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Differentiation Therapy by Epigenetic Reconditioning Exerts Antitumor Effects on Liver Cancer Cells

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Primary liver tumors are mainly represented by hepatocellular carcinoma (HCC), one of the most aggressive and resistant forms of cancer. Liver tumorigenesis is characterized by an accumulation of epigenetic abnormalities, leading to gene extinction and loss of hepatocyte differentiation. The aim of this work was to investigate the feasibility of converting liver cancer cells toward a less aggressive and differentiated phenotype using a process called epigenetic reconditioning. Here, we showed that an epigenetic regimen with non-cytotoxic doses of the demethylating compound 5-azacytidine (5-AZA) promoted an anti-cancer response by inhibiting HCC cell tumorigenicity. Furthermore, epigenetic reconditioning improved sorafenib response. Remarkably, epigenetic treatment was associated with a significant restoration of differentiation, as attested by the increased expression of characteristic hepatocyte markers in reconditioned cells. In particular, we showed that reexpression of these epigenetically silenced liver genes following 5-AZA treatment or after knockdown of DNA methyltransferase 1 (*DNMT1*) was the result of regional CpG demethylation. Lastly, we confirmed the efficacy of HCC differentiation therapy by epigenetic reconditioning using an *in vivo* tumor growth model. In summary, this work demonstrates that epigenetic reconditioning using the demethylating compound 5-AZA shows therapeutic significance for liver cancer and is potentially attractive for the treatment of solid tumors.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents a major cause of death from cancer worldwide and exhibits one of the lowest remission rates.[1,2] HCC follows a typical development and progression scheme, generally affecting patients suffering from a chronic liver disease caused by hepatitis B virus (HBV) and/or C virus (HCV) infection, alcohol abuse, genetic diseases, or liver steatosis.[3] Sorafenib has been considered as one of the molecules approved for the management of HCC and improving patient survival.[4] However, surgical resection and liver transplantation remain the only effective therapeutic options for liver cancer.[5] In addition, HCC prognosis remains poor and recurrence is frequent because of the high metastatic potential of primary hepatic tumors and their strong resistance to chemotherapies. Therefore, the development of new curative alternatives remains critical for liver cancer management.

Epigenetic regulatory mechanisms are essential for orchestrating cellular processes and functions.[6,7] A strong inverse correlation between DNA methylation and transcription has been well reported, because methylation at CpG dinucleotide sites is usually associated with stable gene repression.[8] In addition to its occurrence during normal development, cell differentiation, and tissue-specific gene regulation, the significance of DNA methylation has been extensively described in malignant cells, in which oncogenes and tumor-suppressor genes acquire cancer-specific methylation patterns.[9,10] In the liver, the accumulation of epigenetic abnormalities during precancerous stages frequently alters hepatocyte differentiation and survival, leading to tumorigenesis.[11] Moreover, the aberrant methylation of DNA observed in liver cancer cells is generally responsible for the epigenetic silencing of a large set of genes.[12–14]

In humans, approximately 70% of annotated gene promoters contain CpG-rich regions, which might be affected by "liver disease imprinting."[15] Unlike oncogenic mutations, which are permanent changes in the cancer genome, epigenetic alterations can potentially be reversed.[16] For this purpose, demethylating compounds show promise in erasing the silencing of critical genes and reversing the phenotypes of tumor cells epigenetically locked in a dedifferentiated state. Various data support this idea and have shown that

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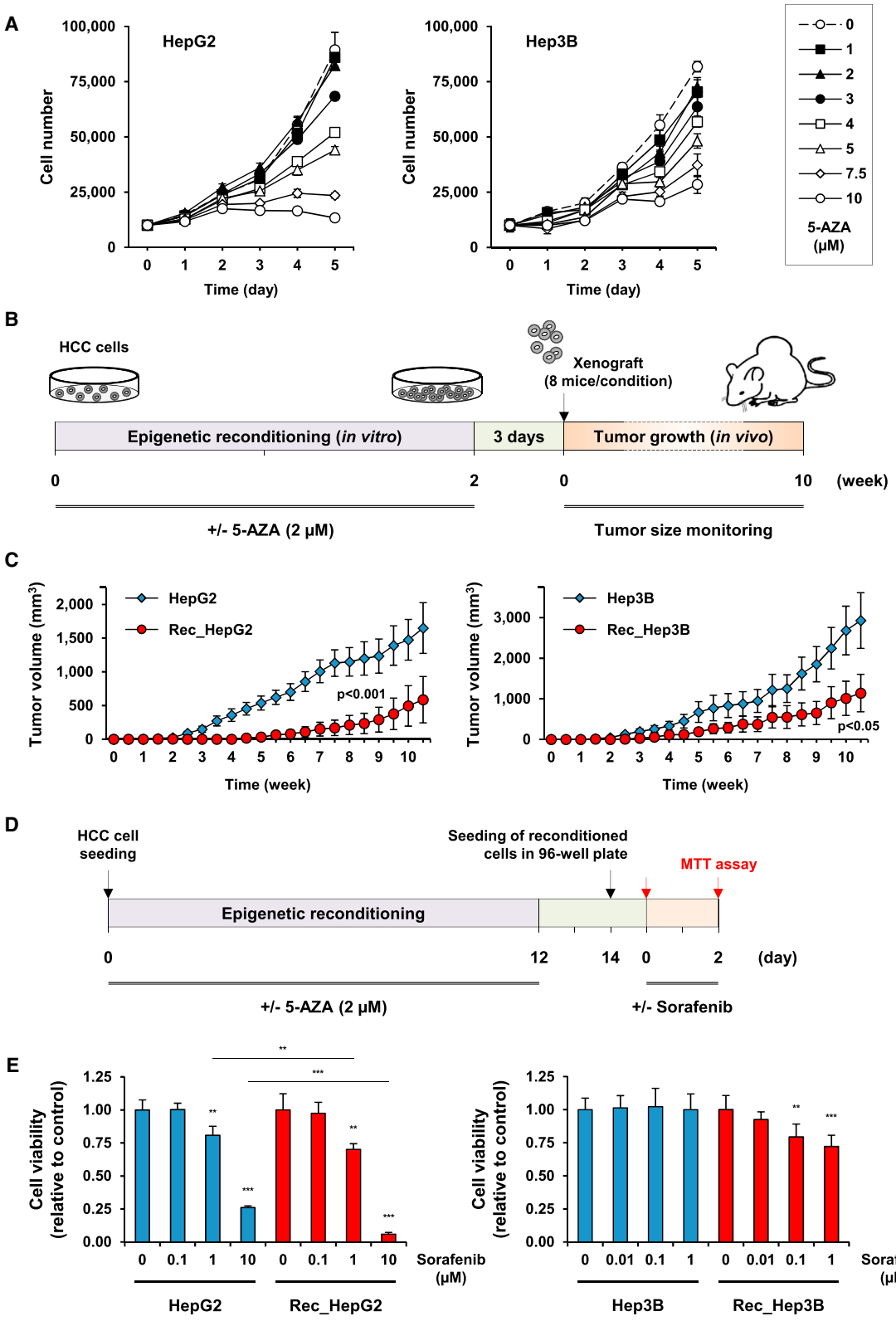

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hypomethylating drugs are attractive for epigenetically reconditioning cells derived from melanomas,[17] gliomas,[18] pancreatic adenocarcinomas,[19] epithelial tumors,[20] and hepatomas.[21,22] We recently reported that a sustained exposure to the demethylating agent 5-azacytidine (5-AZA) efficiently promoted the reexpression of drug-metabolizing enzymes and increased detoxification functions in HCC cells.[23]

In this study, we aimed to investigate the significance of liver cancer differentiation therapy using 5-AZA-based epigenetic reconditioning. We first evaluated HCC cell proliferation in response to 5-AZA treatment *in vitro* to determinate the optimal concentration for cell reconditioning. Next, HCC tumor growth was analyzed *in vivo* after the engraftment of epigenetically reconditioned cells into mice. We also examined whether the epigenetic reconditioning procedure could potentiate the cytotoxic effects of sorafenib on HCC cells. To confirm the differentiation and reactivation of liver-specific genes in the epigenetically reconditioned cells, we analyzed the expression and methylation profiles of characteristic genes related to hepatocyte phenotype. Importantly, the functional specificity of DNA methyltransferase (DNMT) enzymes in controlling liver cancer cell differentiation was explored. Lastly, we assessed the effects of 5-AZA epigenetic treatment on HCC cell differentiation and tumor growth *in vivo*.

