattered CpGs were methylated in the matching liver DNA (Fig. 2*C*). Thus, methylation of *miR-1-1* CGI-81 in HCCs is a tumor-specific event.

In mammals, the genomic methylation pattern is initiated and maintained by three essential DNA methyltransferases namely DNMT1, DNMT3A, and DNMT3B. In cancer cells, depending upon the promoter, DNMT1 alone or in concert with DNMT3A/B maintain methylation profile of methylated loci. To identify the enzyme involved in the aberrant methylation of miR-1 in cancer cells, we took advantage of a colon cancer cell line with targeted disruption of DNMTs and analyzed methylation profile of CGI-81 in the wild-type, *DNMT1−/−*, *DNMT3B−/−*, and DKO (*DNMT1−/− DNMT3B−/−*) cells. Almost 50% digestion with each enzyme indicates that miR-1 is methylated in the parental HCT116 cells (Fig. 2*D, top*). It is noteworthy that in DNMT3B null cells, *Aci I* completely and *Taq I* partially cleaved the amplicon. In contrast, the PCR product was minimally (~8%) cleaved in DNMT1 null cells and was totally resistant to digestion in DKO cells, indicating that DNMT1 plays a key role in aberrant methylation of miR-1 in HCT116 and probably in HCC cells. Real-time RT-PCR analysis showed that the disruption of DNMT1 alone could induce miR-1 expression in nonexpressing parental HCT cells, which was further up-regulated in DKO cells (Fig. 2*D, bottom*). miR-1 was induced at a low level also in

DNMT3B−/− cells. These results strongly suggest inverse correlation between CGI-81 methylation and *miR-1-1* activation.

Ectopic expression of miR-1 reverses cancer cell–specific phenotype of HCC cells. Because the demethylating agent reactivated methylated *miR-1-1* gene in HCC cells, the next series of experiments were performed to determine the function of miR-1 in HCC cells. miR-1 is abundantly expressed in the heart and smooth muscle where it inhibits cell cycle progression of cardiac progenitors and promotes their differentiation (38, 39). We entertained the possibility that the low-level expression of miR-1 in other tissues such as liver (40) may be involved in controlling proliferation and/or maintaining differentiated state of the hepatocytes. If this is true, ectopic expression of miR-1 in nonexpressing HCC cell lines should reverse their cancer cell–specific phenotype. Because miR-1 level is undetectable in HCC cell lines tested (Fig. 1), we transiently transfected cells with hsa-pre-miR-1 (miR-1) or negative control RNAs (that do not have homology to any mammalian RNA) and analyzed phenotypes of these cells. Overexpression of miR-1 in HepG2 cells increased mature miR-1 expression ~5000-fold, which was significantly less than its constitutive level in the heart (Fig. 3*A*). The proliferation rate of miR-1–expressing cells was markedly reduced compared with those transfected with the control RNA. MTT assay showed ~25% reduction in growth of miR-1–expressing cells at all time points tested (Fig. 3*B, left*). Significant decrease in replication potential as measured by incorporation of $^3H_1$-thymidine in HepG2 cells upon ectopic expression of miR-1 confirmed its growth inhibitory potential (Fig. 3*B, right*). Furthermore, clonogenic survival of these cells was reduced by ~60% (P = 0.005) upon ectopic expression of miR-1 (Fig. 3*C*). Reproducible results were obtained with three different batches of transfected cells as well as with two different control miRs (data not shown).

To rule out the possibility that the growth inhibitory property of miR-1 is restricted to HepG2 cells, we studied its function in another HCC cell line (Hep3B). The expression of mature miR-1 was elevated 155-fold in cells transfected with miR-1 (Supplementary Fig. S2*A*). Ectopic expression of miR-1 led to ~20% (Supplementary Fig. S2*B*) and ~75% reduction (Supplementary Fig. S2*C*) in growth of Hep3B cells and replication potential of miR-1–expressing cells, respectively, compared with the cells expressing control miRs. As observed for HepG2 cells, ectopic miR-1 reduced clonogenic survival of Hep3B cells by 38% (P = 0.018; Supplementary Fig. S2*D*). Taken together, these results indicate significantly reduced ability of miR-1–overexpressing HCC cells to maintain their tumorigenic properties.

