Carcinogenesis, 2020, Vol. 41, No. 2, 130–138

doi:10.1093/carcin/bgz065
Advance Access Publication September 9, 2019
Original Article

ORIGINAL ARTICLE

Metadherin–PRMT5 complex enhances the metastasis of hepatocellular carcinoma through the WNT–β-catenin signaling pathway

Kai Zhu[1,2,†], Yuanfei Peng[1,†], Jinwu Hu[1,†], Hao Zhan[1,†], Liuxiao Yang[1], Qiang Gao[1], Hao Jia[3], Rongkui Luo[4], Zhi Dai[1], Zhaoyou Tang[1], Jia Fan[1,2,5] and Jian Zhou[1,2,5,*]

[1]Department of Liver Surgery and Transplantation, Liver Cancer Institute, Zhongshan Hospital, Fudan University, Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Shanghai, China, [2]Key Laboratory of Medical Epigenetics and Metabolism, Institute of Biomedical Sciences, Fudan University, Shanghai, China, [3]Faculty of Basic Medicine, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, [4]Department of Pathology, Zhong Shan Hospital, Fudan University, Shanghai 200032, China and [5]Shanghai Key Laboratory of Organ Transplantation, Shanghai, China

*To whom correspondence should be addressed. Tel: +86 21 6404 1990, Fax: +86 21 6403 7181; Email: zhou.jian@zs-hospital.sh.cn
†These authors contributed equally to this work.

Abstract

Accumulating data suggest that metadherin (MTDH) may function as an oncogene. Our previous study showed that MTDH promotes hepatocellular carcinoma (HCC) metastasis via the epithelial-mesenchymal transition. In this study, we aim to further elucidate how MTDH promotes HCC metastasis. Using Co-immunoprecipitation (co-IP) and mass spectrometry, we found that MTDH can specifically bind to protein arginine methyltransferase 5 (PRMT5). Further functional assays revealed that PRMT5 overexpression promoted the proliferation and motility of HCC cells and that knockout of PRMT5 impeded the effect of MTDH. The immunohistochemistry assay/tissue microarray results showed that when MTDH was overexpressed in HCC cells, PRMT5 translocated from the nucleus to the cytoplasm, with the subsequent translocation of β-catenin from the cytoplasm to the nucleus and upregulation of the WNT–β-catenin signaling pathway. Further *in vivo* experiments suggested that PRMT5 and β-catenin played a pivotal role in MTDH-mediated HCC metastasis. We therefore concluded that the MTDH–PRMT5 complex promotes HCC metastasis by regulating the WNT–β-catenin signaling pathway.

Introduction

Surgical resection and liver transplantation are curative interventions for hepatocellular carcinoma (HCC). However, high recurrence rates after surgical intervention compromise the outcome of patients with HCC (1). This predicament highlights the need for a better understanding of the molecular mechanisms underlying HCC invasion and metastasis.

Accumulating data have suggested that metadherin (MTDH) may function as an oncogene. MTDH overexpression is observed in several tumor types, such as HCC, breast cancer, malignant glioma and so forth (2–6), and is associated with a poor clinical outcome. MTDH plays key roles in carcinogenesis (7,8), angiogenesis (9), metastasis (2,10) and chemo resistance (11,12). For instance, Lee *et al.* (13) revealed that MTDH promotes tumor progression through the phosphorylation of PI3K/Akt; moreover, MTDH enhances the expression of angiogenic factors, such as hypoxia-inducible factor 1-α (9), and matrix metalloprotease-9 (5), and promotes the transformation of endothelial cells to carcinoma-associated fibroblasts (14).

Our previous study demonstrated that MTDH promotes HCC metastasis *via* the epithelial-mesenchymal transition (EMT) (6). MTDH inhibition in HCC cells leads to the downregulation of N-cadherin and Snail, and the upregulation of E-cadherin. Furthermore, increased cytoplasmic β-catenin and reduced nuclear β-catenin have also been observed when MTDH was

Received: December 27, 2018; **Revised:** March 3, 2019; **Accepted:** May 7, 2019

Abbreviations

ATCC	American Type Culture Collection
Co-IP	co-immunoprecipitation
cDNA	complementary DNA
EMT	epithelial-mesenchymal transition
GST	glutathione S-transferase
HCC	hepatocellular carcinoma
IHC	immunohistochemistry assay
LEF	lymphoid enhancer factor
MS	mass spectrometry
MTDH	metadherin
PRMT5	protein arginine methyltransferase 5
TCF	T-cell factor

knocked down. However, the underlying mechanism has not been fully investigated.

Materials and methods

Cells and clinical samples

L02 [an immortal hepatic cell line, American Type Culture Collection (ATCC)], HepG2 (ATCC), Huh7 (ATCC), MHCC97-L (97L) and HCCLM3 (LM3) (established at the Liver Cancer Institute, Zhong Shan Hospital, Fudan University, Shanghai, China) cells were used in this study. All the cell lines were tested once purchased, using the Applied Biosystems 3730xl DNA Analyzer. Data were analyzed using GeneMapper 5.0 software (Applied Biosystems). A tissue microarray of 323 HCC samples was used in the immunohistochemistry assay (IHC) (6). The IHC and evaluation of the intensity was performed following our former procedure (6). For nuclear and cytoplasmic expression pattern, a revised scoring system was used (15): Nuclear and cytoplasmic staining was evaluated separately by staining intensity (0, 1+, 2+ and 3+) and the fraction of positive tumor cells. The final score of either nuclear of cytoplasmic expression was calculated by Σ intensity $\times$ fraction. The distribution was considered 'nuclear' if the score of nuclear expression was higher.