RESULTS

Exposure to Non-cytotoxic Doses of 5-AZA Inhibits HCC Cell Tumorigenicity

To investigate the therapeutic potential of epigenetic reconditioning, we first evaluated the effect of the DNA demethylating compound 5-AZA on the proliferative abilities of the human HCC cells HepG2 and Hep3B. We used 5-AZA in this study because it has been reported to exhibit reduced DNA damage compared with 5-aza-2′-deoxycytidine (5-AZA-dC, decitabine).[24] Cell growth assessment revealed a clear dose-response effect with a gradual decrease in cell proliferation and marked toxicity in cells treated with high doses of 5-AZA (Figure 1A). No significant cell growth inhibition and morphological abnormalities were observed up to 2 μM 5-AZA. Notably, we also did not observe cytotoxicity when culturing normal human hepatocytes in the presence of 5-AZA (data not shown). The optimal concentration for a 5-AZA regimen was determined based on MTT assays to limit the toxic effects of the epigenetic drug. Accordingly, we then applied a reconditioning protocol using 2 μM 5-AZA for 2 weeks with daily replacement and assessed the *in vivo* tumorigenicity of HCC cells after ectopic engraftment into mice (Figure 1B). Remarkably, xenograft monitoring revealed an important inhibition of tumor progression by the reconditioned HepG2 and Hep3B cells compared with cells that were not treated with 5-AZA prior to implantation (Figure 1C).

Epigenetic Reconditioning Improves the Cytotoxic Effect of Sorafenib

We assessed whether epigenetic reconditioning could modify the sensitivity of liver cancer cells to sorafenib. HepG2 and Hep3B cells were reconditioned with 5-AZA (2 μM) for 2 weeks before treatment with increasing concentrations of sorafenib for 48 hr (Figure 1D). Cell viability measurements showed that HepG2 cells exhibited a response to sorafenib from 1 μM (Figure 1E). We observed that epigenetic reconditioning was able to promote this cytotoxic effect by 10.6% ± 4.3% and 20.1% ± 1.3% after 48 hr of treatment with 1 and 10 μM sorafenib, respectively ($p < 0.01$ and $p < 0.001$, reconditioned versus control cells, t test). By contrast, Hep3B cells were resistant to sorafenib up to 1 μM. Interestingly, the viability of reconditioned Hep3B cells was significantly decreased by sorafenib in a concentration-dependent manner, with an inhibition ratio equivalent to 20.6% ± 9.8% and 27.9% ± 8.6% after 48 hr of treatment with 0.1 and 1 μM sorafenib, respectively ($p < 0.01$ and $p < 0.001$ compared with the non-treated control cells, t test). While low concentrations of the drug alone had no effect on Hep3B cells, the application of 5-AZA-based epigenetic reconditioning increased the cytotoxicity of sorafenib. The most notable difference between HepG2 and Hep3B cells is their P53 status. HepG2 cells are P53 wild-type, whereas Hep3B cells are P53 deficient.[24]

Expression of Characteristic Hepatocyte Marker Genes Is Restored in Epigenetically Reconditioned HCC Cells

To evaluate the differentiation level of reconditioned liver cancer cells, we analyzed the expression of a cluster of characteristic hepato-specific genes: albumin (*ALB*), solute carrier family 10 member 1 (*SLC10A1*), cytochrome P450 (*CYP*) 3A4, and microRNA-122 (*miR-122*). These four genes were initially selected because of their specific expression levels, which were dramatically reduced in clinical samples from HCC patients ($p < 0.001$, Mann-Whitney *U* test) (Figure 2A). In addition, *in silico* analyses revealed that the *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* genes contained CpG-rich regions surrounding their transcription start sites (Figure S1). Consequently, we evaluated the expression of these four liver markers after epigenetic reconditioning of HepG2 and Hep3B cells with 2 μM 5-AZA for 12 days (Figure 2B). qRT-PCR data revealed that these genes

Figure 1. HCC Cell Tumorigenicity and Sorafenib Response after Exposure to Non-cytotoxic Doses of 5-AZA

(A) Time- and dose-dependent cytotoxicity of 5-AZA in the human HCC cell lines HepG2 and Hep3B. Twenty-four hours after seeding, cells were treated with 5-AZA at the indicated concentrations for 5 days. The number of cells was estimated at the indicated times using cell viability assays. The data represent the mean ± SD. (B) Experimental design for assessing HCC tumor growth *in vivo* after epigenetic reconditioning. HepG2 and Hep3B cells were treated with 2 μM 5-AZA for 2 weeks (*in vitro*). After 3 days without 5-AZA, the reconditioned and control cells were subcutaneously implanted into athymic nude mice. The tumor nodules were monitored twice a week for 11 weeks (*in vivo*). (C) Tumor growth assay. The data represent the mean ± SEM. The p value was calculated with a t test to statistically evaluate the difference in tumor size between the control (n = 8) and reconditioned HCC cells (n = 8). (D) Experimental design for measuring sorafenib cytotoxicity in HCC cells after epigenetic reconditioning. (E) Effect of sorafenib treatment on the growth of reconditioned HepG2 and Hep3B cells. Cell viability was measured after 48 hr of treatment at the indicated concentrations. Histograms show the mean ± SD, and statistical significance is indicated as $^{**}p < 0.01$ and $^{***}p < 0.001$ as determined by t tests. 1 μM 5-AZA = 244.2 ng/mL.

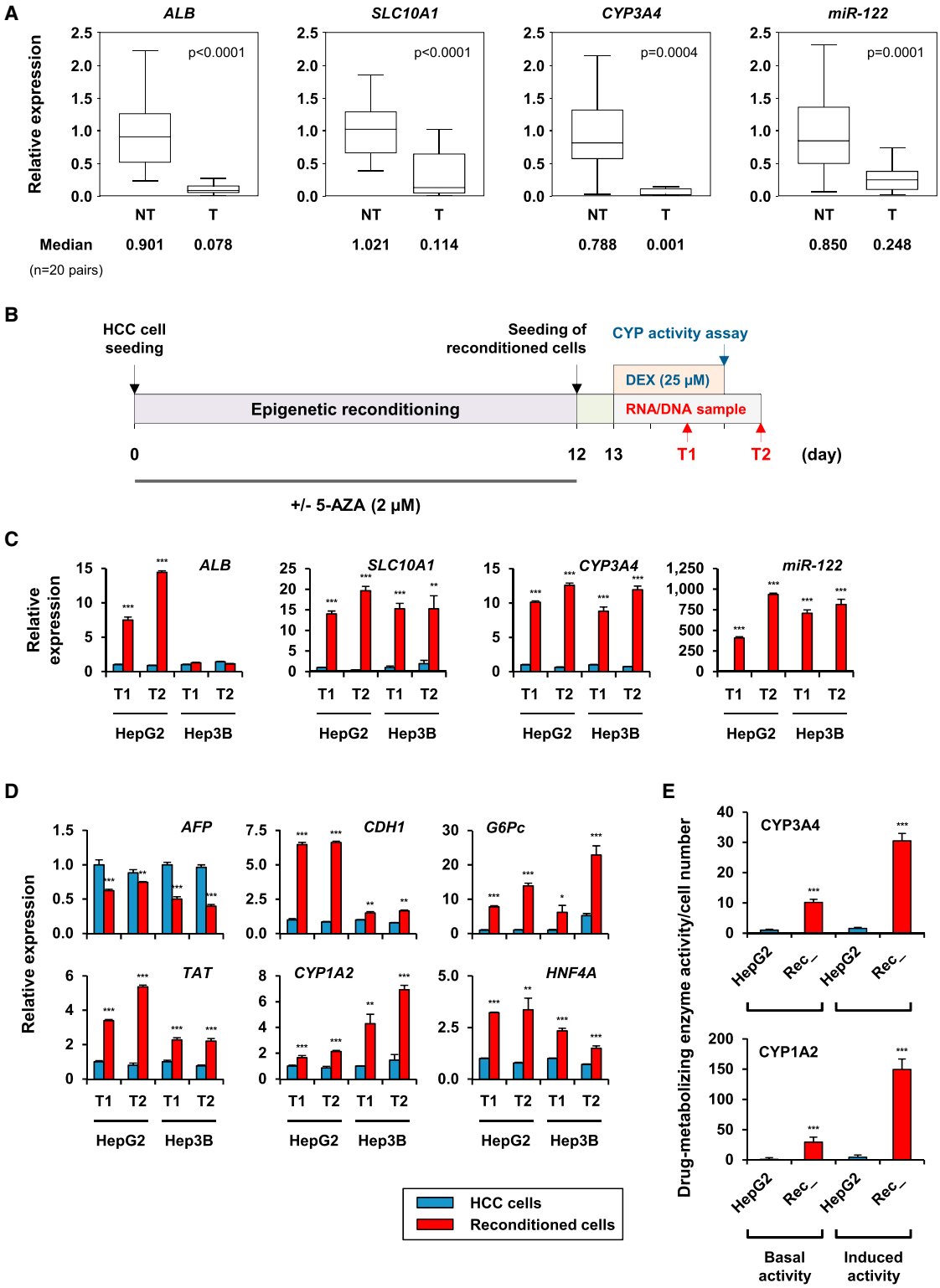

Figure 2. Hepatospecific Gene Expression and Drug-Metabolizing Activity in Epigenetically Reconditioned HCC Cells