Ectopic expression of miR-1 inhibits growth of HCC cells by inducing apoptosis and inhibiting cell cycle progression. To elucidate the mechanism of miR-1–mediated inhibition of HCC cell growth, we analyzed cell cycle profile of HepG2 cells transfected with hsa-miR-1 and control RNA. Fluorescence-activated cell

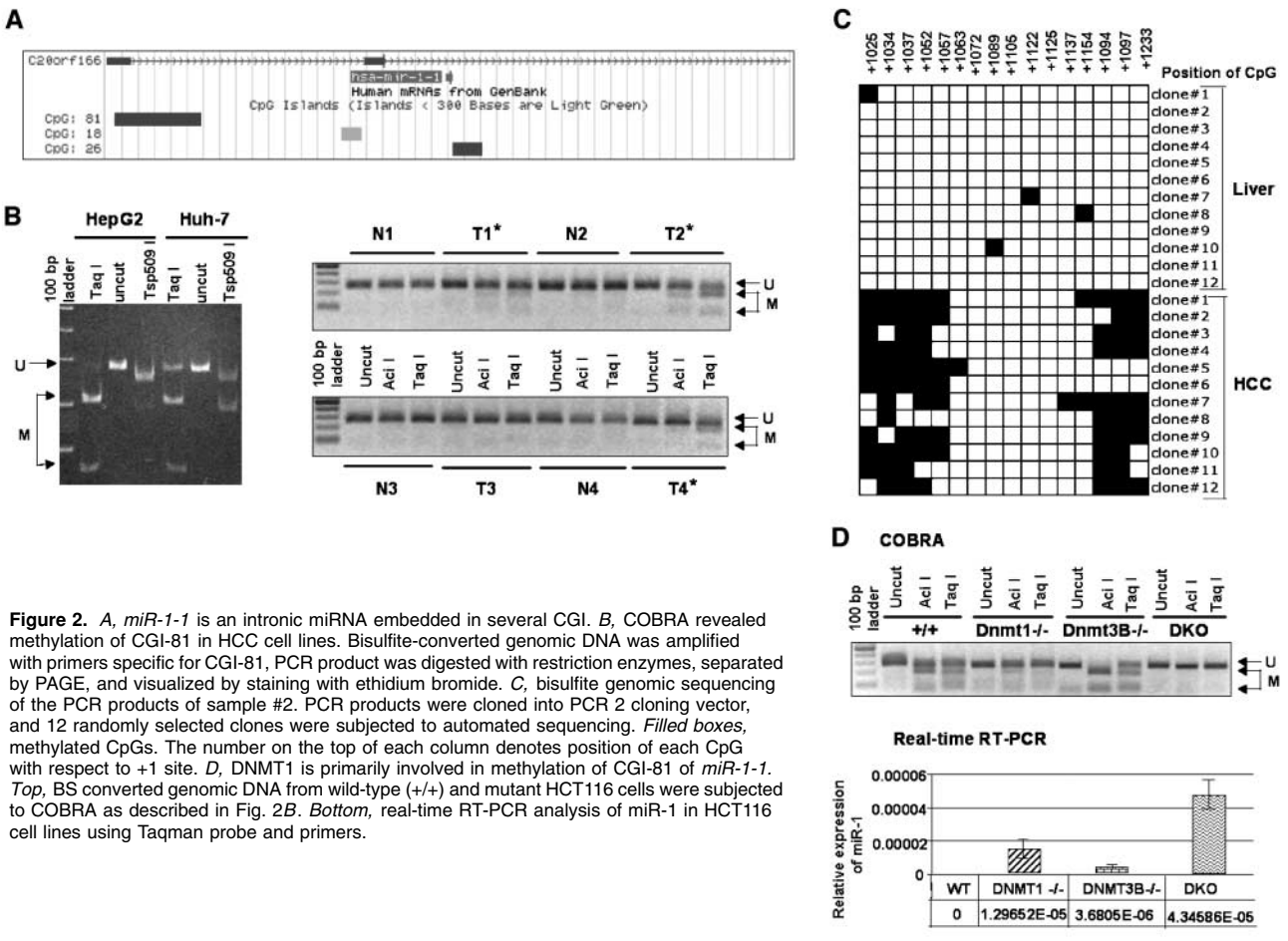

Figure 2. *A, miR-1-1* is an intronic miRNA embedded in several CGI. *B,* COBRA revealed methylation of CGI-81 in HCC cell lines. Bisulfite-converted genomic DNA was amplified with primers specific for CGI-81, PCR product was digested with restriction enzymes, separated by PAGE, and visualized by staining with ethidium bromide. *C,* bisulfite genomic sequencing of the PCR products of sample #2. PCR products were cloned into PCR 2 cloning vector, and 12 randomly selected clones were subjected to automated sequencing. *Filled boxes,* methylated CpGs. The number on the top of each column denotes position of each CpG with respect to +1 site. *D,* DNMT1 is primarily involved in methylation of CGI-81 of *miR-1-1*. *Top,* BS converted genomic DNA from wild-type (+/+) and mutant HCT116 cells were subjected to COBRA as described in Fig. 2*B*. *Bottom,* real-time RT-PCR analysis of miR-1 in HCT116 cell lines using Taqman probe and primers.

