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Sirtuin7 Oncogenic Potential in Human Hepatocellular Carcinoma and Its Regulation by the Tumor Suppressors MiR-125a-5p and MiR-125b

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Sirtuins are nicotinamide adenine dinucleotide oxidized form (NAD^+)-dependent deacetylases and function in cellular metabolism, stress resistance, and aging. For sirtuin7 (SIRT7), a role in ribosomal gene transcription is proposed, but its function in cancer has been unclear. In this study we show that SIRT7 expression was up-regulated in a large cohort of human hepatocellular carcinoma (HCC) patients. SIRT7 knockdown influenced the cell cycle and caused a significant increase of liver cancer cells to remain in the G_1/S phase and to suppress growth. This treatment restored $p21^{WAF1/Cip1}$, induced Beclin-1, and repressed cyclin D1. In addition, sustained suppression of SIRT7 reduced the *in vivo* tumor growth rate in a mouse xenograft model. To explore mechanisms in SIRT7 regulation, microRNA (miRNA) profiling was carried out. This identified five significantly down-regulated miRNAs in HCC. Bioinformatics analysis of target sites and ectopic expression in HCC cells showed that miR-125a-5p and miR-125b suppressed SIRT7 and cyclin D1 expression and induced $p21^{WAF1/Cip1}$-dependent G_1 cell cycle arrest. Furthermore, treatment of HCC cells with 5-aza-2′-deoxycytidine or ectopic expression of wildtype but not mutated p53 restored miR-125a-5p and miR-125b expression and inhibited tumor cell growth, suggesting their regulation by promoter methylation and p53 activity. To show the clinical significance of these findings, mutations in the DNA binding domain of *p53* and promoter methylation of miR-125b were investigated. Four out of nine patients with induced SIRT7 carried mutations in the *p53* gene and one patient showed hypermethylation of the miR-125b promoter region. *Conclusion*: Our findings suggest the oncogenic potential of SIRT7 in hepatocarcinogenesis. A regulatory loop is proposed whereby SIRT7 inhibits transcriptional activation of $p21^{WAF1/Cip1}$ by way of repression of miR-125a-5p and miR-125b. This makes SIRT7 a promising target in cancer therapy. (HEPATOLOGY 2013;57:1055-1067)

Sirtuins, also designated as class III histone deacetylases, are nicotinamide adenine dinucleotide oxidized form (NAD^+)-dependent deacetylases that target histone and nonhistone proteins and are implicated in the control of a wide range of biological processes such as apoptosis, stress responses, DNA repair, cell cycle, metabolism, and senescence.[1] The importance of sirtuins is demonstrated by their role in several major human pathologic conditions, including cancer, diabetes, cardiovascular disease, and neurodegenerative disease.[2] Mammals express seven sirtuins (denoted SIRT1-7) that have considerably different functions and catalytic activities.[3] The most closely related to yeast Sir2 and the best-characterized sirtuin,

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; CDKN1A, cyclin dependent kinase 1A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAT, histone acetyltransferase; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; miRNA, microRNA; mRNA, messenger RNA; MSP, methylation-specific PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; rDNA, ribosomal DNA; siRNA, small interfering RNA; TSA, trichostatin A; UTR, untranslated region.

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SIRT1 possesses a large number of substrates such as p53, MyoD, FOXO3, nuclear factor kappa B (NF-κB), and others,[4] but little is known about the biological functions of the other mammalian sirtuins.

For SIRT7, evidence has suggested a role in the control of ribosomal RNA (rRNA) expression. SIRT7 localizes mainly in the nucleous, where it binds to the rRNA gene (rDNA) and participates in activation of RNA polymerase I transcription.[5] Another study has demonstrated that SIRT7 is relevant for the reactivation of rDNA transcription at the end of mitosis.[6] In addition, some reports have proposed that SIRT7 is associated with thyroid and breast cancer.[7,8] Based on these findings and that SIRT7 is more abundant in highly proliferative tissues than in lowly proliferative tissues,[9] a role for SIRT7 as a principal activator of proliferation has been proposed. On the contrary, a finding has suggested its role in cancer prevention, proposing that SIRT7 may enable cells to sustain critical metabolic function by inhibiting cell growth even under severe stress conditions.[10] This discrepancy has been a subject of controversy until now, and it prompted our interest to investigate the biological role of SIRT7 in human HCC.

HCC is the third leading cause of cancer-related death and the fifth most common cancer worldwide.[11] Recently, integrated analysis of somatic mutations and focal copy-number changes identified key genes and pathways in HCC, and suggested interactions between mutations in oncogene and tumor suppressor gene mutations related to specific risk factors.[12] It showed that the Wnt/β-catenin pathway was the most frequently altered, with the occurrence of either activating mutations in *CTNNB1* (encoding β-catenin; 32.8%) or inactivating mutations in *AXIN1* (15.2%) or *APC* (1.6%), and that the p53 pathway was identified as the second most frequently altered pathway in HCC, shown by the presence of *p53*-inactivating mutations (20.8%) and homozygous deletions or mutations in *CDKN2A* (8%). However, it is unclear how these genetic changes precisely cause the clinical characteristics observed in individual patients with HCC, and thereby the underlying mechanisms involved in the development and progression of HCC remain poorly understood.

In the present study, to better understand the biological roles of SIRT7 in liver tumorigenesis, SIRT7 expression was evaluated in a subset of human HCC by western blot analysis, and its messenger RNA (mRNA) level was analyzed in a large cohort of HCC patients. Evidence was obtained for SIRT7 overexpression to potently mediate mitotic stimulation of cells by way of transcriptional inactivation of $p21^{WAF1/Cip1}$ and activation of cyclin D1 in liver cancer cells. Additional research identified miR-125a-5p and miR-125b as endogenous regulators of SIRT7; these miRNAs are transcriptionally repressed in HCC. Further research identified inactivation of p53 and promoter methylation and suppression of these regulatory miRNAs to sustain SIRT7 overexpression in HCC. Thus, a mechanism is proposed that makes SIRT7 a promising target in cancer therapy.

Materials and Methods

Gene Expression Data. Gene expression data were acquired from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession numbers GSE25097, GSE14520, GSE17856, and GSE10694). In an attempt to identify the molecular signature associated with oncogenic SIRT7 activity, whole genome expression analysis was applied to mock (negative control shRNA-expressing plasmid) or shRNA (SIRT7 shRNA-expressing plasmid) transfected Hep3B cells. Differential miRNA expression analysis was performed to identify miRNAs that are significantly down-regulated in HCC. Primary gene expression and miRNA microarray data were submitted to the GEO database (http://www.ncbi.nih.gov/geo/), and the accession numbers are GSE33234 and GSE39678, respectively.

Statistical Analysis of Microarray Data. For gene expression analysis, BeadStudio (v. 3.0) was used for the data acquisition and calculation of signal values on an Illumina expression microarray. Normalization of microarray data and hierarchical clustering were performed by using GenPlex (v. 3.0). Sets of differentially expressed genes were identified by a parametric test (Welch's t test). A threshold P value in combination with fold change was applied. Expression profiles of the gene set with a fold change deregulation of more than 1.5-fold and $P < 0.05$ were used to find the differentially expressed genes. For miRNA expression

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Additional Supporting Information may be found in the online version of this article.