(A) Expression levels of four characteristic liver marker genes (*ALB*, *SLC10A1*, *CYP3A4*, and *miR-122*) in clinical samples. Boxplots illustrate the differential gene expression between 20 primary HCC samples (T) and their corresponding paired non-tumor tissues (NT). Mann-Whitney *U* tests were used to calculate p values. (B) Experimental design

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were significantly induced, indicating a restoration of hepatic differentiation in the reconditioned HCC cells (Figure 2C). Interestingly, *miR-122* levels were strongly increased in the reconditioned HepG2 and Hep3B cells ($p < 0.001$, t test), whereas this major tumor-suppressor miRNA was barely detectable in the non-reconditioned cells. There was an apparent absence of *ALB* induction in the reconditioned Hep3B cells. However, *ALB* levels were already elevated in non-reconditioned Hep3B cells by approximately 7.5-fold relative to the expression levels observed in non-reconditioned HepG2 cells, arguing for an absence of *ALB* epigenetic silencing in this cell line. In addition to the genes delineating this cluster of hepatocyte markers, additional genes with importance in hepatic functions were found to be upregulated in the reconditioned cells, including *CYP1A2*, tyrosine aminotransferase (*TAT*), glucose-6-phosphatase catalytic subunit (*G6Pc*), and hepatocyte nuclear factor 4 alpha (*HNF4A*) (Figure 2D). We also observed an augmentation in the expression level of epithelial cadherin 1 (*CDH1*) after epigenetic reconditioning, as well as a significant downregulation of the oncofetal liver marker alpha-fetoprotein (*AFP*).

To further evaluate the efficacy of epigenetic reconditioning for restoring differentiation in HCC cells, we analyzed the expression levels of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* in three reconditioned HCC cell lines (HepG2, Hep3B, and Huh-7) and compared these data with the expression profiles obtained in normal human hepatocytes from three different donors. Remarkably, three of these four liver markers (*ALB*, *CYP3A4*, and *miR-122*) exhibited consistent augmentation in reconditioned cells, reaching expression levels that were very similar to those observed in human hepatocytes (Figure S2). Notably, our observation demonstrated that 5-AZA treatment had no clear effect on promoting expression of the analyzed genes in human hepatocytes (Figure S3).

Drug-Metabolizing Activity Is Increased in Reconditioned HCC Cells

To confirm the restoration of hepatic differentiation in liver cancer cells after epigenetic reconditioning, we analyzed the activity of drug-metabolizing enzymes in HepG2 cells. Measurements of CYP activity represent the gold standard for evaluating functional differentiation in hepatic cells. Among human detoxification enzymes, CYP3A4 plays the most important role in the metabolism of xenobiotics and commercial drugs.[25] As described above, HepG2 cells first underwent an epigenetic reconditioning regimen for 2 weeks (Figure 2B). Next, we measured the activity levels of two of the phase I drug-metabolizing enzymes, CYP3A4 and CYP1A2, after treatment with dexamethasone (25 μM) for 72 hr. CYP measurements showed that both basal and dexamethasone-induced CYP3A4 and CYP1A2 activities were strongly enhanced in reconditioned cells ($p < 0.001$, t test) (Figure 2E). These results were remarkable because they demonstrated a significant promotion of hepatic differentiation in the reconditioned HCC cells, as well as a recovery of hepatic detoxification functions. We confirmed the promotion of drug-metabolizing activity using another HCC cell line (Huh-7) after epigenetic reconditioning. Thus, prominent increases in both the basal and dexamethasone- and phenobarbital sodium-induced CYP3A4 activities were observed in reconditioned Huh-7 cells (Figure S4).

Hepatocyte Marker Gene Reexpression Is Associated with CpG Demethylation

Next, we investigated whether the reexpression of the cluster of four hepatocyte marker genes used to characterize the reconditioned cells was a direct consequence of DNA demethylation in response to 5-AZA exposure. As previously mentioned, we identified several CpG sites associated with the regulation of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* gene expression (Figure S1). Combined bisulfite restriction analysis (COBRA) evidenced a prominent hypermethylation of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* genes in HCC cells compared with human hepatocytes (Figure 3A), which was consistent with the altered expression of these genes observed in clinical samples from HCC patients (Figure 2A). Notably, the HepG2 cells exhibited a methylation level close to 100% for all of these hepatocyte markers. Using an ELISA-based method, we found that 5-AZA treatment dramatically reduced the global level of DNA methylation in reconditioned cells (Figure 3B). More importantly, the COBRA data revealed an extensive demethylation of the CpG sites analyzed in *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* after reconditioning (Figure 3C). The absence of epigenetic silencing was confirmed in Hep3B cells for the *ALB* gene, because no CpG sites analyzed by COBRA were found to be methylated before 5-AZA treatment. In contrast, the reexpression of *ALB* observed in reconditioned HepG2 cells was clearly correlated with a significant decrease in CpG methylation levels.

DNMT1 Invalidation Promotes Hepatic Differentiation

To clarify the respective roles of the DNMT enzymes and to determine their functional specificity or redundancy in liver cancer cells, we first measured the global levels of DNA methylation in HepG2 cells after the respective knockdown of *DNMT1*, *DNMT3A*, and *DNMT3B* (Figure 3D). The ELISA data showed that *DNMT1* silencing significantly reduced DNA methylation in the cells, with approximately 50% inhibition 8 days after transfection ($p < 0.05$ and $p < 0.01$ using siDNMT1_A and siDNMT1_B, respectively, t test). Conversely, no modification in the DNA methylation levels

for evaluating liver gene expression and drug-metabolizing activity in HCC cells after epigenetic reconditioning. (C) Expression levels of selected hepatospecific genes in epigenetically reconditioned HepG2 and Hep3B cells. Total RNA was extracted after reconditioning with 2 μM 5-AZA for 12 days and 3 and 5 days of culture without 5-AZA (T1 and T2, respectively). The relative mRNA expression levels were determined by RT-qPCR. Non-reconditioned HCC cells were used as controls. (D) Relative levels of the *AFP*, *CDH1*, *G6Pc*, *TAT*, *CYP1A2*, and *HNF4A* mRNAs measured by RT-qPCR in the control and reconditioned HCC cells. (E) Evaluation of CYP3A4 and CYP1A2 enzyme activity. CYP activities were induced by treatment with 25 μM dexamethasone for 72 hr before assessment. All data shown in the figure represent the mean ± SD. Statistically significant differences in gene expression and CYP activity levels were achieved at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (t test).