Cell transfection

The genomic complementary DNA (cDNA) of 293 T cells was used as a template for amplification of the protein arginine methyltransferase 5 (PRMT5) using the primer: Forward: 5′-ataggatcc (BamHI) ATGCGGGGTCCGAACTCGGGGA-3′; Reverse: 5′-atactcgag (XhoI) CTAGAGGCCAATGGTATATGAG-3′.

The PCR fragment was cloned to pcDNA3-FLAG. The PRMT5 cDNA were subcloned from pcDNA3-FLAG-PRMT5 via PCR and inserted into the pWPI.1 vector, which using the primer: Forward: 5′-cacagatct (Bgl II) CCCACCATGGACTACAAAGACGA-3′; Reverse: 5′-ataacgcgt (MluI) CTAGAGGCCAATGGTATATGAG-3′. Same procedures were followed in the amplification of MTDH, its truncations, and β-catenin, primers used are listed in the Supplementary Table 1, available at *Carcinogenesis* Online.

PRMT5 knockdown was achieved using the short hairpin RNA (shRNA)-mediated stable silencing method. Three sequences of short hairpin RNA targeting PRMT5 were designed (Supplementary Table 1, available at *Carcinogenesis* Online). The second sequence was used based on results of western blot.

Co-immunoprecipitation

Myc tagged MTDH full-length (MTDH-Myc) and its truncations (Supplementary Table 1, available at *Carcinogenesis* Online) were inserted into pcDNA3.1-Myc vector. The human PRMT5 cDNA were inserted into pcDNA3.1-Flag vector (PRMT5-Flag). The human β-catenin cDNA was inserted into pcDNA3.1-HA vector (β-catenin-HA).

The plasmids were transiently transfected into 293 T cells, and at 24 h after transfection, the cells were lysed in buffer A. Co-immunoprecipitations (co-IP) were performed with anti-Myc, anti-Flag and anti-HA antibodies. Antibodies used in these assays are listed in Supplementary Table 1, available at *Carcinogenesis* Online.

2D-LC-MS/MS

Total cell lysates extracted from MTDH-Myc stably expressed cells were subjected to affinity purification. The purified protein complex was resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver stained, then the bands of interest were retrieved and analyzed by mass spectrometry (MS): The gel pieces were digested with trypsin, then vacuum-dried and resuspended in acetonitrile for MS analysis. 2D-LC-MS/MS analyses were performed using a Nano Aquity UPLC system (Waters, Milford, MA) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany), equipped with an online nanoelectrospray ion source (Michrom Bioresources, Auburn, CA). A BEH300 C18 reverse phase (75-mm inner diameter, 20 cm) capillary column was used in this study. Under 19-kV accelerating voltage, MS was performed in reflection mode with an *m/z* range of 400–2000 Da. All MS/MS data were identified using SEQUEST (v.28; BioWorks 3.3 software package; Thermo Electron) against the Human International Protein Index (IPI) database (IPI human v3.45 FASTA with 71 983 entries). The search results were checked by examination of the Xcorr (cross-correlation) and the DCn (delta normalized correlation) scores.

Glutathione S-transferase pull down

GST pull down assay was applied as described previously (16). The glutathione S-transferase (GST)–MTDH protein GST-MTDH (aa 49–69) was generated by PCR amplification of the DNA fragment encoding amino acid residues 49–69 of human MTDH. The pulled down mixture was analyzed by SDS–PAGE.

Luciferase reporter assay

293 T cells were seeded 24 h before transfection in 24 wells plates at 50–60% confluence. The TCF1/TCF7 promoter reporter constructs were co-transfected along with MTDH, PRMT5 and shβ-catenin or vector using Lipofectamine 3000 (Invitrogen). After 24 and 36 h, luciferase activity was assessed using the Dual-Luciferase Reporter reagent (Promega) following the manufacturer's instructions. Renilla luciferase was used for normalization.

Metastasis assays *in vivo*

Male athymic BALB/c nude mice were purchased from Shanghai Institute of Material Medicine, Chinese Academy of Science, and were raised in specific pathogen-free conditions. Four cell lines (mock-luc, MTDH-PRMT5-luc, MTDH/PRMT5/shβ-catenin-luc or shβ-catenin-luc) were created using LM3 cells. 2 × 107 cells were injected in the tail vein of nude mice. The pulmonary metastases (bioluminescence) were detected using an IVIS live-imaging system (Caliper IVIS Lumina XR) every week for 4 weeks. D-luciferin (150 μl) was injected intravenously before detection. After 4 weeks, the mice were killed and the total number of metastases was counted. Six mice per group were used in each experiment, and animal care and experimental protocols were conducted in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission.

Statistical analysis

Statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL). The correlation between PRMT5 and MTDH expression was evaluated using the Pearson chi-square test. Two-tailed value of $P < 0.05$ was used to indicate a significant result.

Primers and antibodies used in this study are listed in the Supplementary Table 1, available at *Carcinogenesis* Online.

Results

PRMT5 is identified to be closely associated with MTDH

To identify MTDH associated proteins in HCC, we affinity-purified MTDH-interacting proteins in MTDH-overexpressing HepG2 cells (MTDH) and their parental counterparts (Control) using co-IP. The purified protein complex was resolved on SDS–PAGE and silver stained, and a new 72-kDa band was observed (Figure 1A). The band was then retrieved and analyzed by MS. A large number of proteins were identified (Table 1), among which PRMT5 had the highest unique peptide score.