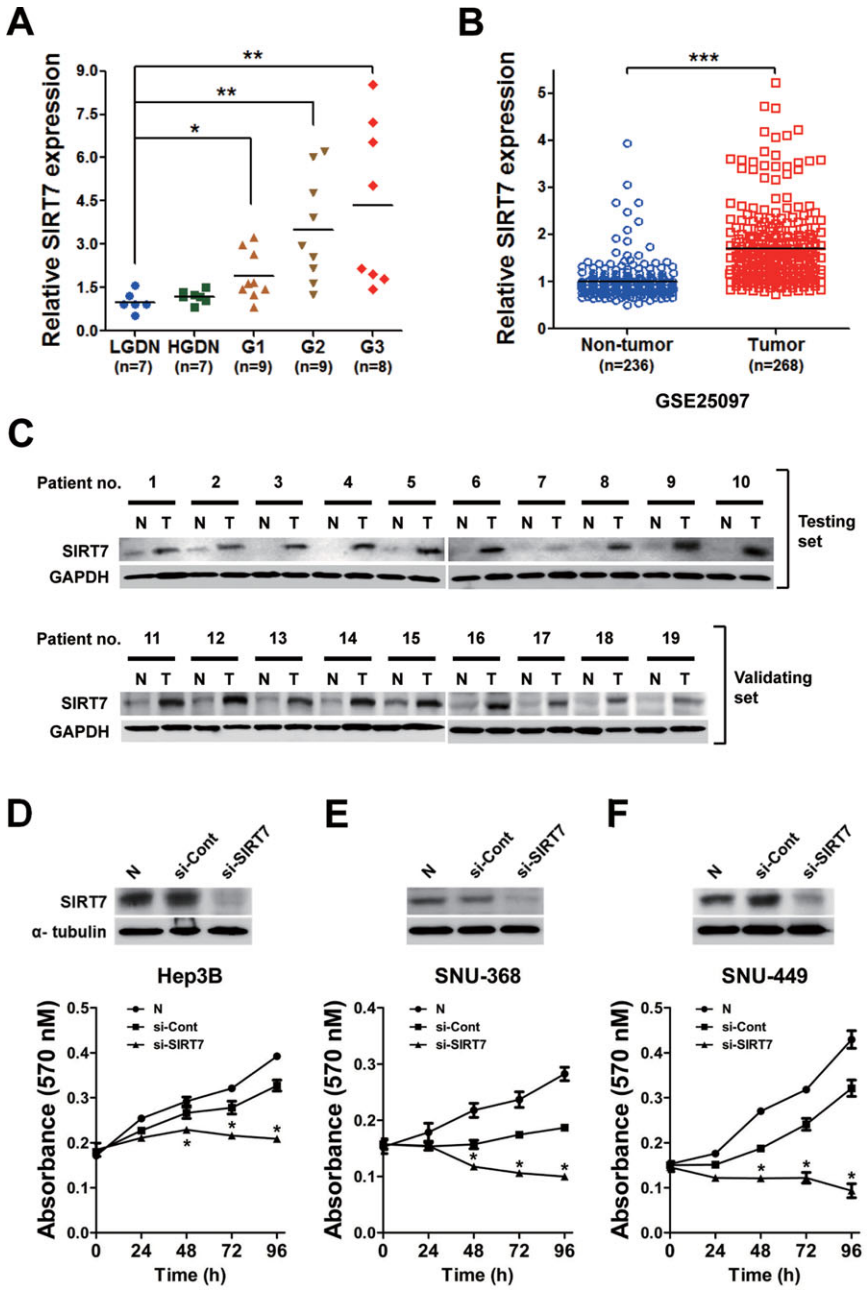

Fig. 1. SIRT7 overexpression in HCCs and its association in hepatocellular malignant proliferation. (A) Recapitulated is the gene expression of *SIRT7* mRNA based on microarray analysis of preneoplastic nodules to overt HCC. LGDN, low-grade dysplastic nodule; HGDN, high-grade dysplastic nodule; $G_{1\text{-}3}$, Edmondson grades I-III. *P* values were generated using the unpaired Student's *t* test (*$P < 0.05$, **$P < 0.001$ versus LGDN). (B) *SIRT7* gene expressions in liver cancer tissues (Tumor) and noncancerous liver tissues (Non-tumor) as determined by gene expression array. Data from NCBI, GEO database (accession number; GSE25097), unpaired Student's *t* test. ***$P < 0.0001$ versus Non-tumor. (C) Analysis of SIRT7 expression in human HCC and adjacent noncancerous tissues by western blot. T, HCC tissue; N, adjacent noncancerous liver tissue. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal loading control. Effects of SIRT7 knockdown on cellular growth rates of (E) Hep3B, (F) SNU-368, and (G) SNU-449 cells. MTT assay was performed after transfection at indicated times. Cell lysates were obtained from cells transiently transfected with either SIRT7 siRNA (si-SIRT7) or negative control siRNA (si-Cont). N, nontransfected cells. Data are presented as mean ± standard error of mean (SEM), unpaired Student's *t* test. *$P < 0.01$ versus si-Cont. All measurements were performed in triplicate and each experiment was repeated at least two times.

analysis, flagged spots were excluded from analysis, unless specified otherwise. Signal intensities within each array were normalized using the Quantile algorithm. Then we used a dataset of genes that satisfied the filtering criteria (genes having more than 50% missing data of each class). Finally, 510 miRNAs were subjected to unsupervised hierarchical clustering analysis. Hierarchical clustering was performed using Cluster and TreeView 2.3 (Stanford University). Euclidean correlation, median centering, and complete linkage were applied during all clustering applications.

Full descriptions of additional Materials and Methods are given in the Supporting Information.

Results

SIRT7 Is Aberrantly Expressed in HCC and Its Inactivation Causes Growth Inhibition. We previously reported comprehensive gene expression data of human HCC tissues including preneoplastic lesion to different pathological grades of HCC.[13] From these data, regulation of SIRT7 was evident and appeared to correlate with the multistep histopathological process. As shown in Fig. 1A, expression of SIRT7 was gradually increased from premalignant lesions (low- and high-grade dysplastic nodules) to overt cancer (Edmondson grades 1-3). To generalize our finding,

we recapitulated *SIRT7* gene expression from the large cohorts of HCC patients that are available from the GEO database (accession numbers GSE25097, GSE14520, and GSE17856) and data are given as scatterplots. Consistently, *SIRT7* gene expression was significantly up-regulated in all three different HCC cohorts (Fig. 1B; Supporting Fig. 1A,B). Increased expression of SIRT7 protein was confirmed by immunoblotting of 10 randomly selected human HCC tissues (testing set), and further validated with an additional set (validating set) of nine HCC tissues (Fig. 1C). Human liver cancer cell lines also exhibited relatively high expression of SIRT7 protein as compared with the immortalized normal hepatic liver cells, LO2, MiHA, and THLE-3 (Supporting Fig. 1C). To better understand the molecular functions of SIRT7 in HCC tumorigenesis, SIRT7 knockdown was attempted by way of RNA-interference and studied in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays. SIRT7 knockdown resulted in a significant reduction in SIRT7 protein expression and in reduced proliferation rates of the Hep3B, SNU-368, and SNU-449 liver cancer cells, respectively (Fig. 1D-F). This antigrowth effect could be partially explained by the disruption of cell growth regulation, such as cell cycle arrest, cellular senescence, or apoptosis, on SIRT7-targeting. Thus, we next explored the effects of SIRT7 knockdown on cell cycle regulation and cell death mechanism.

Whole Genome Expression Scans Identified Deregulated Gene Targets by Oncogenic SIRT7. In an attempt to identify molecular targets associated with oncogenic SIRT7 activity, whole genome expression analysis was applied to mock (negative control shRNA-expressing plasmid) or shSIRT7 (SIRT7 shRNA-expression plasmid) transfected Hep3B cells. Such analysis revealed SIRT7 knockdown to restore expression of $p21^{WAF1/Cip1}$ and to influence the expression of genes involved in cellular growth and death pathways (Supporting Fig. 2A,C). This result implies that SIRT7 inactivation may disturb the G_1/S phase by deregulating cell cycle regulatory proteins. To clarify the role of SIRT7 in cell cycle progression, SIRT7 knockdown Hep3B and SNU-449 cells were treated with nocodazole. This treatment synchronizes the cells in the G_2/M phase. After release from nocodazole block, the proportions of cells in the G_1-phase were determined by flow cytometry. SIRT7 knockdown caused a significant increase of liver cancer cells in the G_1/S phase and delayed cell cycle transition, suggesting that the proliferative defect and/or growth retardation of liver cancer cells by SIRT7 inactivation, at least in part, is due to interference with the cell cycle (Fig. 2A; Supporting Fig. 2D). We then observed that SIRT7 knockdown selectively induced $p21^{WAF1/Cip1}$ expression, and simultaneously suppressed the expression of cyclin D1 among G_1/S cell cycle regulators, and that SIRT7 knockdown also selectively induced the proautophagy factor Beclin-1 and LC3B-II conversion in Hep3B cells by western blot analysis (Fig. 2B,C; Supporting Fig. 2B).