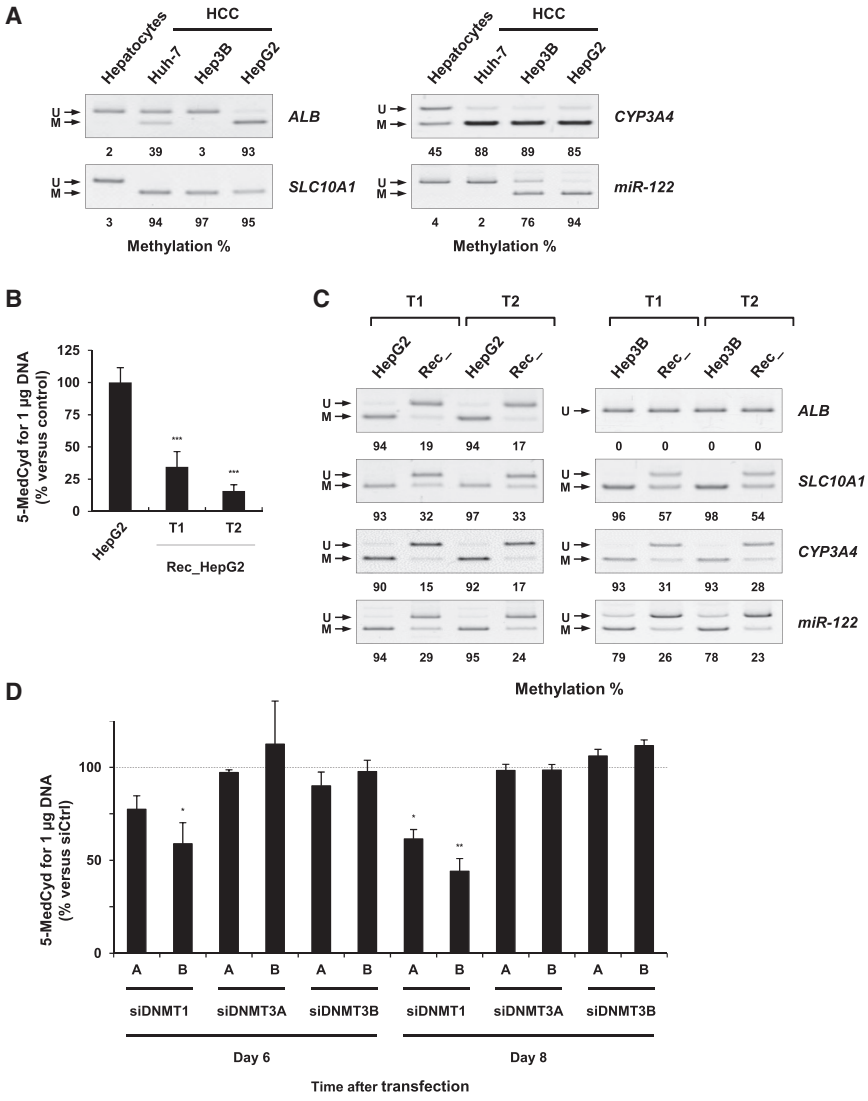

Figure 3. Methylation Profiles of Liver Marker Genes in Reconditioned Cells

(A) Comparison of the methylation levels of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* between human hepatocytes and HCC cells. COBRA was performed to evaluate the CpG methylation ratios (%) of gene promoter regions. The CpG sites were identified by *in silico* analysis (Figure S1). (B) Effect of 5-AZA treatment on the global DNA methylation level in HepG2 cells. The levels of 5′-methyl-2′-deoxycytidine (5-MedCyd) in the DNA samples were quantified with an ELISA-based method at the indicated times. (C) Methylation status of the *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* genes in reconditioned HepG2 and Hep3B cells, as evaluated by COBRA. (D) Methylated DNA levels after selective knockdown of *DNMT1*, *DNMT3A*, and *DNMT3B* in HepG2 cells. The histograms show the 5-MedCyd ratios from siDNMT-transfected cells relative to those of cells transfected with control siRNAs. Histograms shown in the figure represent the mean ± SD. Statistical significance versus control cells (non-reconditioned and siCtrl-transfected cells): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (t test). Representative data from three COBRA experiments are shown for each gene. M, methylated; U, unmethylated.

was observed after *DNMT3A* and *DNMT3B* repression. We analyzed the expression levels of *DNMT1* in biopsies from liver cancer patients by qRT-PCR and found that *DNMT1* was overexpressed in HCC tumors compared with their adjacent non-neoplastic tissues ($p = 0.024$, Wilcoxon test) (Figure 4A). Furthermore, inhibition of DNMT1 at the protein level in response to 5-AZA treatment was confirmed by immunoblotting in HepG2 cells (Figure 4B).

Next, we evaluated the consequences of *DNMT1* silencing on HCC cell differentiation and hepatic functions. First, we found that *DNMT1* inhibition was correlated with a significant augmentation of drug-metabolizing activity as evidenced by CYP3A4 and CYP1A2 assays (Figures 4C and 4D). Furthermore, the restoration of hepatic differentiation was confirmed in the transfected cells on the basis of their increased expression of the four hepatocyte markers previously characterized in epigenetically reconditioned cells after 5-AZA treatment (Figure 4E). Other hepatospecific genes were significantly upregulated in the *DNMT1*-knockdown HepG2 and Hep3B cells (Figure S5). Importantly, the COBRA data revealed that the reexpression of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* was correlated with a substantial demethylation of regional CpG sites (Figure 4F). Using a similar approach, we analyzed the expression and methylation profiles of these hepatospecific genes in HepG2 cells after *DNMT3A* and *DNMT3B* silencing. We found that the expression levels of the four markers remained unchanged after *DNMT3A* (Figure S6) and *DNMT3B* inhibition (Figure S7). Furthermore, COBRA data showed that neither *DNMT3A* nor *DNMT3B* knockdown was able to reverse the epigenetic silencing of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* (Figure S8).

Epigenetic Reconditioning Shows Efficacy for HCC Differentiation Therapy *In Vivo*

To strengthen our *in vitro* data and to support the potential of HCC differentiation therapy by epigenetic reconditioning, we tested whether 5-AZA could modify the malignant phenotype of liver cancer cells *in vivo* using an HCC tumor growth model in mice. A pilot dose-response experiment indicated that 5-AZA concentrations up to 3 mg/kg did not affect animal survival (Figure 5A) or body weight (Figure 5B) after 5 weeks of treatment. Next, HepG2 and Hep3B cells were subcutaneously implanted into mice, and 5-AZA treatment was started when tumors reached a minimal size of 100 mm^3. The epigenetic reconditioning protocol consisted of an intraperitoneal injection

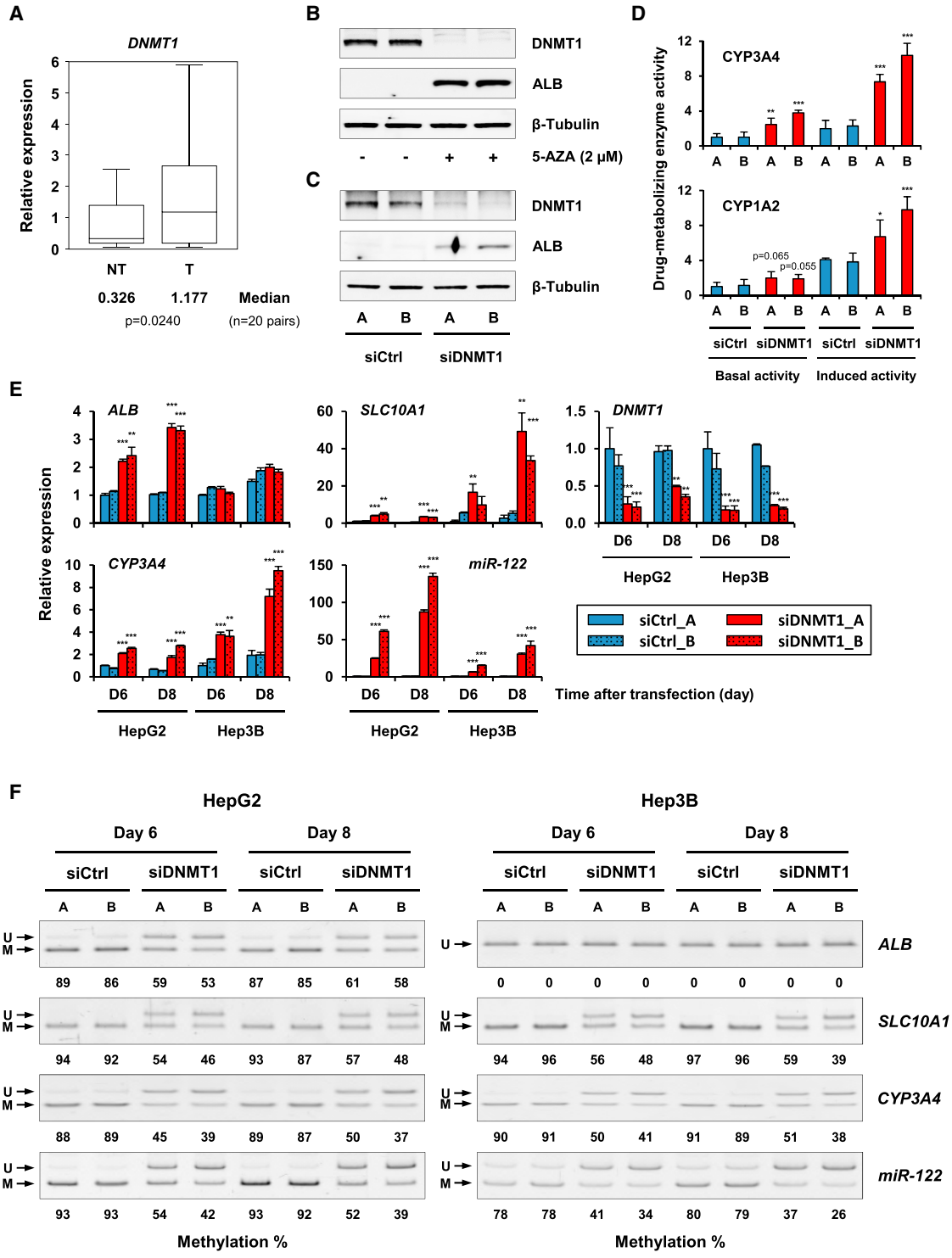

Figure 4. Evaluation of Hepatic Differentiation in HCC Cells after *DNMT1* Knockdown

(A) Expression of *DNMT1* in clinical liver samples. The expression levels of *DNMT1* in 20 primary HCC tumors and their corresponding paired non-tumorous tissues were determined by RT-qPCR. The Wilcoxon signed-rank test was used to evaluate the p value. DNMT1 and ALB protein levels in (B) epigenetically reconditioned and (C) *DNMT1*-knockdown HepG2 cells. Proteins were extracted 8 days after transfection. β-Tubulin was used as a loading control for immunoblots. Two distinct siRNAs were used to specifically target *DNMT1* (siDNMT1_A and siDNMT1_B), and two scrambled siRNAs were used as negative controls (siCtrl_A and siCtrl_B). (D) CYP3A4 and CYP1A2 activity

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of 3 mg/kg 5-AZA, six times/week (Figure 5C). After 4 weeks of treatment, the mice were euthanized according to the animal care guidelines of our institute, and their tumors were collected. After resection, the tumors were markedly smaller in the mice that received injections of the demethylating agent (Figure 5D). As presented in Figure 5E, xenograft monitoring showed a significant inhibition of HepG2 and Hep3B tumor progression after 3 weeks of 5-AZA treatment ($p < 0.05$, t test; n = 8 mice per group).