PRMT5 belongs to the family of methyltransferases, which catalyze arginine methylation (17). PRMT5 is associated with

multiple benign and malignant cellular processes and has emerged as a pivotal molecule in tumorigenesis and metastasis (18–21). We therefore selected PRMT5 for further investigation. We performed western blot analysis to compare the expression levels of PRMT5 in one hepatic cell line (L02) and four HCC cell lines with different metastatic potentials (HepG2, Huh7, 97L and LM3) (Figure 1B). The results showed that PRMT5 expression was low in the normal hepatic cell line (L02) and low metastatic HCC cell lines (HepG2 and Huh7), but high in the high metastatic HCC cell lines (97L and LM3). We then treated LM3 with a lentivirus targeting PRMT5 to generate an LM3-knockdown (LM3-KO) cell line in which PRMT5 was stably downregulated compared with that in the parental cell lines. Furthermore, we generated a 97L-overexpressing (97L-OE) cell line in which the level of PRMT5 expression was higher than that in the parental 97L cell line (Figure 1Cand D). Proliferation assays using CCK-8 revealed that the PRMT5 knockdown resulted in a significant decrease in the proliferation rate of the HCC cells and vice versa (Figure 1E). We also performed two-chamber migration assays to compare *in vitro* motility between the parental and interfered HCC cells, and the results suggested that PRMT5 enhanced the motility of HCC cells (Figure 1F). These abilities of PRMT5 were reminiscent of those of MTDH observed in our previous study (6).

To verify the connection between MTDH and PRMT5, we generated an MTDH-overexpressing (MTDH) cell line that expressed a higher level of MTDH than that in the parental cell line, and then, we treated the MTDH cells with a lentivirus targeting PRMT5 to generate MTDH-shPRMT5, in which PRMT5