sorting (FACS) analysis of propidium iodide–stained cells at different days showed that in control cells at day 0, nearly 65% of the cells were in G_1 phase (Supplementary Fig. S3; Fig. 3*D*). At day 2, a large portion of the control cells from the G_1 phase progressed to S and G_2-M phases. At this time point, a dramatic decrease (~20%) in the number of cells in G_1 phase was observed compared with that at day 0 (Supplementary Fig S3; Fig. 3*D*). Furthermore, ~14% and 5% increase in the cell numbers were observed in S and G_2-M phase, respectively, indicating a significant (~20%) distribution in growth phase. In contrast, the miR-1–overexpressing cells exhibited very little cell cycle progression at day 2. Only ~2% decrease in cell number was noticed in G_1 phase compared with that at day 0. At this time point, the number of cells in S phase decreased by 4% and ~2% more cells were accumulated in the G_2-M phase. Again, a relatively large proportion (~5-fold and ~16-fold at day 0 and day 2, respectively, compared with control) of the miR-1–overexpressing cells were apoptotic (sub-G_0 phase) compared with that in nonexpressing cells (~0.4%; Supplementary Fig. S3; Fig. 3*D*). These data revealed that both cell cycle arrest and induction of apoptosis contribute to growth inhibitory property of miR-1.

FOXP1 and MET are targets of miR-1. Next, we explored the underlying molecular mechanism of antitumorigenic property of miR-1 in HCC cells. Because miRNAs primarily mediate their biological functions in animal cells by impeding expression of target genes, we searched for its potential targets that exhibit oncogenic properties. Different target prediction algorithms (MiRanda, TargetScan, and Pictar) identified forkhead box transcription factor FOXP1 as a potential target of miR-1. It harbors 3 miR-1 recognition sites in its 3′-UTR among which two are in close proximity (Fig. 4*A*). FoxP1, a member of F box family of ubiquitously expressed transcription factors, plays a critical role in development (41, 42). This dysregulated factor can act as an oncoprotein or a tumor suppressor depending upon the cellular context. To assess whether miR-1 can directly alter the expression of FOXP1, a region (1-2192) of the 3′-UTR of FOXP1 mRNA, containing two putative miR-1 binding sites in close proximity (752–780 and 795–827) and the 3′UTR depleted of these sites, were cloned into a firefly luciferase reporter vector pIS0 (43). These constructs were cotransfected into Hep3B cells along with pre-miR–1 or control RNA. SV40-βGAL plasmid was cotransfected to monitor transfection efficiency. Ectopic miR-1 significantly (60%) reduced luciferase activity driven by the wild-type FOXP1 3′-UTR (pIS0-FoxP1-3′UTR) compared with the control (Fig. 4*B*). In contrast, pre-miR-1 could not inhibit luciferase activity of pIS0-FoxP1-Δ3′-UTR lacking both miR-1 sites. MiR-1 had no significant effect on pIS0, the parental vector (data not shown). These results suggest that miR-1 can block translation of a chimeric protein harboring two miR-1 complimentary sites of FoxP1 in its 3′-UTR. To confirm that FOXP1 is indeed the target of miR-1 in HCC cells, we measured endogenous FoxP1 level in HCC cells expressing ectopic miR-1 or control RNA. Western blot analysis of whole cell extracts showed that the steady-state level of FoxP1 was reduced by ~40% and ~60% in Hep3B and HepG2 cells, respectively, by ectopic miR-1 (Fig. 4*C* and *D*).

MET (hepatocyte growth factor receptor), a proto-oncogene, which harbors two conserved miR-1 cognate sites, is another target predicted by different databases. MET is a receptor tyrosine kinase (RTK) family of oncogenes overexpressed in many human cancers (44–46). This RTK consists of disulfide-linked subunits of 50 kDa (α) and 145 kDa (β) processed from the same precursor polypeptide (170 kDa). As observed for FoxP1, the luciferase activity of the chimeric pIS0-MET-3′-UTR was inhibited specifically by ectopic miR-1 (data not shown). Western blot analysis showed that the endogenous MET level was significantly diminished in