To confirm that epigenetic reconditioning was effective *in vivo*, we analyzed the changes in expression of the characteristic genes related to hepatic functions and hepatocyte differentiation in resected tumors. The data were fully consistent with the observations made of the cells reconditioned by 5-AZA treatment *in vitro*. Specifically, we observed that *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* expression levels were very low in the untreated mice, which confirmed the undifferentiated status of the tumors generated with HepG2 and Hep3B cells (Figure 6A). In contrast, the epigenetic treatment led to a significant upregulation of these hepatic markers. Spearman's rank correlation analyses showed that the increased expression of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122* was highly correlated with the regional demethylation of the CpG sites associated with their respective promoters, as evidenced by COBRA data (Figure 6B). Furthermore, other major hepatospecific genes, such as *G6Pc*, *TAT*, *CYP1A2*, and *HNF4A*, and the tumor-suppressor gene *CDH1* were found to be upregulated in the HCC tumors after epigenetic treatment (Figure 6C), which further argued for the restoration of hepatic differentiation.

DISCUSSION

Our present study provides evidence for an efficient differentiation therapy via the epigenetic reconditioning of liver cancer cells. We report that administration of non-cytotoxic doses of 5-AZA reverses the tumor properties of HCC cells and restores hepatic differentiation. Furthermore, our results show that the altered expression of characteristic hepatocyte genes observed in HCC cells is associated with epigenetic silencing through DNA hypermethylation. We demonstrate that this cancer imprinting can be efficiently reversed by epigenetic treatment or by *DNMT1* knockdown.

CpG methylation is primarily controlled by three major DNMT enzymes.[6,26] Our data implicated DNMT1, rather than DNMT3A and DNMT3B, in the maintenance of the undifferentiated phenotype of HCC cells, which was reversed by epigenetic reconditioning. *DNMT1* gene expression is known to be increased in livers affected by the hepatitis viruses[27] and, more dramatically, in HCC tumors, in which *DNMT1* upregulation correlates with poor prognosis.[28] Park and colleagues[29] showed that the experimental introduction of the oncogenic HBx protein from HBV increased total DNMT activity by upregulating *DNMT1* and *DNMT3A*, and selectively promoted the regional hypermethylation of specific tumor-suppressor genes. Moreover, genome-wide DNA methylation profiling identified another group of tumor suppressors that were epigenetically silenced in HCC patients infected with HCV.[30] In accordance with our findings, Robert and collaborators[31] found that the DNMT1 enzyme is required to maintain global methylation and aberrant CpG methylation in colon tumor cells. The authors also demonstrated that specific depletion of *DNMT1*, but not of *DNMT3A* and *DNMT3B*, significantly promoted the ability of 5-AZA-dC to reactivate the tumor-suppressor genes silenced by hypermethylation. Recently, Liu and colleagues[32] reported that *DNMT1* knockdown or 5-AZA-dC treatment sensitized drug-resistant HCC cells to sorafenib treatment.

The cytidine analog 5-AZA and its deoxy derivative 5-AZA-dC were initially synthesized and tested as cytotoxic drugs in the 1960s and were later demonstrated to have DNA demethylating activity.[33] The use of these hypomethylating agents has shown efficacy in treating myelodysplastic syndromes[34] and has gained attention for the treatment of solid tumors.[35,36] Tsai and coworkers[20] reported that transient exposure of leukemic and epithelial tumor cells to low doses of 5-AZA and 5-AZA-dC was sufficient to produce an antitumor response. In addition, other DNA methylation inhibitors, such as Zebularine (ZEB),[37] RG108,[38] and Nanaomycin A,[39] represent valuable chemical options for reactivating tumor-suppressor genes and tissue-specific genes in human cancer cells. In liver cancer the Thorgeirsson group[22] reported that ZEB treatment could modulate the fraction of cancer stem cells. Notably, the effect of the drug appeared to be dependent on cell density, because low-density ZEB-derived spheres showed the acquisition of stemness potential and enhanced tumor-initiating ability, whereas high-density ZEB-derived cells had reduced stemness, showed low tumor-initiating potential, and exhibited upregulation of liver differentiation-related genes.

Given the short half-life of previously developed epigenetic drugs, second-generation DNA hypomethylating agents are promising. For example, guadecitabine (SGI-110) is a novel demethylating compound that was designed as a dinucleotide of decitabine and deoxyguanosine, and is more resistant to cytidine deaminase degradation. SGI-110 efficiently impedes HCC progression in pre-clinical models alone[40] or in combination with sorafenib.[41] Interestingly, Jueliger and colleagues[40] demonstrated that SGI-110 inhibited the growth of HCC cells overexpressing the histone H2A variant macroH2A1, an oncogene upregulated in liver cancer cells that synergizes with DNA methylation in silencing tumor-suppressor genes and preventing the inhibition of cell growth by decitabine.[42]

assay in the *DNMT1*-knockdown cells. Five days after transfection, HepG2 cells were treated with 25 μM dexamethasone for 72 hr, and the induced activities were measured. (E) Relative expression of *ALB*, *NTCP*, *CYP3A4*, and *miR-122* following *DNMT1* silencing in HepG2 and Hep3B cells. Gene expression levels were measured 6 and 8 days after transfection. (F) COBRA analysis of the *ALB*, *NTCP*, *CYP3A4*, and *miR-122* genes after *DNMT1* knockdown. Genomic DNA was extracted from HepG2 and Hep3B cells 6 and 8 days after cell transfection. Representative data from three COBRA experiments are shown for each gene. The data represent the mean ± SD. Statistical significance relative to the siCtrl-transfected cells: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (t test). M, methylated; U, unmethylated.