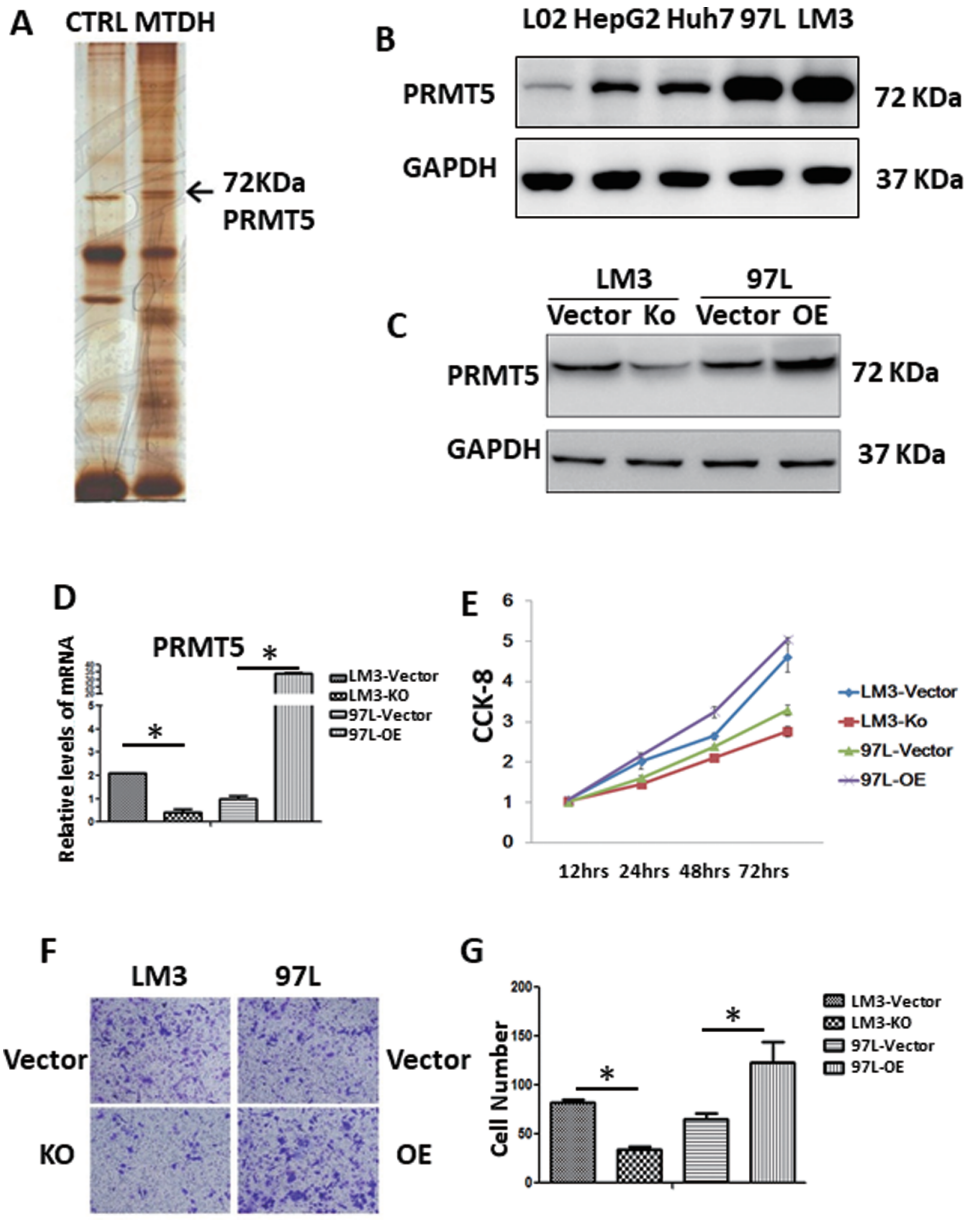

Figure 1. PRMT5 is closely associated with MTDH. (**A**) The SDS–PAGE and silver staining of the purified protein complexes extracted from MTDH-overexpressing and control cells. (**B**) Western blot analysis of PRMT5 in hepatic and HCC cell lines. (**C, D**) Efficiency of PRMT5 knockdown (KO) and overexpression (OE) according to western blot (C) and qRT-PCR (D) analysis. (**E**) Proliferation assays reveals that PRMT5 knockdown results in a significant decrease in the HCC cell proliferation rate and vice versa. (**F, G**) The results of the two-chamber migration assay show that the number of PRMT5-KO-treated cells that migrated through the transwell was far fewer than that of the cells in the control group. In addition, more PRMT5-OE-treated cells than control cells migrated through the transwell (*$P < 0.05$).

was stably downregulated compared with that in the parental cell lines (Figure 2A). MTDH overexpression led to a marked increase in the proliferation rate; however, when PRMT5 was knocked down, the proliferation rate returned to normal (Figure 2B). The two-chamber migration assays also showed that PRMT5 knockdown hampered the motility of the HCC cells, even when MTDH was overexpressed (Figure 2C). These results suggest that the function of MTDH is mediated by PRMT5.

Table 1. MS analysis of MTDH-associated proteins

Protein name (annotation)	Unique peptides
PRMT5 (protein arginine N-methyltransferase 5)	20
HSPA9 (cDNA FLJ51907, highly similar to Stress-70 protein, mitochondrial)	20
RPN1 (dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1 precursor)	18
HSPA5 (78-kDa glucose-regulated protein)	17
AGPS (alkyldihydroxyacetonephosphate synthase, peroxisomal)	7
GTPBP1 (GTP-binding protein 1)	4
ATAD3B (isoform 1 of ATPase family AAA domain-containing protein 3B)	3
KIAA0020 (Pumilio-domain-containing protein KIAA0020)	3

Proteins with highest score of unique peptide are listed.

A single domain in the 49–69 region of MTDH is essential for PRMT5 binding.

To further explore the interaction between MTDH and PRMT5, we performed co-IP assays with either an MTDH (Figure 3A) or a PRMT5 (Figure 3B) antibody, and the co-eluted proteins were detected by western blot assays with either a PRMT5 or an MTDH antibody. Both proteins were detected in the co-eluted protein complex, which confirmed that PRMT5 was closely associated with MTDH.

MTDH is a single-pass transmembrane protein (22) that can be divided into three truncations (Figure 3C): the 1–48 domain is the extracellular region, the 49–69 domain is the transmembrane region, and the 70–582 domain in the cytoplasmic region. To identify the region in MTDH that mediated the interaction between MTDH and PRMT5, we first generated a series of Myc-epitope-tagged truncations of MTDH. We co-expressed full-length Flag-PRMT5 and Myc-MTDH in 293 T cells, and co-IP assays were performed with Flag and Myc antibodies. Notably, the full-length protein and the 49–69 domain of MTDH were bound to PRMT5, whereas the 1–48 and 70–582 domains were not (Figure 3D), suggesting that the amino acid residue 49–69 was critical for PRMT5 binding.

To further determine whether PRMT5 directly bound to the 49–69 domain of MTDH, we expressed GST-MTDH and Flag-PRMT5 proteins in *Escherichia coli* and purified these proteins for *in vitro* binding assays. Co-IP assays were performed using a GST antibody, and the co-eluted Flag-PRMT5 was detected with a PRMT5 antibody (Figure 3E). Indeed, PRMT5 could bind to both the full length protein and the 49–69 domain of MTDH,