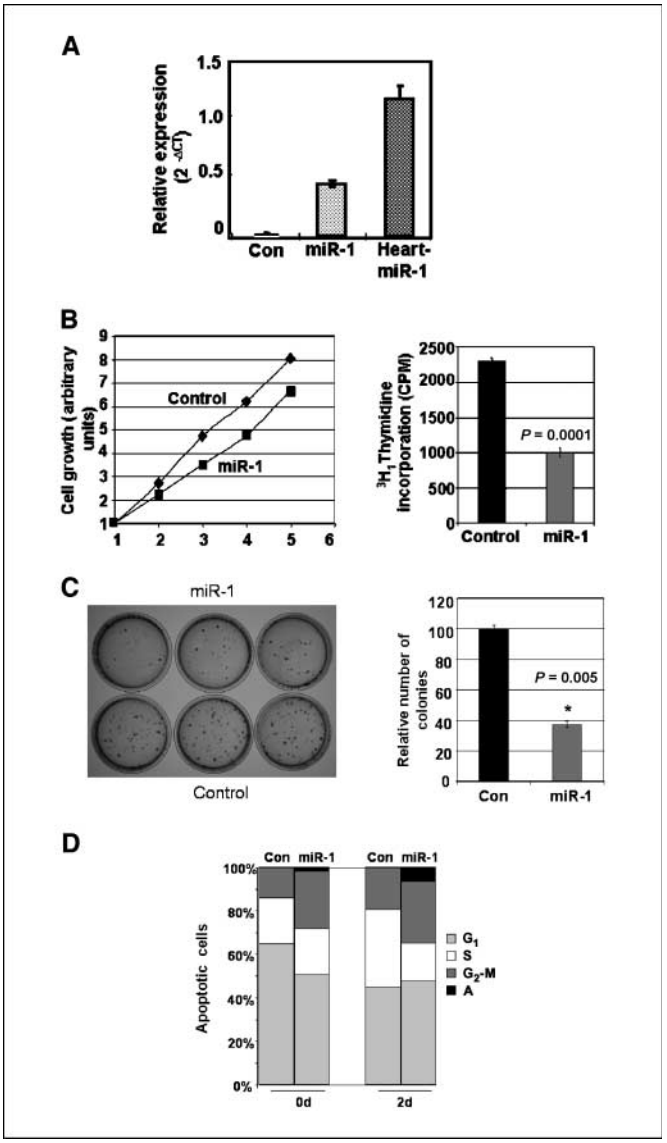

Figure 3. Ectopic expression of miR-1 reduces growth, replication potential, and clonogenic survival, and induces apoptosis and cell cycle arrest of HepG2 cells. Cells were transfected with (100 nmol/L) hsa-pre-miR-1 or control RNA. After 24 h, cells were distributed to perform experiments *A* to *D*. *A,* total RNA isolated from transfected cells 48 h posttransfection, and heart was subjected to real-time RT-PCR analysis. *B,* cells (10,000/well) were distributed in 96-well plates, and cell growth was measured every 24 h for 5 d using MTT assay (*left*). *Right,* cells were serum starved overnight 48 h posttransfection followed by addition of serum and $^3H_1$-thymidine. Incorporation of $^3H_1$-thymidine into DNA after 4 h was measured in a scintillation counter. *C,* single cells (1,000) were seeded onto a 60-mm plate and allowed to grow for 10 d, and the colonies formed were stained with crystal violet and photographed. The total number of colonies in three plates was counted, and the number of colonies formed by cells transfected with control miR was assigned a value of 100. Each experiment (*A–C*) was performed in triplicate with two different batches of transfected cells. *D,* FACS analysis of the control and miR-1–overexpressing Hep3B cells. An equal number of cells (5×10^5) transfected with hsa-miR-1 or control RNA were plated into 10-cm plates and allowed to grow for 2 d. Cells were then fixed, stained with propidium iodide, and analyzed in a flow cytometer. The cell cycle analysis was performed in duplicate with two different batches of transfected cells, and representative percentage of cells present in different phases of cell cycle is shown.

both Hep3B and HepG2 cells upon ectopic expression of miR-1 (Fig. 4*C* and *D*).

HDACs play an important role in cancer development, and several HDAC inhibitors are in clinical trials for treating a variety of malignancies (28, 47). HDAC4 is a validated target of miR-1 (39). We confirmed that HDAC4 level was indeed reduced in HCC cells expressing miR-1 (Fig. 4*C* and *D*). These results, taken together, show that the expressions of three key targets of miR-1, namely, FOXP1, MET, and HDAC4, are negatively regulated by miR-1 in HCC cells.