Recent studies showed that SIRT7 could be a positive regulator of the RNA polymerase I transcription machinery and its levels are high in metabolically active tissues, such as liver, spleen, testis, and types of carcinoma.[9] Because altering the protein synthesis machinery, such as ribosome biogenesis, are essential cellular processes that are governed by malignant progression, we explored the biological function of SIRT7 in the protein synthesis machinery of liver cancer cells. Initially, we found that SIRT7 is a nucleolar sirtuin of liver cancer cells by detecting exclusive expression and enzymatic activity of SIRT7 in nuclear fraction of Hep3B cells (Fig. 2D). Note that deacetylase activity of SIRT7 on p53 as a substrate was significantly increased in immunoprecipitates of SIRT7 antibody to nuclear fractions of Hep3B cells. We then evaluated the efficiency of ectopic protein synthesis of Hep3B cells and compared that of SIRT7 inactivating Hep3B cells because the rDNA transcription is related to the translation capacity of cells. To this end, Hep3B cells were transfected with various expression plasmids such as pME18S-HDAC2 (HDAC2-expressing vector), pcDNA3.1_SIRT1 (SIRT1-expressing vector), pCMV-Neo-Bam p53 wt (wildtype p53-expressing vector), and pcDNA3.1_HDAC6 (HDAC6-expressing vector). All ectopic plasmids were successfully expressed and detected by immunoblotting with each indicated antibody. Notably, SIRT7 knockdown suppressed the protein expression of these ectopic plasmids. Note that SIRT7 inactivation suppressed wildtype HDAC6 expression, a potent tubulin deacetylase, and thereby recovered the acetylated-α-tubulin status of SRIT7 knockdown cells. To generalize this finding, we performed the same experiments in three different liver cancer cell lines, SNU-368, SNU-449, and Huh7 cells. As expected, SIRT7 knockdown suppressed the protein synthesis of ectopic plasmids in these liver cancer cells as compared with control (non- or negative control siRNA-transfected) cells (Fig. 2E). In addition, we performed gene set enrichment analysis from the deregulated genes by SIRT7 in Hep3B cells to dissect

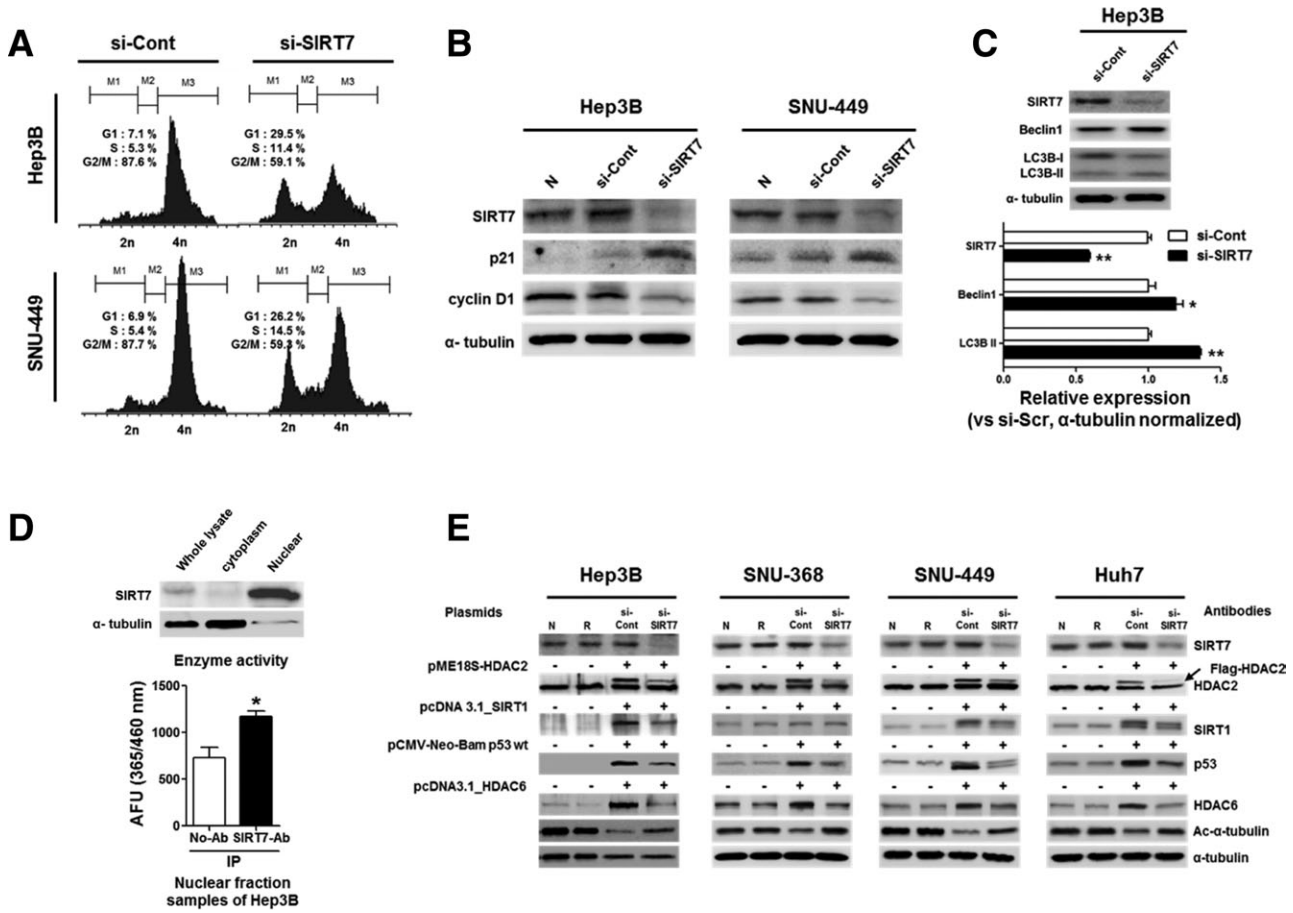

Fig. 2. Targeted-inactivation of SIRT7 inhibits hepatocellular malignant proliferation by selective modulating cell cycle, autophagy-related proteins, and protein synthesis machinery in HCC cells. (A) Cell cycle profiles of SIRT7 siRNA (si-SIRT7) or negative control siRNA (si-Cont) transfected Hep3B and SNU-449 cells followed by treatment with nocodazole. Percentage in each histogram indicates the portion of cells remaining in each cell cycle phase. (B) Effect of SIRT7 knockdown on cell cycle proteins in Hep3B and SNU-449 cells. $p21^{WAF1/CIP1}$ and cyclin D1 protein levels were analyzed by immunoblotting in Hep3B and SNU-449 cells transfected with SIRT7 siRNA (si-SIRT7) or negative control siRNA (si-Cont). (C) Knockdown of SIRT7 induced Beclin1 expression and LC3B-II conversion in Hep3B cells. Upper panel, expression levels of SIRT7, Beclin1, LC3B-II, and α-tubulin were determined by western blot analysis. Bottom, expression ratios were determined by densitometric analysis. Indicated protein band intensities were normalized to averaged α-tubulin band intensities of two independent experiments (unpaired Student's t test, $^*P < 0.05$ and $^{**}P < 0.001$ versus si-Cont). (D) SIRT7 is localized in nuclei and deacetylates p53 as substrate. Cytoplasmic and nuclear fractions were prepared from Hep3B cells, followed by immunoblotting with anti-SIRT7 antibody. A nuclear fraction was then used for the deacetylase activity assay with p53 as substrate (unpaired Student's t test, $^*P < 0.05$ versus No-Ab, no antibody treatment). (E) SIRT7 knockdown inhibits ectopic protein synthesis machinery in HCC cells. The efficiency of protein synthesis in SIRT7 knockdown Hep3B cells was compared with that of control siRNA (si-Cont) transfected Hep3B cells followed by transfection with indicated plasmid (pME18S-HDAC2, pCMV-Neo-Bam p53 wt, pcDNA3.1_SIRT1, and pcDNA3.1_HDAC6). The same experiments were performed in SNU-367, SNU-449, and Huh7 liver cancer cells. The loaded samples were normalized to the α-tubulin, serving as loading control.

signaling pathways that are enriched by SIRT7 in liver cancer cells. From this analysis, the nucleic acid metabolic process and protein modification process were identified as signaling pathways enriched by SIRT7 in Hep3B cells. We also noted that all the expressions of these two gene sets were down-regulated in SIRT7 knockdown Hep3B cells (Supporting Fig. 3). These results support our finding that SIRT7 may play a role in protein synthesis machinery in HCC tumorigenesis.

Identification of Endogenous MiRNAs to Control SIRT7 Regulation in Liver Cancer. It has been demonstrated that all the known processes involved in cancer, including apoptosis, proliferation, survival, and metastasis, are regulated by small regulatory noncoding RNAs consisting of ~19-25 nucleotides; e.g., miRNAs.[14] Therefore, the fact that SIRT7 is up-regulated in HCC led us to hypothesize that SIRT7 expression is balanced by endogenous miRNAs that control *SIRT7* mRNA translation in normal hepatic liver cells. Loss or suppression of miRNAs targeting SIRT7 may cause aberrant overexpression of SIRT7, and thereby confer oncogenic potential for the hepatocellular malignant proliferation and transformation. Therefore, to identify miRNAs that deregulated in HCC, we