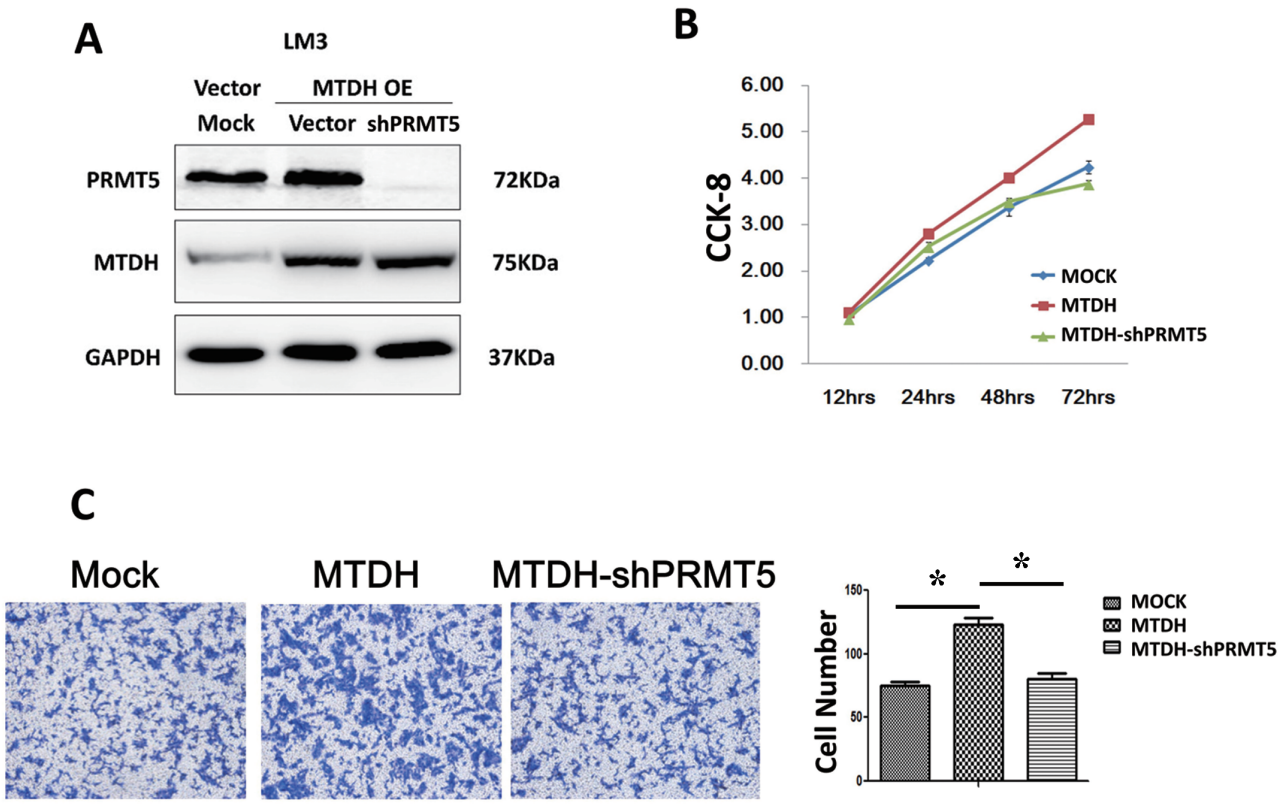

Figure 2. PRMT5 knockdown impedes the effect of MTDH. (**A**) Efficiency of MTDH overexpression (MTDH) and PRMT5 knockdown in MTDH-OE (MTDH-shPRMT5) according to western blot. (**B**) Proliferation assays reveal that PRMT5 knockdown inhibits the proliferation rate of MTDH cells. (**C**) The results of the two-chamber migration assay show that fewer MTDH-shPRMT5-treated cells than control MTDH cells migrated through the transwell (*$P < 0.05$).