FoxP1, MET, and HDAC4 levels are down-regulated in HCC cells upon treatment with 5-azacytidine. If activation of miR-1 is one of the mechanisms by which 5-AzaC mediates its growth inhibitory function, it is expected that the expression of miR-1 targets should decrease in cells upon treatment with the DNA hypomethylating agent. To test this possibility, we monitored the levels of miR-1 targets in the drug-treated cell extracts by immunoblot analysis. The results showed a dose-dependent decrease in FoxP1, MET, and HDAC4 protein levels, albeit at different levels, in all six HCC cell lines treated with the 5-AzaC (Fig. 5*A–D*). The differential response is likely due to different origin of these cell lines and involvement of additional factors in the down-regulation of these miR-1 targets.

FoxP1 and MET are overexpressed in human primary HCC. Next, we measured the expression of these miR-1 targets in 11 human primary HCC samples by Western blot and immunohistochemical analyses. The results showed significant up-regulation of FoxP1 and MET in majority of the HCCs compared with the matching liver tissues (Fig. 6*A* and *B*). The levels of FoxP1 and MET increased in nine and seven HCC samples, respectively, compared with the corresponding matching livers. HDAC4 was elevated in six HCC samples. Immunohistochemical analysis of HCC sections also revealed that these proteins are expressed in malignant hepatocytes (a representative picture is shown in Supplementary Fig. S4). These data suggest that the up-regulation of oncogenic FoxP1 and MET contributes to hepatocarcinogenesis and down-regulation of miR-1 is likely to be one of the factors that contributes to this process. The present study also showed that the therapeutic intervention with DNA hypomethylating agents could inhibit HCC cell growth by reducing expression of oncogenes such as MET and FoxP1. The up-regulation of HDAC4 in primary HCCs suggests the usefulness of combination therapy (DNMT and HDAC inhibitors) against liver cancers.

Discussion

Like many growth regulatory genes, the genes encoding some mircroRNAs contain CGIs that are susceptible to methylation-mediated silencing. The major objective of the present study was to identify growth regulatory miRNAs and to determine whether re-expression of these miRs by treatment with DNA hypomethylating agents or other epigenetic drugs can inhibit growth of HCCs by altering expressions of specific target genes. Recent studies have shown that DNA demethylating agents can re-express silenced