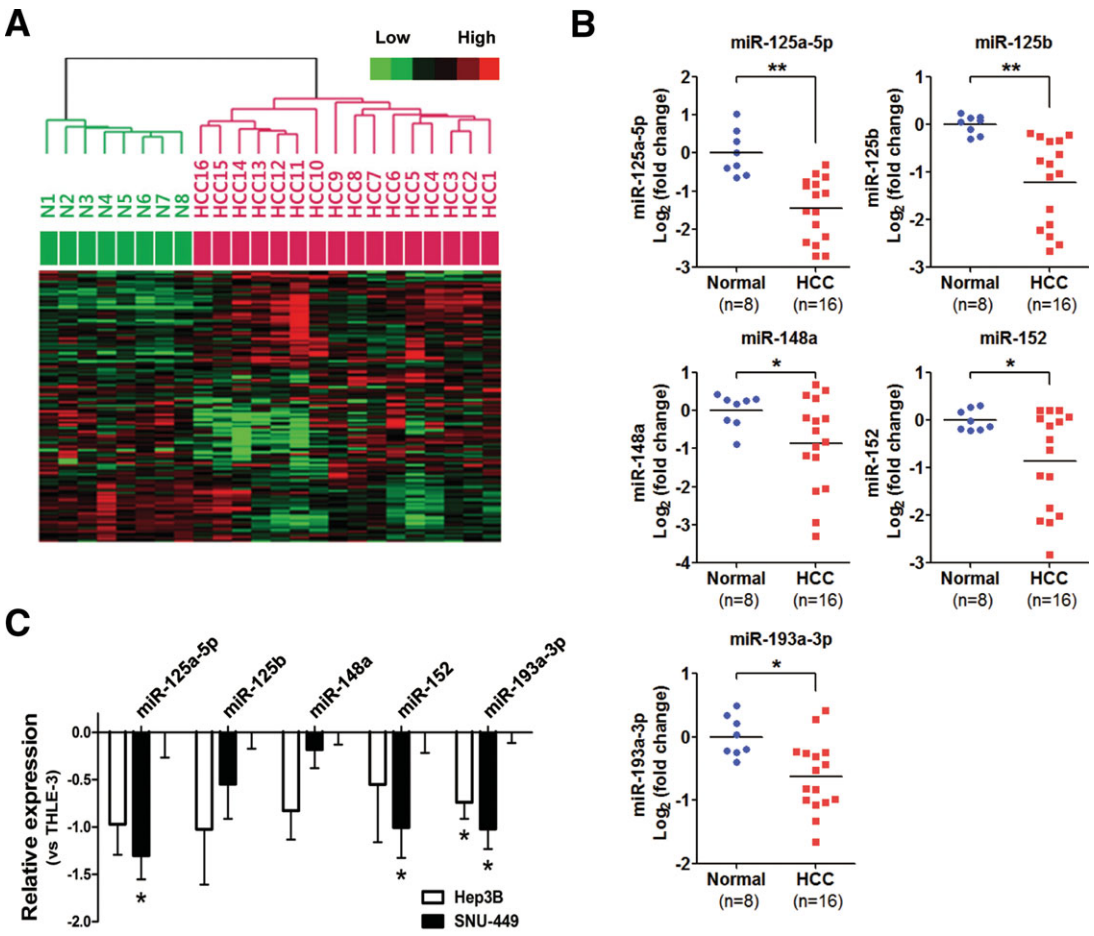

Fig. 3. Identification of miRNAs targeting SIRT7 in liver cancer. Differential miRNA expression analysis was performed in a subset of human HCC tissues. Total RNAs were isolated from normal liver tissues (n = 8) and HCC tissues (n = 16), and then subjected to whole genome miRNA microarray. (A) Result of unsupervised hierarchical clustering analysis of 510 miRNAs that were filtered by minimum selection criteria of 1,371 miRNAs. The level of miRNA expression is presented as a heatmap. Red, relatively higher miRNA expression; green, relatively lower miRNA expression; black, no difference. N, normal hepatic liver tissue; HCC, hepatocellular carcinoma tissue. (B) Potential SIRT7 targeting miRNAs were selected by using predictive program, miRanda. Expression of five miRNAs (miR-125a-5p, miR-125b, miR-148a, miR-152, and miR-193a-3p) targeting SIRT7 are presented as scatterplot graphs (unpaired Student's *t* test, $*P < 0.05$ and $**P < 0.001$ versus normal). (C) Endogenous expression of five miRNAs targeting SIRT7 in HCCs. Total RNAs were isolated from Hep3B, SNU-449, and THLE-3, and then real-time PCR analysis was performed. The expression of five miRNAs was compared with that of THLE-3. Data were analyzed using the ΔC_t approach and expressed as both miRNA/U6 ratio ($2^{-(Ct\ Target\ miRNA\ -\ Ct\ U6)}$) and $\log_2$ fold change (HCC/normal liver tissue). *P* values were generated using the unpaired Student's *t* test ($*P < 0.05$ versus THLE-3).

performed miRNA expression profiling analysis in a subset of human HCCs. Unsupervised hierarchical clustering analysis of 510 miRNAs that were selected by minimum filtering criteria of 1,371 miRNAs of eight normal liver tissues and 16 HCCs resulted in two distinct clusters within dendrogram and suggested characteristic miRNA signature associated with HCC tumorigenesis (Fig. 3A). Then, a target prediction program, miRanda (http:// www.microrna.org), was used to predict and identify miRNAs that possibly target the endogenous SIRT7 in HCC. From this, we were able to identify five miRNAs (miR-125a-5p, 125b, 148a, 152, and 193a-3p) that are significantly down-regulated in HCC (Fig. 3B). To confirm the repression of these miRNAs in HCC, quantitative real-time polymerase chain reaction (qRT-PCR) analysis for five miRNAs in Hep3B and SNU-449 cells was performed, and the results compared with that of THLE-3, a normal hepatic liver cell line (Fig. 3C). As expected, the expressions of these five miRNAs were repressed in both Hep3B and SNU-449 cells with some variations.

MiR-125a-5p and MiR-125b Regulate Endogenous SIRT7 Expression. Next, to determine whether SIRT7 is selectively regulated by these miRNAs by way of direct interaction with the 3′-untranslated region (UTR) of *SIRT7* mRNA, we cloned the 3′-UTR of *SIRT7* into a reporter vector linking luciferase open