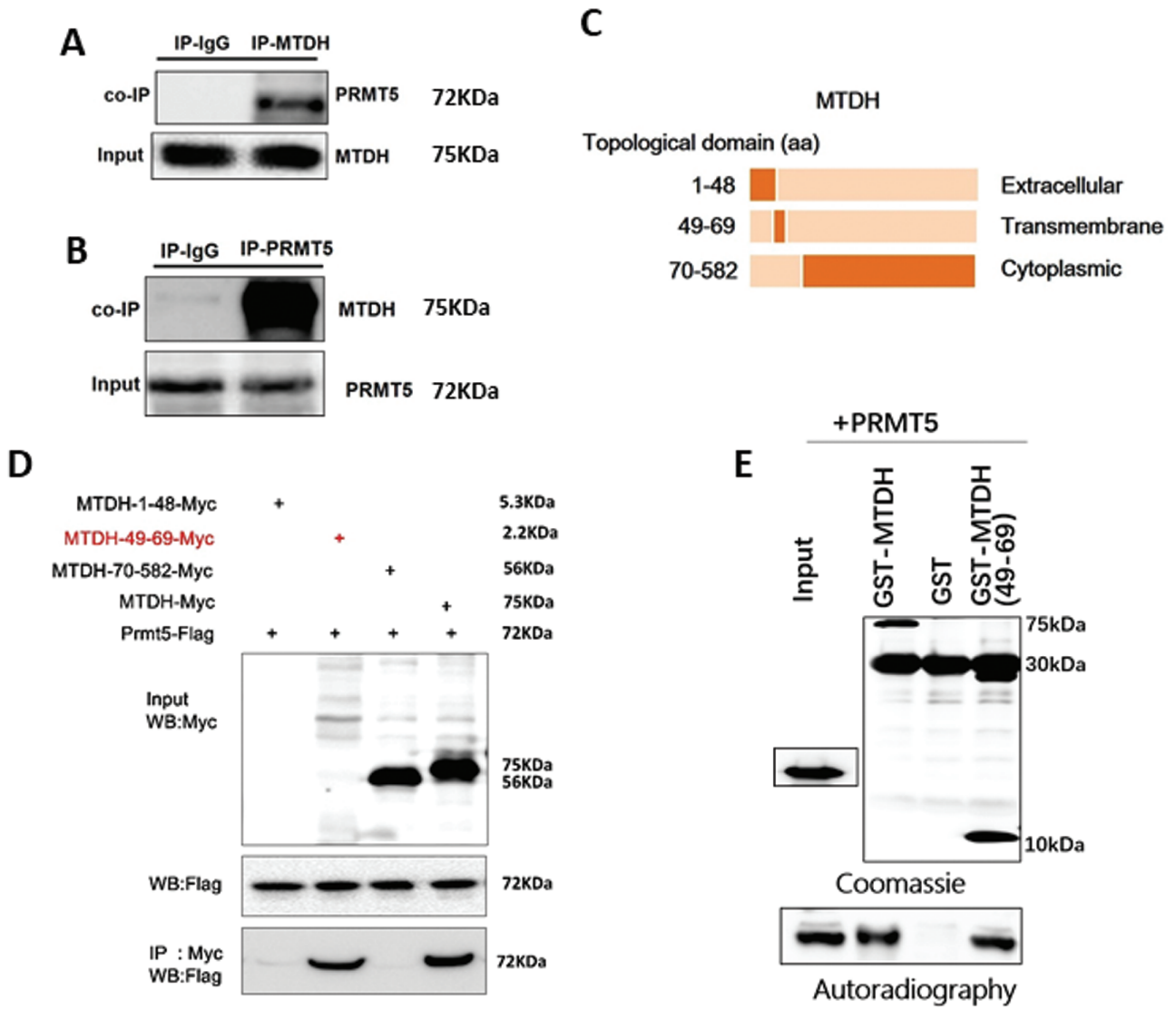

Figure 3. A single domain in the 49–69 region is essential for PRMT5 binding. (**A**) PRMT5 is detected in the purified protein complex extracted with the MTDH antibody. (**B**) MTDH is detected in the purified protein complex extracted with the PRMT5 antibody. (**C**) Diagrammatic structure of MTDH. (**D**) PRMT5 binds to the full-length protein and the 49–69 domain but not to the 1–48 or 70–582 domain of MTDH when co-expressed in 293 T cells. Cell lysates were prepared from 293 T cells transiently co-expressing Myc-MTDH (or truncations of MTDH) and Flag-PRMT5. The co-IP assays were carried out with Flag antibody, and the co-eluted proteins were visualized by western blot with Myc antibodies. (**E**) An *in vitro* GST assay shows that PRMT5 binds to both the full-length protein and the 49–69 domain of MTDH.

suggesting that PRMT5 formed a complex by directly interacting with the 49–69 domain of MTDH.

PRMT5 overexpression activates the WNT–β-catenin signaling pathway

To find the downstream targets of PRMT5, we performed RNA-seq assays to identify differentially expressed genes in 97L-Vector VS 97L-PRMT5-OE cells (Figure 4A). We observed that overexpression of PRMT5 resulted in the upregulation of several downstream molecules of the WNT pathway (LEF1, Cyclin D1 and TCF1/TCF7) (23). Further quantitative real-time PCR and western blot analysis confirmed that the expression levels of these proteins were higher when PRMT5 was overexpressed and lower when PRMT5 was knocked down (Figure 4B and C), indicating that a high level of PRMT5 may activate the WNT signaling pathway.

Our previous study showed that β-catenin is closely associated with MTDH in HCC (6). β-Catenin is a crucial factor in the canonical WNT pathway (23–25). We therefore explored the role of β-catenin in MTDH/PRMT5-mediated HCC metastasis. We generated a cell line (MTDH-PRMT5) that expressed higher levels of both MTDH and PRMT5 than those in the parental cell line, and after that, we treated the cells with a lentivirus targeting β-catenin to generate MTDH-PRMT5-shβ-catenin, in which β-catenin was stably downregulated. Luciferase reporter assay was performed on these cell lines to test the effect of MTDH/PRMT5/β-catenin on TCF1/TCF7 transcriptional activity. The results revealed that MTDH and PRMT5 markedly enhanced the activity of TCF1/TCF7, and the activity was impeded when β-catenin was suppressed (Figure 4D).

The proliferation rate and two-chamber migration assays showed that β-catenin knockdown suppressed the growth rate (Figure 4E) and motility (Figure 4F) of MTDH-PRMT5 cell lines, indicating that β-catenin mediates the function of MTDH and PRMT5 in HCC cells.