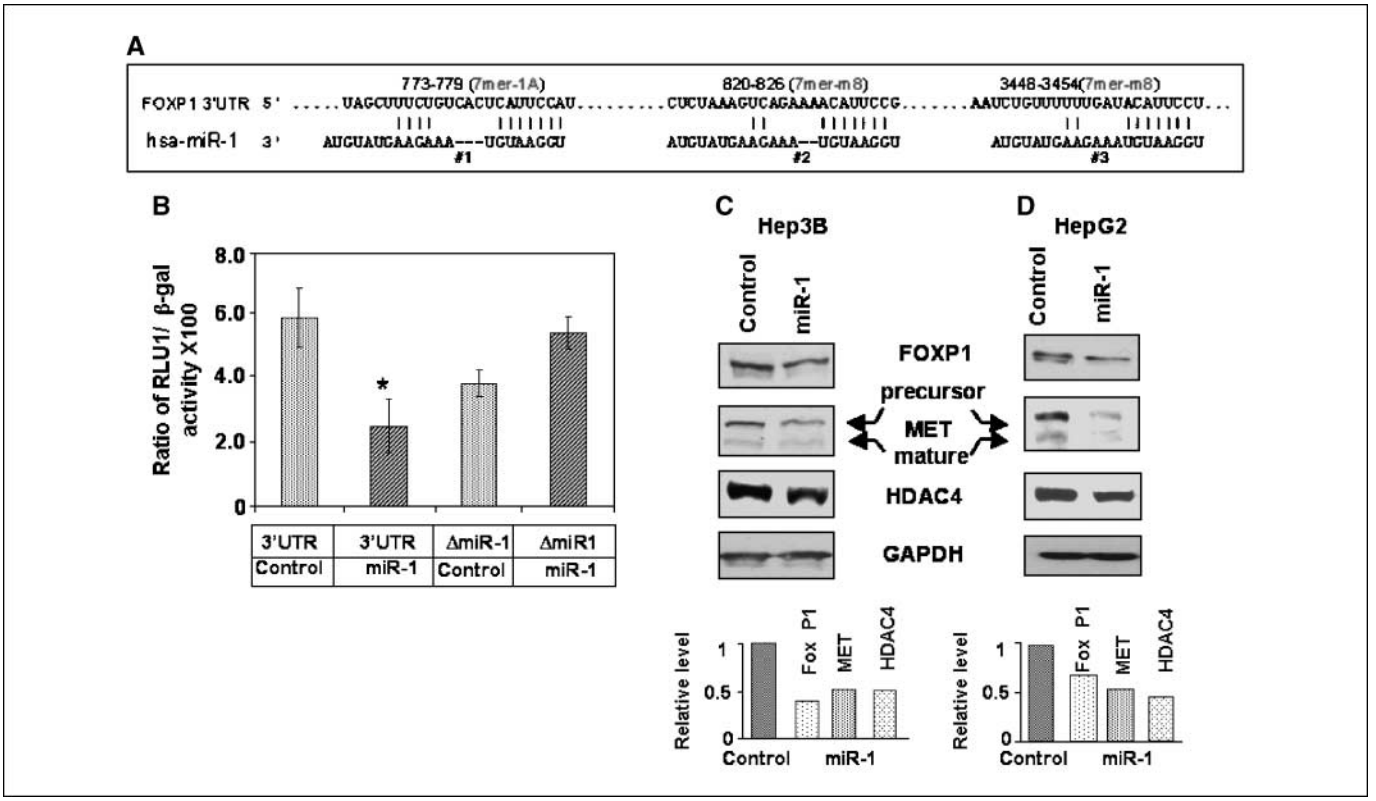

Figure 4. FoxP1 is a validated target of miR-1. *A,* the 3′-UTR of FoxP1 harbors 3 miR-1 binding sites. *B,* luciferase activity controlled by 3′UTR of FoxP1 is inhibited by ectopic expression of pre-miR-1. Hep3B cells were cotransfected with firefly luciferase-3′UTR (FoxP1) and pre-miR-1 or control RNA (60 nmol/L) along with SV40-β-gal (as internal control). After 48 h, luciferase and β-galactosidase activities were measured in cell extracts. FoxP1-3′UTR deleted of miR-1 sites were used as control. *C* and *D,* extracts from cells transfected with pre-miR-1 or control miR for 48 h were subjected to Western blot analysis with antibodies as indicated. The signal in each band in the scanned X-ray film was quantified using KODAK imaging software, and the levels were normalized to that of GAPDH.