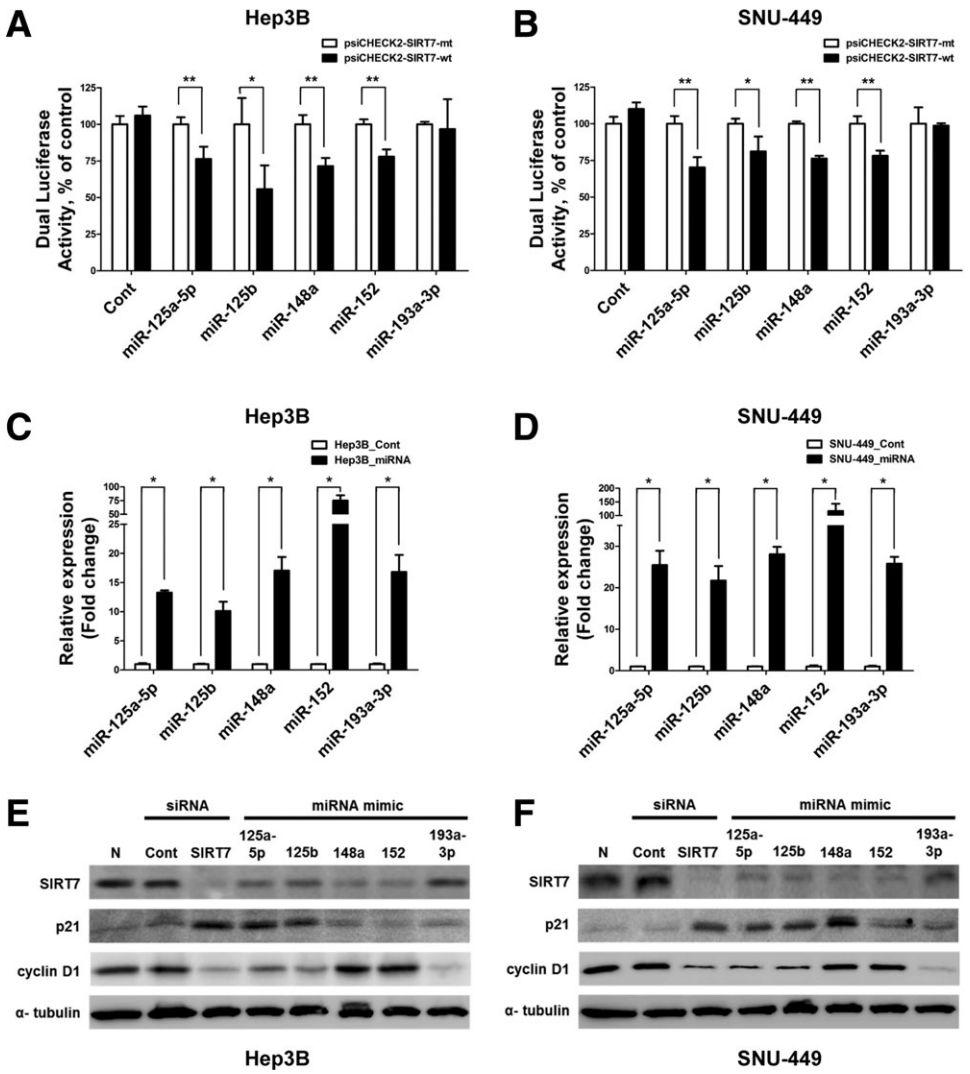

Fig. 4. MiR-125-a-5p and miR-125b are endogenous regulators for SIRT7 expression in HCC tumorigenesis. For *in vitro* validation of miRNAs targeting SIRT7, Hep3B (A) and SNU-449 (B) cells were transfected in the psiCHECK2-SIRT7-wt or psiCHECK2-SIRT7-mt (see Materials and Methods in Supporting Information), and cotransfected with each indicated miRNA, or negative control miRNA (Cont). After 24 hours of transfection, dual-luciferase assays were performed. The data represent the mean ± SEM of four determinations from two independent transfections (unpaired Student's *t* test, $*P < 0.05$, $**P < 0.001$ versus respective controls). Experimental manipulation of miRNA levels in Hep3B (C) and SNU-449 (D) cells. Each cell line was transfected with the indicated mimic miRNA (Hep3B_miRNA or SNU-449_miRNA) or negative control miRNA (Hep3B_Cont or SNU-449_Cont). Quantitative real-time RT-PCR was performed to examine the levels of miRNA in these cells. The results shown are means ± SEM and are representative of at least three independent experiments (unpaired Student's *t* test, $*P < 0.0001$ versus respective control). MiR-125a-5p and miR-125b regulate endogenous SIRT7 expression. Hep3B (E) and SNU-449 (F) cells were transfected with either SIRT7 siRNA, negative control siRNA, or indicated mimic miRNA. After 48 hours of transfection, cell lysates were obtained for western blot analysis of SIRT7, $p21^{WAF1/CIP1}$, cyclin D1. α-tubulin served as a loading control.

reading frame downstream to generate psiCHECK2-SIRT7_3′-UTR wildtype (psiCHECK2-SIRT7-wt). We also cloned 3′-UTR of random mutation sequences of the *SIRT7* gene to generate a mutant-type (psiCHECK2-SIRT7-mt) reporter vector (Supporting Fig. 4A). Then each vector was cotransfected with these miRNAs into Hep3B and SNU-449 cells. The results of dual-luciferase reporter assays of psiCHECK2-SIRT7-wt plasmid with five miRNAs were compared with that of psiCHECK2-SIRT7-mt and are depicted as bar graphs (Fig. 4A,B). It was found that miR-125a-5p, miR-125b, miR-148a, and miR-152 were able to suppress reporter gene activity in both Hep3B and SNU-449 cells, whereas miR-193a-3p had no effect, therefore indicating that these four miRNAs are able to regulate SIRT7 expression in HCC cells *in vitro*. Thus, we assessed whether ectopic expression of these five miRNAs mimics the effects of SIRT7 knockdown by siRNA directed against SIRT7 in liver cancer cells. Note that a high level of miRNA expression was detected in both Hep3B and SNU-449 cells after ectopic transfection of five miRNAs (Fig. 4C,D).

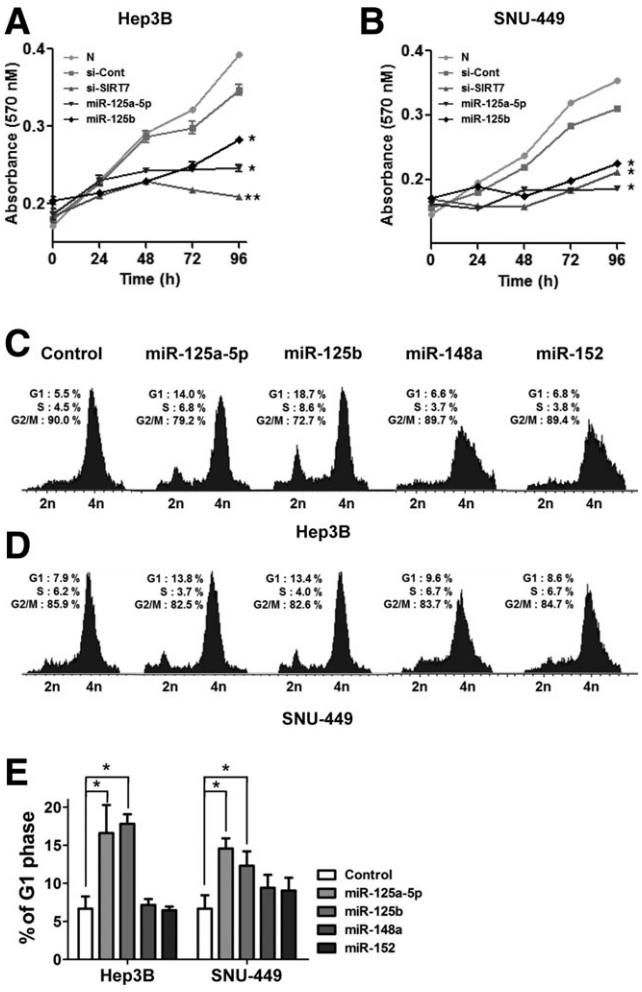

Fig. 5. MiR-125a-5p and miR-125b function as tumor suppressors by inhibiting oncogenic SIRT7 in HCC. Ectopic expression of miR-125a-5p and miR-125b causes growth retardation in liver cancer cells. Hep3B (A) and SNU-449 cells (B) were plated in 24-well plates after transfection with miRNA-specific mimics (miR-125a-5p, miR-125b) or SIRT7 siRNA (si-SIRT7) or negative control siRNA (si-Cont). Cell proliferation was assessed at indicated hours using the MTT assay. Data represent the mean and standard errors of two independent experiments (unpaired Student's *t* test, $*P < 0.05$ and $**P < 0.01$ versus respective control). Ectopic expression of miR-125a-5p and miR-125b induces G_1 cell cycle arrest in Hep3B (C) and SNU-449 (D) cells. Cells were transfected with indicated miRNA, and then treated with nocodazole. After release from nocodazole block, the proportions of cells in the G_1-phase were determined by flow cytometry. Percentage in each histogram indicates the portion of cells remaining in each cell cycle phase. A typical result of three performed experiments is shown. (E) Bar graph indicates the percent of cells remaining in G_1 phase in Hep3B and SNU-449 cell lines transfected with each indicated miRNA. The results represent the mean ± SEM of three independent experiments (unpaired Student's *t* test, $*P < 0.05$ versus control).

Consistent with the results of the luciferase assays, miR-125a-5p, miR-125b, miR-148a, and miR-152 were able to suppress endogenous SIRT7 expression, as SIRT7 siRNA did in both Hep3B and SNU-449 cells (Fig. 4E,F). However, it was found that only miR-125a-5p, miR-125b selectively recovered $p21^{WAF1/Cip1}$ and suppressed cyclin D1, as SIRT7 siRNA did in both Hep3B and SNU-449 cells. In addition, it was found that expressions of both miR-125a-5p and miR-125b were significantly down-regulated in a large cohort of HCC patients (Supporting Fig. 4B,C). Although it is not clear why miR-148a and miR-152 did not affect these cell cycle proteins, these result suggest that miR-125a-5p and miR-125b are endogenous regulators for SIRT7 in HCC tumorigenesis.

To clarify that both miR-125a-5p and miR-125b are the endogenous regulators of SIRT7 in liver cancer cells, we investigated whether ectopic expressions of miR-125a-5p and miR-125b cause growth retardation by way of cell cycle arrest, as SIRT7 inactivation does in liver cancer cells. Both ectopic expressions of miR-125a-5p and miR-125b showed a significant growth inhibition in Hep3B and SNU-449 cells by MTT assays (Fig. 5A,B). In addition, when we assessed the effect of these miRNAs on cell cycle distribution, miR-125a-5p and miR-125b induced G_1 arrest compared to control (negative control sequence of miRNA) or other miRNAs, miR-148a and miR-152 (Fig. 5C,D). Quantitative analysis of the G_1 phase indicated that both ectopic miR-125a-5p and miR-125b-expressing cells showed a significantly higher portion of G_1 phase cells than that of control or miR-148a or miR-152-expressing cells (Fig. 5E). Overall, these results demonstrated that both miR-125a-5p and miR-125b are direct suppressors of endogenous SIRT7 and may function as tumor suppressors in HCC tumorigenesis.