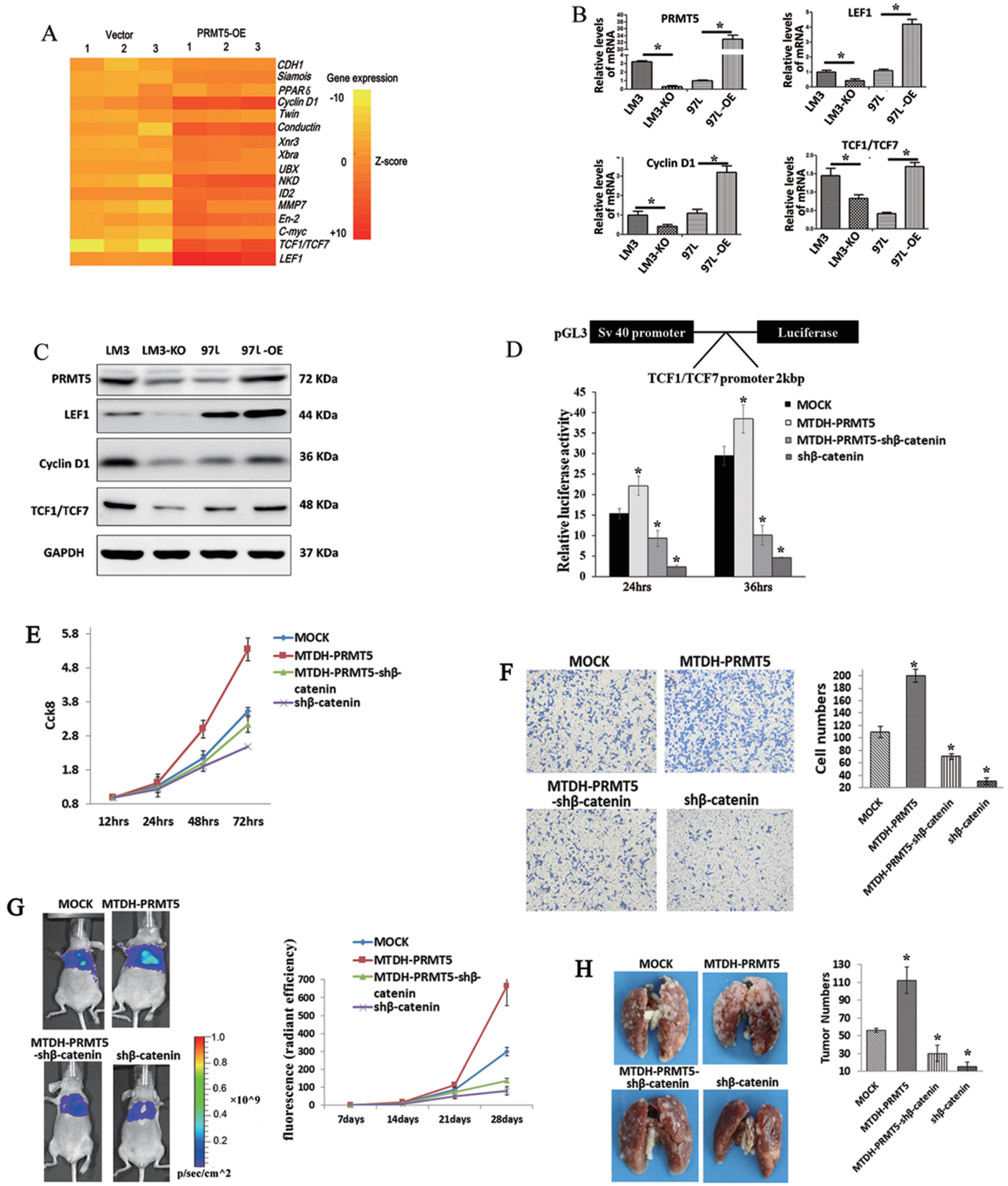

Figure 4. PRMT5 overexpression activates the WNT–β-catenin signaling pathway. (**A**) RNA-seq analyses showed that PRMT5 overexpression in 97-H cells resulted in the upregulation of a group of WNT signaling target genes. (**B**) qRT-PCR analysis of LEF1, Cyclin D1 and TCF1/TCF7 in PRMT5-KO and PRMT5-OE cells compared with their parental counterparts. (**C**) Western blot results of LEF1, Cyclin D1 and TCF1/TCF7 in the four groups of cells. (**D**) TCF1/TCF7 promoter driven luciferase reporter assay on MOCK, MTDH-PRMT5, MTDH/PRMT5/shβ-catenin, and shβ-catenin cells. (**E**) Proliferation assays reveal that β-catenin knockdown inhibits the proliferation rate of MTDH-PRMT5 cells. (**F**) The results of the two-chamber migration assay show that fewer MTDH-PRMT5-shβ-catenin treated cells than control MTDH-PRMT5 cells migrated through the transwell. (**G**) Representative bioluminescent images of mice that received mock-luc, MTDH-PRMT5-luc, MTDH/PRMT5/shβ-catenin-luc, or shβ-catenin-luc at day 28 after transfer. Data are bioluminescent signal of each group, representing the pulmonary metastases. (**H**) Number of pulmonary metastases is significantly larger in the MTDH-PRMT5 group (*$P < 0.05$).

To explore the effect of MTDH/PRMT5/β-catenin on HCC metastasis, we injected four cell lines (mock, MTDH-PRMT5, MTDH-PRMT5-shβ-catenin or shβ-catenin) in the tail vein of nude mice to create pulmonary metastases. The bioluminescence was detected every week for 4 weeks. After 4 weeks, the mice were killed and the total number of metastases was counted. The results also showed that β-catenin knockdown reduced lung metastases of parental MTDH-PRMT5 cell lines (Figure 4G and H).

PRMT5 and β-catenin competitively bind to MTDH.

To further explore the relationship between PRMT5, β-catenin and MTDH in HCC, we assessed their expression levels in serial sections from the same tissue microarray of 323 patients with HCC (Figure 5A). The IHC results showed that the PRMT5 expression levels in most of the tumor tissues were significantly higher than that in the corresponding adjacent non-tumorous tissues, with PRMT5^{High} accounting for 53.3% (172 of 323) of all patients. The Pearson chi-square test indicated that PRMT5 expression was positively correlated with age, gender, liver cirrhosis, serum alpha fetoprotein (AFP), tumor diameter, Edmonson grade, TNM stage and MTDH expression (Supplementary Table 2, available at *Carcinogenesis* Online).

PRMT5 was localized to both the nuclear and cytoplasmic compartments. However, the distribution varied among patients, with PRMT5^{Nuclear} accounting for 36.8% (119 of 323) of all the patients. The Pearson chi-square test also showed a close correlation between PRMT5 and age, serum AFP, microvascular invasion, Edmonson grade, MTDH expression (Supplementary Table 3, available at *Carcinogenesis* Online). Our former study indicated that β-catenin translocation from the membrane to the