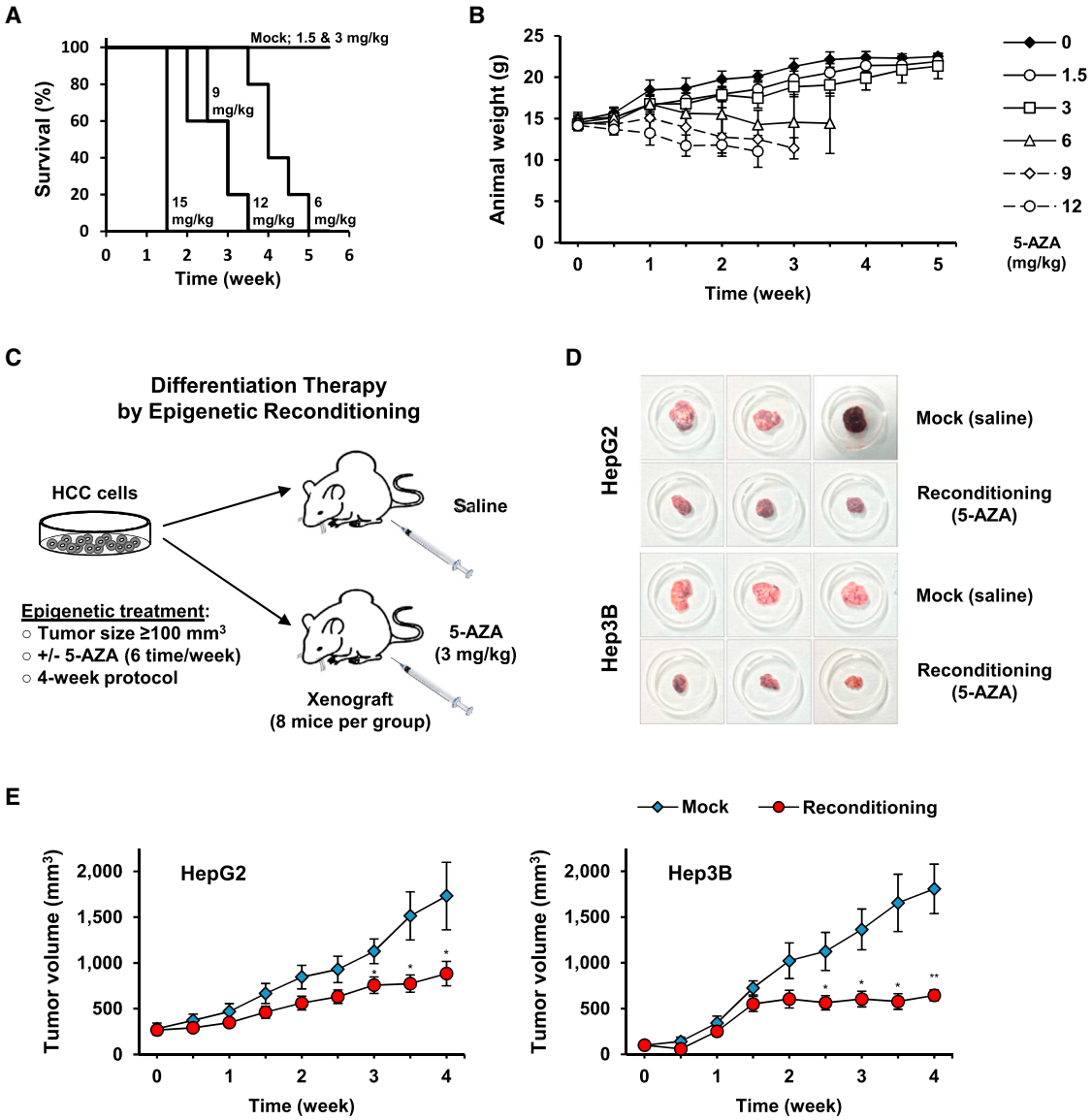

Figure 5. Differentiation Therapy by *In Vivo* Epigenetic Reconditioning: HCC Tumor Growth Assessment

(A) Survival of animals treated with 5-AZA. Each mouse received a daily intraperitoneal injection of 5-AZA diluted in sterile saline solution at concentrations ranging from 1.5 to 15 mg/kg for 5 weeks (six times/week), to determine the maximum tolerated dose of the demethylating drug for *in vivo* administration. Each dose was tested in five mice. (B) Animal weights in response to 5-AZA treatment. The condition and weight of each mouse was monitored twice per week. (C) Schematic outline of the epigenetic therapeutic protocol using an *in vivo* model of HCC tumor growth. HepG2 and Hep3B cells were used to generate tumors in athymic nude mice. *In vivo* cell reconditioning was performed by treating the mice with 5-AZA for 4 weeks (six times/week) at a dose of 3 mg/kg by intraperitoneal injection. Saline solution was injected for the control group. (D) Representative size of the tumors at the end of the experimental protocol. Saline-treated mice: 1,730 ± 368 mm^3 for HepG2 cells and 1,808 ± 269 mm^3 for Hep3B cells. 5-AZA-treated mice: 883 ± 134 mm^3 for HepG2 cells and 644 ± 60 mm^3 for Hep3B cells. (E) HCC tumor growth in response to 5-AZA treatment *in vivo*. The size of the tumor nodules in the reconditioned (n = 8) and control groups (n = 8) was monitored twice a week for 4 weeks. The data show the mean ± SEM. Statistical significance was evaluated with a t tests (*$p < 0.05$; **$p < 0.01$).

Additional works have described the use of demethylating compounds for redifferentiating hepatoma cells and liver progenitors.[23,43] In an interesting study, Dannenberg and coworkers[44] analyzed the expression of approximately 54,000 transcripts by microarray, including several *CYP* genes, in HepG2 cells in the presence or absence of 5-AZA-dC. More recently, He and colleagues[45] demonstrated that 5-AZA treatment significantly induced the expression of *ALB*, *TAT*, and *CYP7A1* in a differentiation model with mouse embryonic hepatic progenitor cells. However, none of these studies evaluated the potential of differentiation therapy by epigenetic

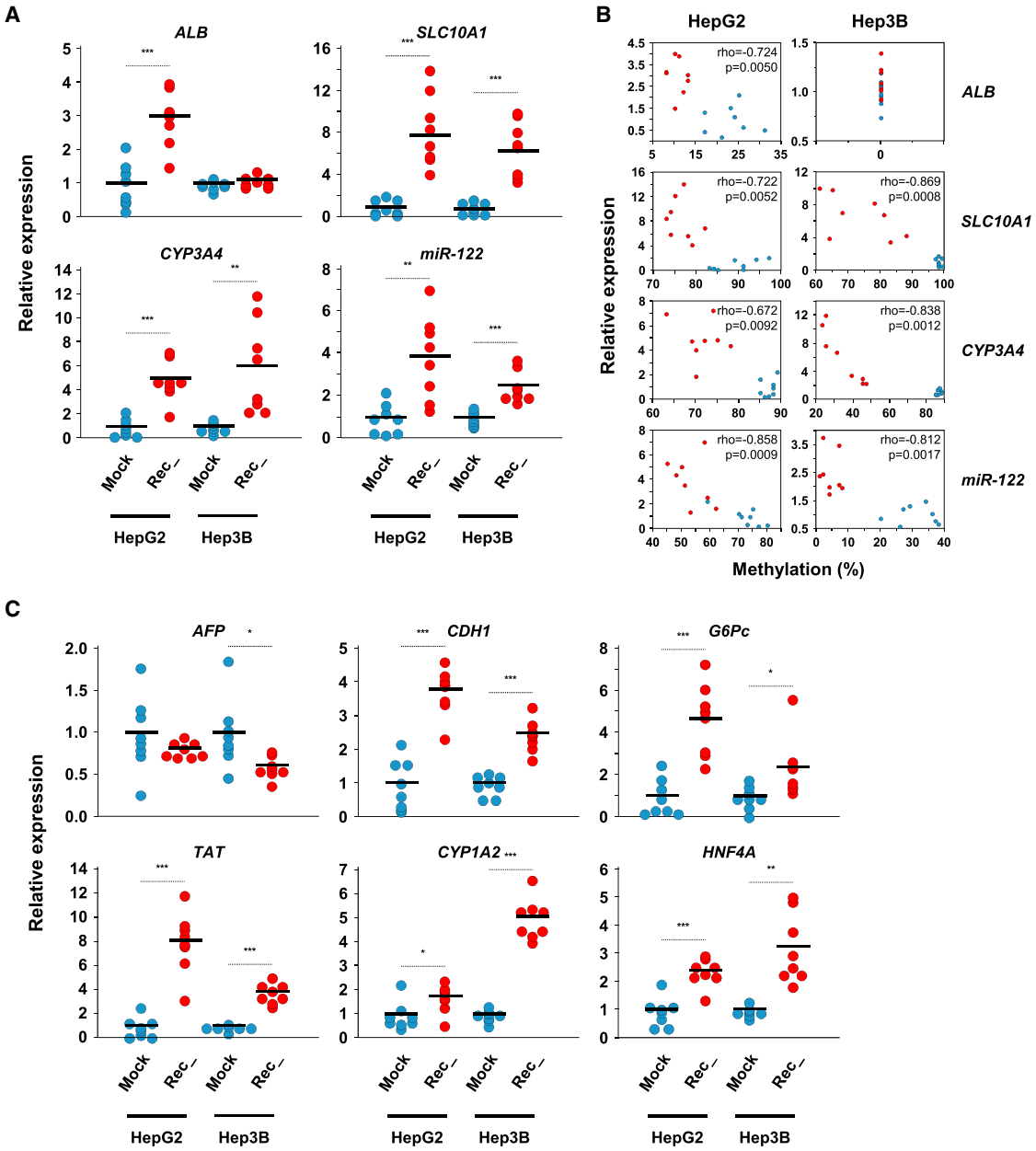

Figure 6. Differentiation Therapy by *In Vivo* Epigenetic Reconditioning: Hepatospecific Gene Expression and Methylation Profiles

(A) Relative expression of the hepatic markers *ALB*, *NTCP*, *CYP3A4*, and *miR-122* after epigenetic reconditioning *in vivo*. Total RNA and genomic DNA were extracted from resected tumors at the end of the treatment for analysis. (B) Scatterplots for Spearman's rank correlation analysis between the gene expression levels and DNA methylation in the HCC tumors. The red and blue plots show the 5-AZA-treated mice (n = 8) and saline-treated mice (n = 8), respectively. (C) Relative expression levels of *AFP*, *CDH1*, *G6Pc*, *TAT*, *CYP1A2*, and *HNF4A* in HCC tumors after 5-AZA treatment. Statistical significance: *p < 0.05; **p < 0.01; ***p < 0.001 (t test). Horizontal bars depict the average expression values.

reconditioning with an *in vivo* model of HCC tumor growth, by analyzing hepatospecific gene expression and methylation profiles. Recently, we reported that 5-AZA-treated HCC cells exhibited increased detoxification functions, which were relatively similar to those observed in human hepatocytes, and extensive storage of lipids and glycogen, which is characteristic of a hepatic phenotype. These cells also exhibited a gradual increase in the expression of a large number of genes associated with lipid metabolism, carbohydrate metabolism, and amino acid metabolic processes.[23]

In the present work, we showed that epigenetic reconditioning using 5-AZA improved the cytotoxic effect of sorafenib against HCC cells.