Inactivating Mechanisms of Tumor Suppressor, MiR-125a-5p and MiR-125b in HCC. Recent studies showed that the expression pattern of miRNAs in cancer could be regulated by various types of regulatory mechanisms, such as DNA methylation, histone modification, and p53-activation.[15,16] These suggestions led us to explore if epigenetic silencing and/or p53 activity would influence transcriptional expression of miR-125a-5p and miR-125b during HCC development and progression. We therefore treated liver cancer cells with either 5-aza-2′-deoxycytidine (5-aza-dC), a potent DNA methylation inhibitor, or trichostatin A (TSA), a histone deacetylase inhibitor, to investigate whether DNA promoter methylation or histone modification restores endogenous expression of miR-125a-5p and miR-125b in HCC cells. The treatment of Hep3B and SNU-449 cells with 5-aza-dC selectively restored expression of miR-125b in both cell lines (Fig. 6A,B), whereas TSA treatment did not affect the expression of either miR-125a-5p or miR-125b in Hep3B and SNU-449 cells (Supporting Fig. 5A,B). To clarify the selective suppression of miR-125b by promoter

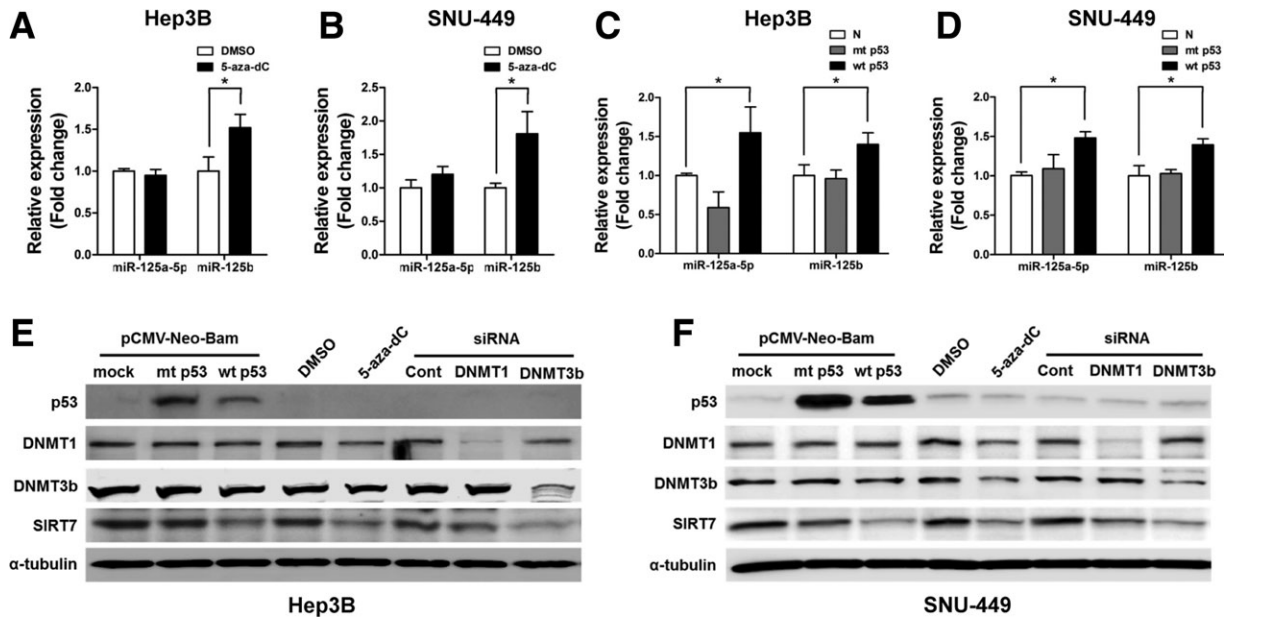

Fig. 6. Expression of endogenous miR-125a-5p and miR-125b is regulated by p53 activity and DNA promoter methylation in HCC cells. Promoter hypermethylation suppresses miR-125b expression in liver cancer. (A) Hep3B cells and (B) SNU-449 cells were treated with 10 μM 5-aza-dC, or 0.1% dimethyl sulfoxide (DMSO) for 120 hours. Quantitative real-time RT-PCR was then performed to assess the levels of miR-125a-5p and miR-125b in these cells. The expression of miRNAs was normalized to that of the small nuclear RNA U6. Data were analyzed using the ΔC_t approach and expressed as $\log_2$ fold change. The results shown are means $\pm$ SEM and are representative of at least three independent experiments (unpaired Student's *t* test, $^*P < 0.05$). Restoration of p53 activity by ectopic expression in liver cancer cells induces miR-125a-5p and miR-125b expression. (C) Hep3B and (D) SNU-449 cells were transfected with mutant type of p53 (mt p53) or wildtype of p53 (wt p53) expressing plasmid. After 48 hours of transfection, quantitative real-time RT-PCR was then performed to analyze expression levels of miR-125a-5p and miR-125b in these cells. The expression of miRNAs was normalized as described above. Data were analyzed using the ΔC_t approach and expressed as $\log_2$ fold change. The results shown are means $\pm$ SEM and are representative of at least three independent experiments (unpaired Student's *t* test, $^*P < 0.05$ versus N, nontransfected cells). Confirmation of the regulatory mechanism of miR-125a-5p and miR-125b targeting SIRT7 in HCC by western blot analysis. (E) Hep3B and (F) SNU-449 cells were transfected with indicated plasmid (mock, pCMV-Neo-Bam; mt p53, pCMV-Neo-Bam-p53 mt; wt p53, pCMV-Neo-Bam-p53 wt), or treated with the indicated drug (0.1% DMSO or 10 μM of 5-aza-dC), or transfected with the indicated siRNA (si-Cont, negative control siRNA; si-DNMT1, siRNA targeting DNMT1; si-DNMT3b, siRNA targeting DNMT3b). Cell lysates were prepared and the expression levels of SIRT7, p53, DNMT1, and DNMT3b were analyzed by western blotting. α-Tubulin served as a loading control.

methylation, the methylation status of miR-125b promoter region was investigated in HCC cells. As expected, Hep3B and SNU-449 cells exhibited high methylated status in the promoter region of miR-125b, whereas THLE-3, normal hepatic liver cell line, was unmethylated. Note that the promoter region of miR-125a-5p was highly methylated in all THLE-3, Hep3B, and SNU-449 cells (Supporting Fig. 6A). We then employed a wildtype p53-expressing plasmid (pCMV-Neo-Bam-p53 wt) to restore p53 activity in HCC cells, because Hep3B cells are *p53*-null and the SNU-449 cell lines expresses mutant p53. It was found that ectopic expression of wildtype p53 caused significant induction of miR-125a-5p and miR-125b expression, and as consequence, suppressed SIRT7 protein expression in both Hep3B and SNU-449 cells, whereas mutant-type p53 expression did not affect SIRT7 expression. In addition, disruption of DNA methylation by either 5-aza-dC treatment or knockdown of DNMT1 and DNMT3b caused induction of miR-125a-5p and miR-125b expression, and thereby suppressed SIRT7 expression in both Hep3B and SNU-449 cells (Fig. 6D-F; Supporting Fig. 6B-E). These results suggest the underlying mechanisms leading to endogenous miR-125a-5p and miR-125b suppression in HCC. Next, to demonstrate the clinical significance of these findings, human HCC tissues were analyzed.

First, we analyzed expressions of miR-125a-5p and miR-125b in a subset of HCCs using qRT-PCR. Endogenous expressions of both miR-125a-5p and miR-125b were significantly down-regulated in HCCs except for one sample, patient number 13 (Fig. 7A,B). These HCC samples were then investigated for *p53* mutation using a single-stranded conformational polymorphism and direct sequencing. From this, we found four (patients 11, 14, 16, and 19) out of nine patients carried mutations in the exons of DNA binding motif of the *p53* gene (Fig. 7C). Then the same tissue samples were investigated for promoter methylation of miR-125b, and found that only in the case of patient