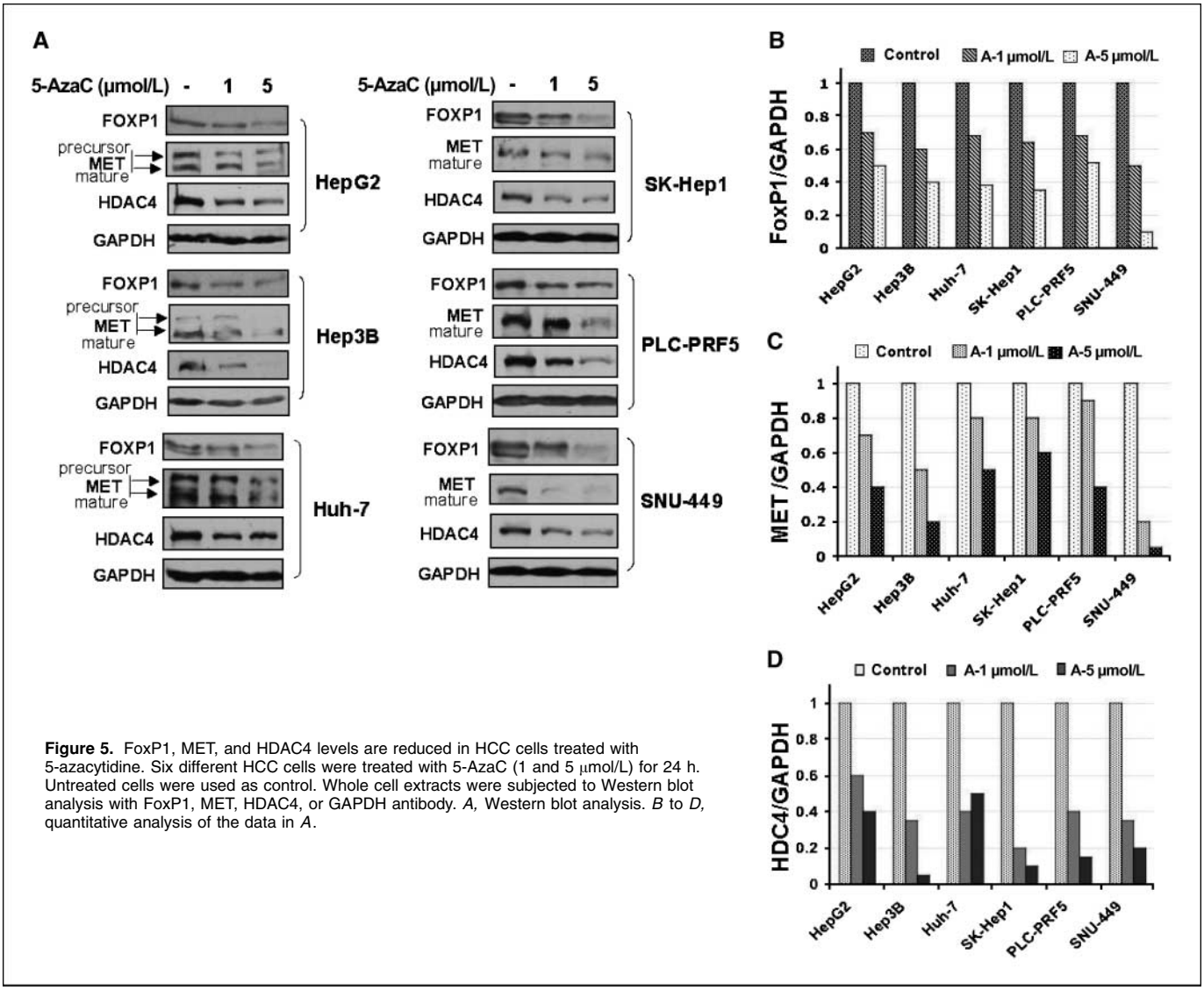

Figure 5. FoxP1, MET, and HDAC4 levels are reduced in HCC cells treated with 5-azacytidine. Six different HCC cells were treated with 5-AzaC (1 and 5 μmol/L) for 24 h. Untreated cells were used as control. Whole cell extracts were subjected to Western blot analysis with FoxP1, MET, HDAC4, or GAPDH antibody. *A,* Western blot analysis. *B* to *D,* quantitative analysis of the data in *A*.

miRs such as miR-127 and miR-124b with growth suppressor properties (12, 13). The present study showed methylation mediated suppression of miR-1 gene and its re-expression upon treatment of HCC cells with 5-AzaC, which suggested its potential function as growth suppressor. Interestingly, disruption of one of the two isoforms of miR-1 (*miR-1-2*) in murine heart resulted in induction of cell cycle regulatory genes with concomitant entry of terminally differentiated cardiomyocytes into cell cycle (39). The methylation-mediated silencing of miR-1 in primary HCCs and up-regulation of its potential oncogenic targets implicated its potential antitumorigenic function in the liver. Indeed, ectopic expression of miR-1 in nonexpressing HCC cell lines at a much lower level than that in the heart reversed many characteristics of cancer cells such as growth, replication potential, clonogenic survival, and resistance to apoptosis. Up-regulation of miR-1 targets such as FoxP1 and MET with oncogenic property in HCCs further explains growth regulatory functions of miR-1 in the liver and probably in other tissues by predisposing these tissues to neoplastic transformation due to loss of miR-1.

MicroRNA-1 is abundantly expressed in cardiac tissue, smooth, and skeletal muscle due to its induction by serum response factor. It promotes differentiation of the heart tissue by reducing expression of repressors such as Hand2 and Hdac4 (39, 48). Cloning of miR-1 from mouse liver RNA by Lagos-Quintana confirmed that it is also expressed in the liver albeit at a lower level (40). Hsa-miR-1 is located in the intron 1 of the putative ORF166 that harbors several CGIs of which CGI-81 is methylated in tumor-specific manner. There was no detectable methylation at other CGIs located upstream or downstream of *miR-1-1* gene in the livers or tumors (data not shown). These results suggest that the altered chromatin structure spanning CGI-81 in cancer cell predisposes it to methylation. Even within CGI-81, methylation was restricted to certain CpGs in the amplicon of HCC sample 2. Methylation may affect expression of miR-1-1 by inhibiting access of one or more transcription factors to their cognate sites in the chromatin context. Indeed, TESS[8] database identified cognate sites for several transcription factors such as USF, and ATF/cAMP-responsive element binding protein, whose DNA binding activities are sensitive to methylation (49).