Sorafenib is an inhibitor of multiple kinases approved for HCC treatment.[4] Two large-scale phase III clinical trials have reported improvement in the survival of advanced HCC patients.[46] However, the survival benefit of sorafenib treatment is limited because HCC cells frequently develop drug resistance.[47] The efficacy of combining 5-AZA and sorafenib has been reported in patients with acute myeloid leukemia.[48] To the best of our knowledge, no study has described the therapeutic potential of a pretreatment model using 5-AZA followed by sorafenib to treat solid tumors. Importantly, our data demonstrate that a sequential therapeutic approach rather than the simultaneous use of a combination of two drugs was appropriate to recondition HCC cells and prime these cells for killing by another anti-cancer agent.

Liver tumorigenesis is characterized by reduced differentiation and extinction of tissue-specific genes, which is often associated with the altered expression of liver-enriched transcription factors.[49] Among the characteristic hepatocyte genes upregulated in reconditioned cells after 5-AZA exposure, we found that *HNF4A* was considerably induced (Figures 2D and 6C). *HNF4A* belongs to the nuclear hormone receptor superfamily, is expressed at high levels in differentiated hepatocytes, and binds to the promoters of approximately 12% of the genes expressed in adult livers.[50] By contrast, the expression of the hepatic lineage marker, *AFP*, which is expressed in fetal livers and is frequently reactivated in liver tumor cells,[51] was downregulated in both the reconditioned HepG2 and Hep3B cells. Another major hepatospecific marker, *miR-122*, was markedly reexpressed in HCC reconditioned cells. This miRNA has been extensively reported as being significantly downregulated in hepatic tumor tissues and HCC cell lines.[52] Of the miRNAs expressed in the liver, *miR-122* is probably the most important because of its essential role in hepatic phenotype maintenance and tumor suppression,[53] as well as its high expression level in adult liver cells.[54] We previously identified *miR-148a* as another hepatic tumor-suppressor miRNA that was also epigenetically silenced by CpG hypermethylation in liver cancer cells.[55] Experimental reexpression of *miR-148a* significantly promoted liver-specific gene expression in mouse fetal hepatoblasts and HCC cells by directly targeting *DNMT1*. Although the mechanisms responsible for the altered expression of miRNAs in liver cancer cells remain to be elucidated,[56,57] our observations involving *miR-122* and *miR-148a* clearly demonstrated a link between the oncogenic silencing of critical tumor-suppressor miRNAs and the hypermethylation of their promoter regions.

Given the non-specificity inherent to most demethylating drugs, clarification of the underlying mechanisms by which 5-AZA treatment influences drug response and tumor growth remains complex. In this work, we focused on two major tumor-suppressor genes that are expressed in the liver, *miR-122* and *CDH1*, with the aim of explaining, in part, the mechanism of action of 5-AZA at the molecular level. Indeed, reexpression of *miR-122* in HCC cells was previously reported to significantly promote the cytotoxic effect of sorafenib and to limit tumor growth.[58] Moreover, the remarkable overexpression of *CDH1* observed in reconditioned HCC cells was consistent with the current literature that describes the regulation of *CDH1* by CpG methylation and the extensive implication of this gene in the suppression of tumor cell invasion and promotion of cell differentiation.[59] Together, these characteristics suggested that the antitumor effect of 5-AZA in HCC cells could be mediated, at least to a certain extent, through reexpression of *miR-122* and *CDH1*, which strongly supports the suitability of epigenetic reconditioning strategies for HCC differentiation therapy.

The establishment of epigenetic-based therapies for solid tumor treatment remains challenging. We tested whether 5-AZA-mediated reconditioning was reversible over time by analyzing the expression profile of hepatospecific genes: (1) in reconditioned HepG2 cells 2 weeks after discontinuation of the 5-AZA treatment (Figure S9) and (2) in HCC tumors generated *in vivo* from reconditioned HepG2 and Hep3B cells 11 weeks after their implantation in mice (Figure S10). With the exception of *miR-122*, which remains overexpressed in tumors generated from reconditioned HepG2 cells (Figure S10), the enhanced expression of *ALB*, *SLC10A1*, and *CYP3A4* was abolished in both HepG2 and Hep3B tumors after 11 weeks. A similar impairment was observed in HepG2 cells *in vitro* 2 weeks after 5-AZA withdrawal for *SLC10A1* and *CYP3A4*, whereas *ALB* and *miR-122* exhibited a sustained expression level 2 days and 2 weeks after 5-AZA treatment was discontinued (Figure S9). These findings suggest that reiterated treatments might be appropriate for ensuring the therapeutic effect of demethylating agents at non-cytotoxic doses over time in liver tumor cells. Consequently, it will be critical to accurately determine the optimal dosage of the demethylating agents and maximize the treatment duration and its frequency to ensure patient response and tolerance. In addition, further investigations will be required to address the specificity of cancer cell reconditioning with regard to undesirable gene reexpression and possible side effects on non-neoplastic cells. Indeed, the broad action spectra of demethylating compounds will make it difficult to precisely evaluate curative gene reexpression, because epigenetic compounds are expected to demethylate the whole genome in a non-specific manner. However, epigenetic drugs seem to preferentially reactivate genes that have been abnormally silenced in cancer cells, as shown in a previous study by Liang and collaborators.[60] In addition, our data showed that the increased expression of key tumor-suppressor genes (e.g., *miR-122*, *CDH1*) and tissue-specific genes (e.g., *ALB*, *SLC10A1*, *HNF4A*, *CYPs*), in response to 5-AZA treatment, was accompanied by the downregulation of other markers related to hepatic cell dedifferentiation, such as *AFP*.

In conclusion, our study demonstrates an effective strategy for HCC differentiation therapy using the demethylating compound 5-AZA. We report that epigenetic reconditioning of liver cancer cells reduces tumorigenicity and improves the cytotoxic effect of sorafenib. In addition, the increased expression of critical hepatocyte markers and tumor-suppressor genes emphasizes the restoration of hepatic differentiation in reconditioned HCC cells. Further investigation will be essential to determining the value of epigenetic reagents for cancer cell reconditioning and for future therapeutic applications.

Nevertheless, we are convinced that DNA demethylating agents represent attractive options for developing epigenetic differentiation therapies with the aim of limiting the aggressiveness of solid tumors.

MATERIALS AND METHODS

HCC Cell Lines, Human Hepatocytes, and Clinical Samples

The human HepG2 and Hep3B cells were purchased from the American Type Culture Collection. The human Huh-7 cells were purchased from the RIKEN Bio Resource Center. All cultured HCC cells were maintained in DMEM (GIBCO) supplemented with penicillin (50 IU/mL; GIBCO), streptomycin (50 μg/mL; GIBCO), and 10% fetal bovine serum (FBS; Thermo Scientific). The human cryopreserved hepatocytes were purchased from XenoTech and maintained in a medium composed of William's Medium E (GIBCO), L-glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL), and 10% FBS, supplemented with hepatic growth factor (HGF; 25 ng/mL; PeproTech), insulin (5 μg/mL; Sigma), and hydrocortisone 21-hemisuccinate (2×10^{-7} M; Sigma). Twenty-four hours after seeding, FBS was removed from the hepatocyte medium. The clinical samples included 20 pairs of primary HCCs and their corresponding non-tumor tissues (see Table S1 for clinical data).

Reagents

The demethylating agent 5-AZA (PubChem CID: 9444) was from Sigma (#A2385). The drug was dissolved in PBS as a 10 mM stock, filtered (0.22 μM), and stored at −20°C in aliquots that were thawed immediately prior to use. The *in vitro* epigenetic reconditioning was performed by addition of 5-AZA to the HCC cells at a concentration of 2 μM. The medium was replaced daily, and 5-AZA was maintained throughout the procedure. Sorafenib (Nexavar; PubChem CID: 216239) was purchased from Santa Cruz Biotechnology (#SC-220125). The compound was dissolved in DMSO as a 10 mM stock solution.

Cell Growth Assay

For the evaluation of the time- and dose-dependent cytotoxicity of 5-AZA, HCC cells were seeded at 10,000 cells/well in 96-well plates. The next day, the medium was changed and cells were treated with the indicated concentrations of 5-AZA for 1–5 days. Cell viability was measured at the indicated times using the Cell Counting Kit-8 (Dojindo), according to the manufacturer's instructions (MTT assay). The absorbance at 450 nm was measured using the Synergy H4 Microplate Reader system (BioTek). For the evaluation of sorafenib cytotoxicity, reconditioned and control cells were seeded in 96-well plates (10,000 cells/well). The next day, the medium was changed, and the cells were cultured in medium containing different concentrations of sorafenib for 48 hr. Treatment with 5-AZA was discontinued 2 days before seeding in 96-well plates, and cells were maintained without 5-AZA until the end of the experiments. Cell viability was measured as mentioned above.