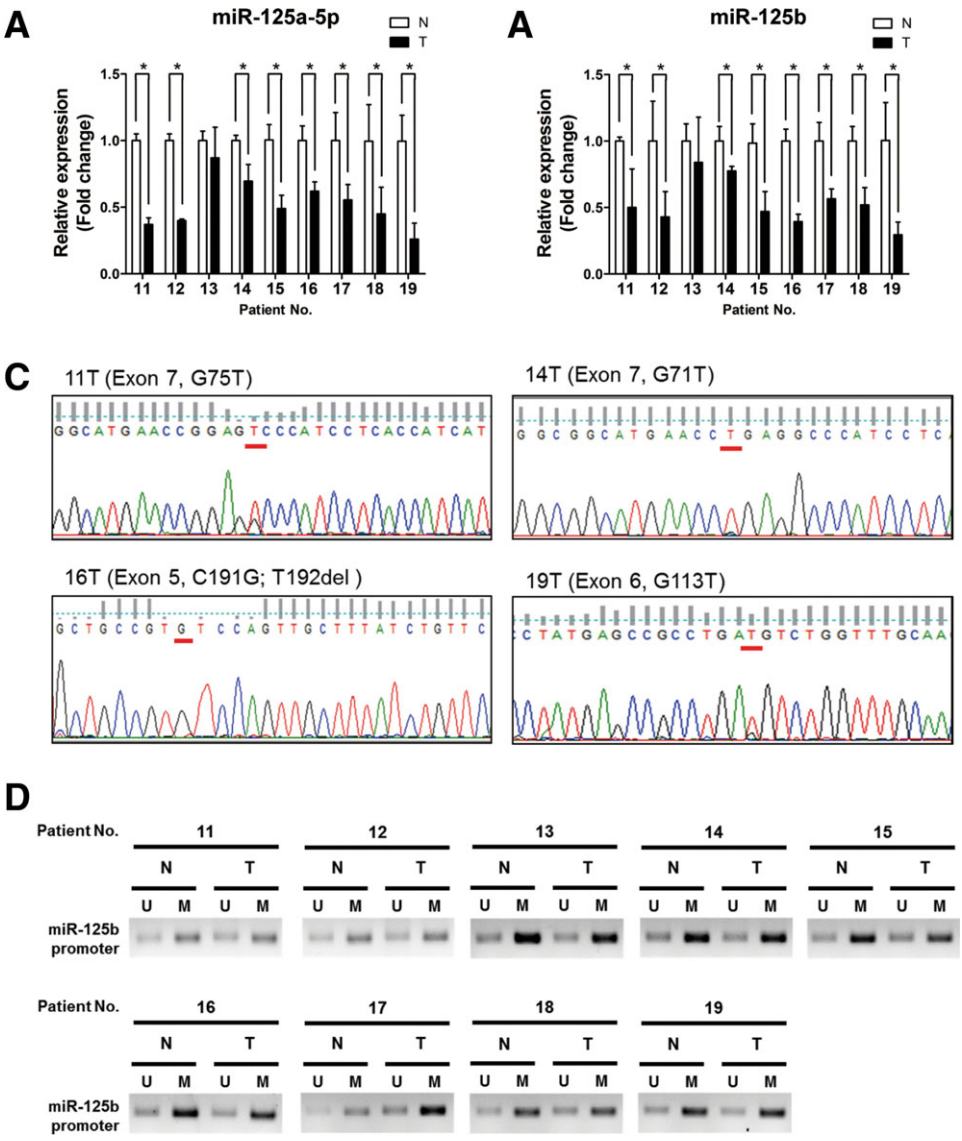

Fig. 7. Identification of *p53* gene mutation and hypermethylation of miR-125b promoter region in human HCCs. Analysis of miR-125a-5p (A) and miR-125b (B) expressions in a subset of human HCCs paired with adjacent noncancerous liver tissue was performed by quantitative real-time RT-PCR. The expression of miRNAs was normalized to small nuclear RNA U6. Data were analyzed using the ΔC approach and expressed as fold change (HCC/noncancerous liver tissue). Results shown are means $\pm$ SEM and are representative of at least three independent experiments (unpaired Student's *t* test, $^*P < 0.05$ versus N, nontumor). (D) Mutational analysis of *p53* gene in human HCC tissues. Direct sequencing of *p53* exons 5, 6, and 7 showing a transversion or a deletion at indicated nucleotide in human HCC (patients 11, 14, 16, and 19). (E) Methylation status in the promoter region of miR-125b in human HCC tissues. Methylation specific PCR analysis was used to determine the methylation status of CpG islands of miR-125b promoter site in a subset of human HCCs (T, patients 11-19) paired with adjacent noncancerous liver tissue (N). M, methylated alleles; U, unmethylated alleles.

17 was the miR-125b promoter region highly methylated as compared to the corresponding noncancerous tissue (Fig. 7D). Based on the methylation specific PCR assay, however, it appears that hypermethylation is not a common mechanism of miR-125b suppression. The findings for patients 12, 15, and 18 are perplexing, as these patients do not carry mutations in the *p53* gene or display hypermethylation in the miR-125b promoter region. Nonetheless, we found that four HCCs have mutations in the DNA binding domain of the *p53* gene and one HCC displayed hypermethylation of miR-125b promoter region out of nine HCCs tested, (Supporting Table 1), therefore suggesting a possible mechanism for regulating endogenous miR-125a-5p and miR-125b in HCC tumorigenesis.

Sustained Suppression of SIRT7 Reduced* In Vivo *Tumorigenic Potential of Hep3B Cells. To investigate whether the stable suppression of SIRT7 leads to suppression of *in vivo* HCC tumorigenesis, we prepared SIRT7-deficient Hep3B cells by establishing stable SIRT7 knockdown cell lines (Hep3B_SIRT7 KD1, Hep3B_SIRT7 KD2, and Hep3B_SIRT7 KD3) and confirmed the suppression of SIRT7 by detecting $p21^{WAF1/Cip1}$ induction and CDK2, cyclin D1 reduction in these cell lines (Fig. 8A). We then assessed the growth rate of the SIRT7-deficient Hep3B cell lines. All three different clones of SIRT7-deficient Hep3B cell line exhibited reduced growth rate as compared to control cells (negative control shRNA expressing Hep3B cell, Hep3B_Mock1, and Hep3B_Mock2) (Fig. 8B). Based on this result, we performed colony-forming and wound-healing assays. The clonal cell growth and cell motility were significantly attenuated by the sustained suppression of SIRT7 in Hep3B cells (Supporting Fig. 7A,B). Lastly, to demonstrate that SIRT7 inactivation elicits a tumor-suppressive effect *in vivo*, we