[8] http://www.cbil.upenn.edu/cgi-bin/tess/tess

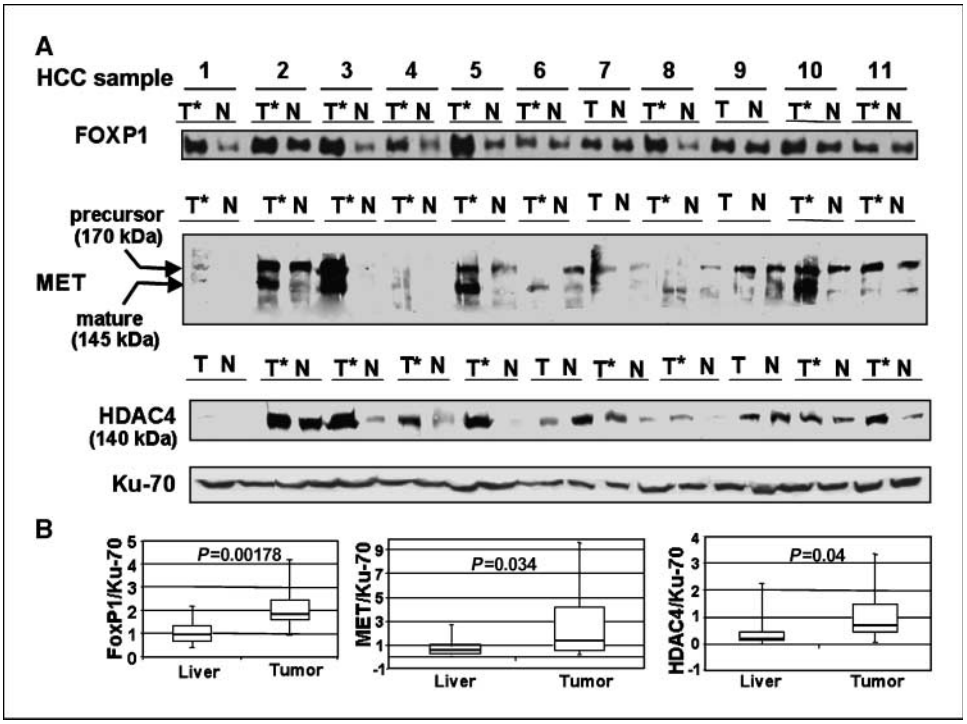

Figure 6. FoxP1, MET, and HDAC4 are elevated in primary human HCCs. *A,* whole cell extracts of HCCs and matching control tissues were subjected to Western blot analysis with antibodies specific for FOXP1, MET, HDAC4, and Ku-70 (used as loading control). *B,* quantitative representation of the data using Box and Whisker plots. A horizontal line in each box represents the median value of each protein level in each group. Box denotes 25th and 75th percentile range of scores, whereas whiskers represent the highest and lowest values.

Prediction of a few oncogenic targets of miR-1 by multiple databases provided the rationale to explore growth suppressor function of miR-1. One such target FoxP1 is unique because it can act as an oncoprotein and tumor suppressor depending upon the tissue type (41). The significant up-regulation of FoxP1 protein in human primary HCCs suggests its potential role in tumorigenesis. MET is an RTK frequently up-regulated in different types of cancers and amplified during the metastatic transition of primary tumors. Many genes that are targets of MET signaling pathway are involved in the regulation of various cellular functions, including mitogenesis, proliferation, angiogenesis, tumor cell invasion, and metastasis. Furthermore, the MET-induced gene expression signature is shared by human HCC and almost all liver metastases (46). It is conceivable that significant down-regulation of MET protein level mediated by ectopic expression of miR-1 in HCC cell lines presumably leads to reduced cell proliferation due to cell cycle arrest, replication potential, clonogenic survival, and induced apoptosis. The down-regulation of these oncoproteins by ectopic miR-1 suggests the potential therapeutic application of miR-1 mimetics against hepatocarcinogenesis.

Finally, the clinical application of HDAC inhibitors underscores the role of HDACs in tumorigenesis. HDAC4 is a class II HDAC that is recruited by sequence-specific transcription factors to repress differentiation-promoting genes (50). It is translocated to the nucleus in response to growth factors through Ras signaling pathway. Thus, the increased expression and/or nuclear translocation of HDAC4 in the liver probably stimulate cellular transformation by promoting dedifferentiation of hepatocytes. In this context, HDAC4 behaves as an oncoprotein. Its nuclear/cytoplasmic export is regulated by phosphorylation. The posttranscriptional regulation of its expression by miR-1 is another mechanism that facilitates tightly controlled expression of this enzyme. The up-regulation of miR-1 with concomitant down-regulation of its targets in HCC cells in response to chromatin modifying agents rationalizes their potential clinical application against liver cancers.

In summary, our results suggest that the activation of silent *miR-1-1* by chromatin modifiers could lead to suppression of target oncogenic proteins that are crucial in the development and progression of human cancer. Future studies in epigenetic regulation of miR-1 expression coupled to downstream signaling pathways is likely to lead to development of novel drug targets in liver cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Methylation Mediated Silencing of MicroRNA-1 Gene and Its Role in Hepatocellular Carcinogenesis

Jharna Datta, Huban Kutay, Mohd W. Nasser, et al.

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