Xenograft Establishment and Tumorigenicity Assay

Female athymic nude mice were purchased at 4–5 weeks old and housed in isolator units under controlled humidity and temperature, with a 12-hr light-dark cycle. The animals received standard sterilized food and water *ad libitum*. The epigenetically reconditioned cells (after *in vitro* reconditioning) and control HCC cells were subcutaneously implanted into the right flanks of the mice at a density of 8×10^6 cells by inoculation in DMEM without serum (100 μL/mouse). The tumor nodules were monitored twice a week by palpation using a digital caliper. The tumor size was determined using the formula (length × width^2)/2 (mm^3). The experiments continued until tumors reached the maximum allowable size. Animal experiments were performed in accordance with the regulations of the National Cancer Center Institutional Animal Care and Use Committee.

Cell Transfection

HepG2 and Hep3B cells were seeded at a density of 50,000 cells/cm^2 in 35-mm-diameter culture dishes and transfected the next day using the TransFectin lipid reagent (Bio-Rad Laboratories). The cells were incubated with the transfection mix containing 100 nM small interfering RNA (siRNA) and 5 μL of TransFectin in a 1.2 mL total volume of serum- and antibiotic-free OptiMEM (Invitrogen) for 5 hr. The human DNMT1 (ID #s4215 and #s4217; siDNMT1_A and siDNMT1_B, respectively) and DNMT3B siRNAs (ID #s4221 and #s4223; siDNMT3A_A and siDNMT3A_B, respectively) were purchased from Ambion. The human DNMT3A siRNAs (ID #4272 and #0197; siDNMT3B_A and siDNMT3B_B, respectively) were purchased from Sigma. The AllStars Negative Control (ID #1027281; siCtrl_A) and Silencer Select Negative Control siRNA (ID #4390843; siCtrl_B) were purchased from QIAGEN and Life Technologies, respectively.

Total RNA and Genomic DNA Isolation

mRNA and miRNA were purified using the miRNeasy Mini Kit (QIAGEN), according to the manufacturer's protocol. Total RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and the integrity of the RNA was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies). Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) and was quantified on a NanoDrop 1000 spectrophotometer.

mRNA and miRNA qRT-PCR

Total RNA was first treated with DNase using the TURBO DNA-free kit (Ambion), to evaluate the gene expression levels. Then, cDNAs were synthesized from 1 μg of purified mRNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. SYBR Green RT-qPCR was performed to evaluate the mRNA levels in each sample (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen) using a Step One Plus Real-time PCR System from Applied Biosystems. After an initial denaturation at 95°C for 2 min, the thermal cycles were repeated 40 times as follows: 95°C for 15 s and 60°C for 30 s. The housekeeping genes glyceraldehyde 3-phosphatase dehydrogenase (*GAPDH*) and ribosomal protein S18 (*RPS18*) were used to normalize the cDNA levels. The sequences of the human primers used for gene amplification are shown in Table S2. For the miRNA analyses, 100 ng of total RNA was

reverse-transcribed using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems). The expression levels of mature *miR-122* were determined by qRT-PCR with the TaqMan Universal PCR Master Mix. The PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. TaqMan probes from Applied Biosystems were used to assess the expression of *miR-122-5p* (ID #002245). The expression levels of *miR-122* were normalized to the endogenous levels of *RNU6B* (ID #001093).

DNA Methylation Analysis

The global levels of genomic DNA methylation were evaluated using the Global DNA Methylation ELISA Kit (Cell Biolabs) according to the manufacturer's recommendations. COBRA[61] was used to assess the methylation status of the specific CpG sites located in the promoter regions of *ALB*, *SLC10A1*, *CYP3A4*, and *miR-122*. An *in silico* analysis using the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu) was performed to identify the CpG sites associated with the transcription start site and polymerase elongation region for each gene (Figure S1). MethPrimer (http://www.urogene.org/methprimer) was used to design the COBRA primers required to amplify the genomic regions containing the CpGs of interest (Table S3). In brief, 1 μg of genomic DNA was subjected to bisulfite modification treatment using the EpiTect Plus kit (QIAGEN). Then, COBRA PCR was performed as follows: after an initial denaturation step at 94°C for 3 min, the following thermal cycles were repeated 40 times: 94°C for 10 s, 55°C for 50 s, and 72°C for 1 min. Each COBRA PCR was performed in a total volume of 10 μL, which contained 0.5 units of Hot Start Taq polymerase (Takara), 10 pmol of primers, and 1 μL of bisulfite-treated DNA. After PCR amplification, 3 μL of amplified products was digested with 3 units of restriction enzyme. Finally, the restriction products were separated by 10% PAGE and visualized by ethidium bromide staining. The bands were densitometrically analyzed using the software ImageJ (v1.50; NIH, USA) to quantify the unmethylated (U) and methylated (M) restriction fragments. The methylation levels were calculated for each locus using the formula $(M \times 100)/(M+U)$ and were expressed as a methylation percentage.

Drug-Metabolizing Enzyme Activity

The drug-metabolizing enzyme activities were determined with the P450-Glo CYP1A2 Assay (#V8772) and the P450-Glo CYP3A4 Assay (#V9002) from Promega, as recommended by the manufacturer. The CYP1A2 and CYP3A4 enzyme activities were induced in HepG2 cells by treatment with dexamethasone (#D2915; Sigma) for 72 hr. CYP3A4 enzyme activity was induced in Huh-7 cells by treatment with dexamethasone or phenobarbital sodium (#162-11602; Wako) for 72 hr. Dexamethasone and phenobarbital sodium were dissolved in H_2O. The CYP activities were normalized with respect to the number of cells present in each well.

Immunoblotting

The proteins were extracted using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific). For each sample, 10 μg of total proteins was resolved by SDS-PAGE (10%) and transferred to nitrocellulose membranes. The membranes were incubated with the following primary antibodies overnight at 4°C: anti-DNMT1 (#sc-10219; Santa Cruz Biotechnology), anti-DNMT3A (#ab71424; Abcam), anti-DNMT3B (#ab119282; Abcam), and anti-Albumin (#ab10241; Abcam). The DNMT and ALB antibodies were used at dilutions of 1/500 and 1/1,000, respectively. A 1/1,500 dilution of the anti-β-Tubulin antibody (#T4026; Sigma) was used as a loading control. The antigen-antibody complexes were visualized by chemiluminescence using the ECL Plus western blotting detection system (GE Healthcare) and scanned with the Fujifilm LAS-3000 imaging system (Fujifilm).

In Vivo Epigenetic Reconditioning

The mice received a daily intraperitoneal injection of 5-AZA diluted in sterile saline solution at concentrations ranging from 1.5 to 15 mg/kg (n = 5 for each concentration) six times/week, to determine the optimal dose of the demethylating drug for *in vivo* administration. The animals' conditions and weights were monitored twice a week. For the *in vivo* epigenetic reconditioning protocol, the HepG2 and Hep3B cells were first implanted in athymic nude mice as described above. After the tumors reached a palpable size ($\geq 100\ mm^3$), the animals were added to the study and randomly separated into two groups. Mice that did not develop appropriate tumors were excluded from the study. Next, the mice received an intraperitoneal (i.p.) injection of 3 mg/kg 5-AZA (n = 8) or saline solution (n = 8) six times/week for 4 weeks. The tumor size was monitored twice a week. The animals were euthanized at the study endpoint dictated by the animal care guidelines of our institute. The tumors were immediately collected and snap frozen in liquid nitrogen for storage until RNA and DNA extraction.

Statistical Analysis

The experimental data are presented as the means ± SD, except for the *in vivo* tumorigenicity assay, in which error bars show the SEM. Student's t test was performed to estimate the statistical significance of the data, except for the clinical sample data and the correlation analysis data. The equality of the variances was tested using an *F*-test. All p values were two-tailed. The statistical significance of the differences in gene expression between the HCC and adjacent non-tumor tissues (clinical samples) was assessed using the Mann-Whitney *U* test. The Wilcoxon test (paired samples) was used to evaluate the statistical significance between the paired samples for *DNMT1* expression. The correlations between the gene expression (qRT-PCR) and DNA methylation levels (COBRA) were assessed by calculating the Spearman's rank coefficient. All statistical analyses were performed using the MedCalc software. The experimental data are representative of at least three independent experiments and were considered statistically significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes ten figures and three tables and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.04.018.

AUTHOR CONTRIBUTIONS

L.G. and T.O. conceived and designed the study; L.G. performed the majority of the experimental work with support from L.C.L., K.Y., I.H., Y.T., H.N., and T.O.; I.H. provided expertise for the methylation analysis; Y.T. collected the clinical samples from HCC patients; L.G. analyzed the data, performed statistical analyses, and wrote the manuscript; T.O. obtained funding for the study.

CONFLICTS OF INTEREST

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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