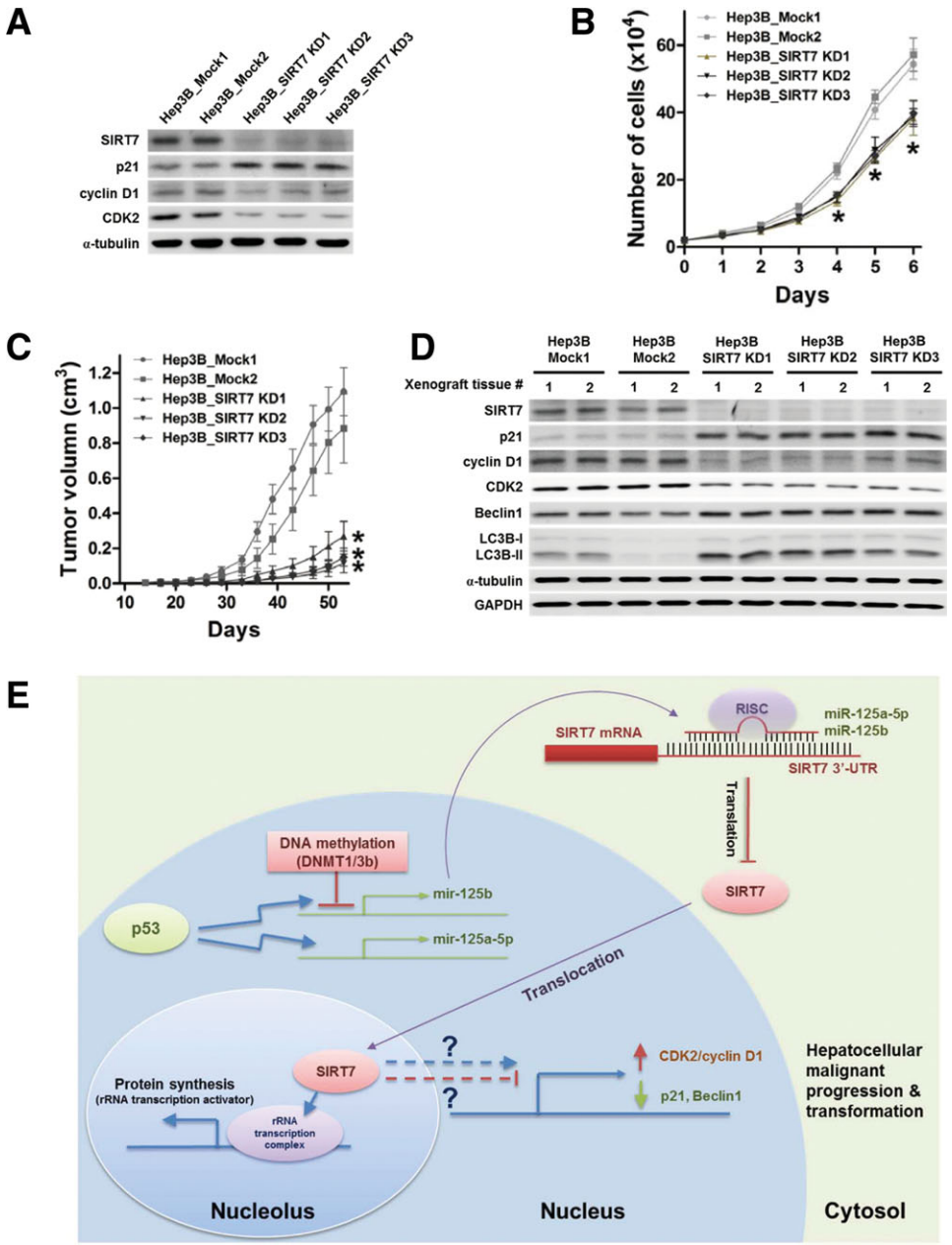

Fig. 8. Sustained suppression of SIRT7 attenuates the tumorigenic potential of Hep3B cells *in vitro* and *in vivo*. (A) Confirmation of SIRT7 suppression by detecting $p21^{WAF1/Cip1}$ induction and CDK2, cyclin D1 reduction in SIRT7-deficient Hep3B cell lines. A typical result of two experiments performed is shown. (B) Time course cell counting analyses of SIRT7-deficient Hep3B cell lines (Hep3B_SIRT7 KD1, 2, and 3) and control cell lines (Hep3B_Mock1 and 2). The cell counts show the results of three independent experiments (unpaired Student's *t* test, $*P < 0.05$ versus Hep3B_Mock1). (C) Tumor growth of animals injected with Hep3B_Mock1 (n = 10), Hep3B_Mock2 (n = 10), Hep3B_SIRT7 KD1 (n = 10), Hep3B_SIRT7 KD2 (n = 7), Hep3B_SIRT7 KD3 (n = 10) cells. Tumor sizes were measured with calipers in three dimensions on the indicated days. Tumor volumes were calculated using the formula: tumor volume (cm^3) = 0.52 × $(W)^2$ × (L), where L is length and W is width. Values shown are mean ± SEM. Data were analyzed by Student's *t* test ($*P < 0.05$ versus Hep3B_Mock1). (D) Western blot analyses of SIRT7 and cell cycle/autophagy-related proteins in xenograft tissues derived from animals injected with SIRT7-deficient cell lines (Hep3B_SIRT7 KD1, 2, and 3) or control (Hep3B_Mock1 and 2). Representative xenograft tissues were sacrificed after 52 days of growth. A typical result of two experiments performed is shown. (E) A model consistent with our findings. A regulatory loop is proposed whereby SIRT7 regulates transcriptional activation of cell cycle, autophagy proteins by way of repression of miR-125a-5p and miR-125b. Regulatory mechanisms of tumor suppressor, miR-125a-5p and miR-125b by way of DNA promoter methylation and wildtype p53 activity are also shown.

subcutaneously injected these cells into athymic nude mice. The overall tumor growth rate and average volume at sacrifice were significantly reduced in SIRT7-deficient Hep3B cells (Fig. 8C; Supporting Fig. 7C). In addition, immunoblot analysis confirmed the induction of $p21^{WAF1/Cip1}$, Beclin-1, and LC3B-II conversion, and the suppression of CDK2 and cyclinD1 in SIRT7-deficient xenograft tissues (Fig. 8D).

Discussion

Sirtuins posttranscriptionally modulate the function of many cellular proteins that undergo reversible acetylation-deacetylation cycles, affecting physiological responses that have implications for treating diseases of aging.[17] Emerging research into sirtuins show their wider role to influence regulatory molecules and pathways in complex manners. A recent breakthrough in SIRT7 research showed that SIRT7 activates RNA polymerase I transcription and deacetylates p53.[5] *Sirt7*-knockout mice have been developed and found to have shorter lifespans with enhanced inflammatory cardiomyopathy.[10] SIRT7 is a nuclear protein that is associated with active rDNA and interacts with RNA polymerase I. SIRT7 overexpression increase rDNA transcription, whereas its down-regulation causes the opposite effect.[5] The rDNA transcription is one of the essential cellular processes, which contains ribosome biogenesis and translation that are governed at numerous levels in cancer progression. An overexpression of SIRT7 has been detected in thyroid and breast cancers,[7,8] and its levels are related to tumor progression. However, no underlying mechanisms for enhanced SIRT7 expression and the consequences of its aberrant regulation have been suggested in these malignancies. In addition, although SIRT7 is abundant in metabolically active tissues,[9] no detailed analysis of biological roles of SIRT7 in liver malignancy, such as HCC, has been conducted to date. In a previous study, we examined large-scale gene expression changes between histopathological grades in human HCCs.[13] Based on these microarray data, we noted that SIRT7 expression was gradually increased from precancer to overt cancer, and we confirmed its up-regulation in an additional subset of human HCCs and in various liver cancer cell lines (Fig. 1; Supporting Fig. 1). These results led us to speculate that SIRT7 plays a role in HCC tumorigenesis. Subsequently, we found that SIRT7 inactivation selectively induced $p21^{WAF1/Cip1}$ expression and concomitantly suppressed cyclin D1 expression in HCC cells (Fig. 2A,B; Supporting Fig. 2A,B). It is not clear whether SIRT7 overexpression leads to the epigenetic suppression of $p21^{WAF1/Cip1}$ *per se* or if other processes also mediate this phenomenon; nonetheless, the present study demonstrates for the first time that SIRT7 can modulate the expression of cell cycle proteins, $p21^{WAF1/CIP1}$ and cyclin D1. This cooperative suppression of $p21^{WAF1/Cip1}$ and induction of cyclin D1 expression by SIRT7 may exert a very potent mitotic stimulation causing uncontrolled cell growth during HCC progression. Furthermore, we found that SIRT7 inactivation suppressed ectopic protein expression, thus implying a role in the protein synthesis machinery during HCC tumorigenesis (Fig. 2E). Overall, our results suggest that these mechanisms are closely interconnected, act in a synergistic manner, and offer a growth advantage to tumor cells. Recently, Barber et al.[18] demonstrated that SIRT7 maintains critical features that define cancer cells by removing the acetylation mark on lysine 18 of histone H3. That study clearly supports our suggestion of oncogenic SIRT7 in hepatocellular malignant proliferation and transformation. However, regulations of SIRT7 activity or expression leading to oncogenic SIRT7 overexpression have not yet been studied.

Many studies have shown that miRNA expression is deregulated in cancer and both loss and gain of miRNA function contribute to cancer development.[19] Thus, we hypothesized that certain miRNAs targeting SIRT7 are down-regulated in HCC, and identified five miRNAs that are significantly down-regulated in HCC from the large-scale miRNA expression analysis of human HCCs (Fig. 3). Additional research demonstrated that both miR-125a-5p and miR-125b are direct suppressors of SIRT7 and may function as tumor suppressors by controlling aberrant expression of SIRT7 in HCC tumorigenesis (Figs. 4, 5). Recently, miR-125b was reported as a tumor suppressor by suppressing tumor angiogenesis, cell proliferation, and metastasis.[20,21] MiR-125a-5p was also found to be an independent prognostic factor and inhibit proliferation of gastric cancer.[22] We then found that p53 activity and promoter methylation could modulate the expression of miR-125a-5p and miR-125b, and the suppression of these regulatory miRNAs led to sustained SIRT7 overexpression in HCC. In addition, mutations in the DNA binding domain of *p53* gene and promoter methylation of miR-125b in HCC patients supported the clinical significance of these findings (Figs. 6, 7).

Although further research for additional suppression mechanisms of these miRNAs in liver cancer has to be done, our results propose a regulatory loop whereby SIRT7 inhibits transcriptional activation of $p21^{WAF1/Cip1}$ by way of repression of miR-125a-5p and miR-125 in HCC tumorigenesis. In normal hepatocytes, the SIRT7 level can be balanced by endogenous miR-125a-5p and/or miR-125b and inhibit *SIRT7* mRNA translation. However, once inactivation of mutation of the p53 gene or hypermethylation of the promoter region of these miRNAs occur, it suppresses endogenous expression of these miRNAs and

thereby causes aberrant regulation of SIRT7. This aberrant overexpression of SIRT7 may contribute to hepatocellular malignant proliferation and transformation by way of activation of the protein synthesis machinery or accelerating the cellular growth rate by transcriptional activation of cell cycle components or preventing autophagic cell death during HCC tumorigenesis (Fig. 8E).

Taken together, in the present study we showed that SIRT7 is up-regulated in HCCs, and that targeted-inactivation of SIRT7 inhibits *in vitro* and *in vivo* HCC tumorigenesis by selective modulating cell cycle, autophagy-related proteins, and protein synthesis machinery in HCC cells, speculating that development of specific modulators of SIRT7 may be crucial for helping control tumor progression or even for reversing cancer phenotype. In this regard, we demonstrated that miR-125a-5p and 125b function as tumor suppressors by regulating abnormal activity of SIRT7 in human HCC. Altogether, these finding define a central role for SIRT7 in HCC tumorigenesis and suggest that miRNAs, miR-125a-5p and miR-125, have potential therapeutic value for the treatment of liver cancer.

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