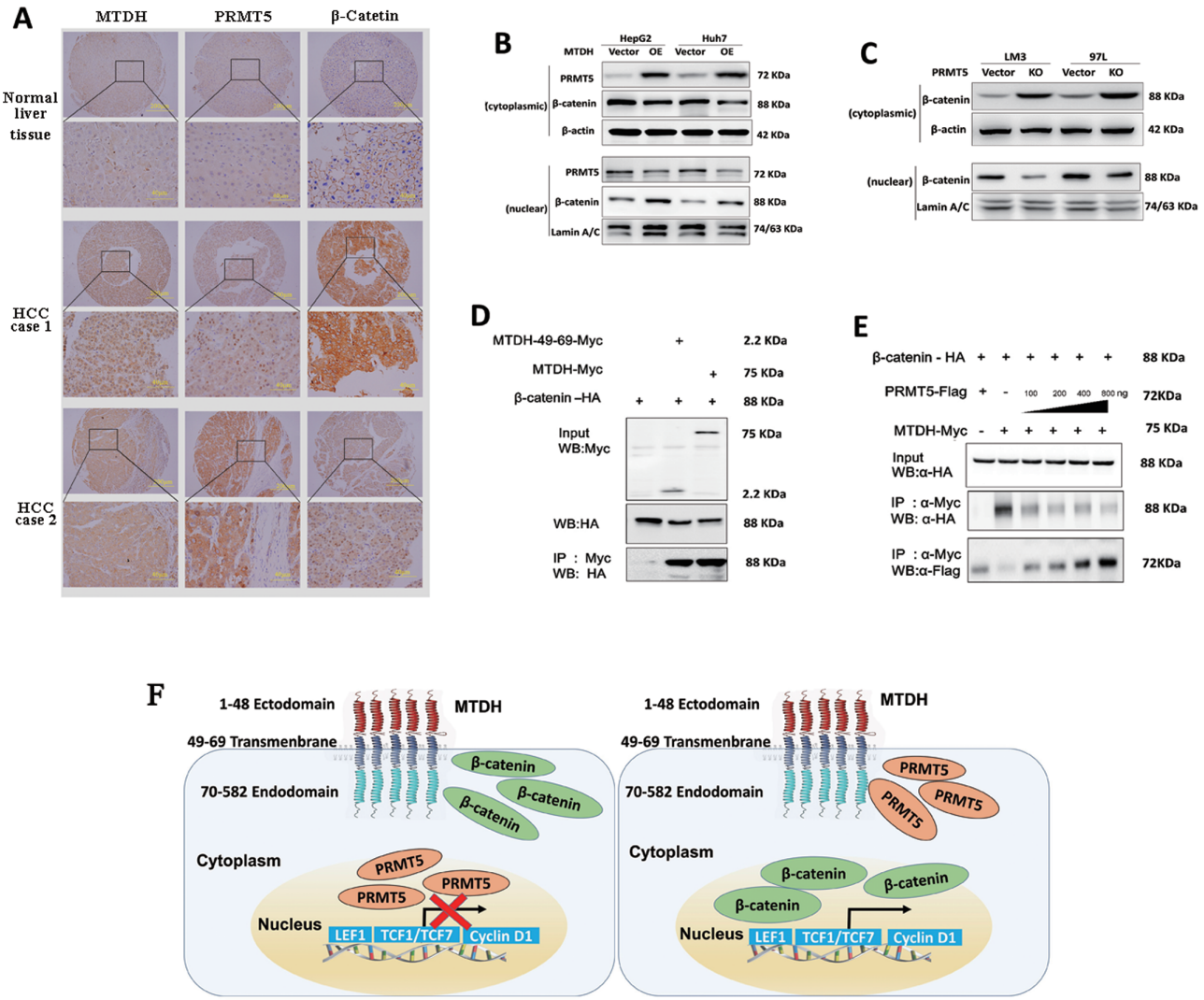

Figure 5. Correlation between PRMT5, β-catenin and MTDH in HCC. (**A**) IHC results of serial sections from the same tissue microarray (TMA). In normal liver tissue, PRMT5 expression is low, and PRMT5 is primarily localized to the nucleus, whereas β-catenin is localized to the membrane. HCC Case 1: PRMT5 is localized to the nucleus; and β-catenin is localized to the membrane. HCC Case 2: PRMT5 is localized to the cytoplasm; whereas β-catenin is localized to the nucleus. (**B**) Western blot analysis of PRMT5 and β-catenin in the cytoplasm (β-actin as the control) and nucleus (Lamin A/C as the control) of the cultured cells shows that the overexpression of MTDH leads to increased PRMT5 and reduced β-catenin in the cytoplasm, with reduced PRMT5 and increased β-catenin in the nucleus. (**C**) PRMT5 knockdown in HCC cells leads to increased cytoplasmic β-catenin, and decreased nuclear β-catenin. (**D**) β-catenin binds to the full-length protein and the 49–69 domain of MTDH. Cell lysates were prepared from 293 T cells transiently co-expressing Myc-MTDH (or the 49–69 domain of MTDH) and HA-β-catenin. Co-IP assays were performed with HA and Myc antibody. (**E**) Increasing the amount of the PRMT5 gradually abolishes the interaction between MTDH and β-catenin. (**F**) The working model shows that the PRMT5 and β-catenin competitively bind to the same domain of MTDH, when β-catenin is released from MTDH, it will translocate from the cytoplasm to the nucleus, and participated in WNT–β-catenin signaling pathway.

nucleus could be induced by MTDH overexpression in HCC (6). We therefore compared the distribution of PRMT5 and β-catenin in the tissue microarray. The results showed that when PRMT5 was localized to the nucleus, β-catenin was localized to the membrane, and vice versa ($P < 0.001$) (Figure 5A, Supplementary Table 3, available at *Carcinogenesis* Online).

We then stably overexpressed MTDH in HepG2 and Huh7 cells, and separated the cell extracts into nuclear and cytoplasmic fractions. As evidenced by western blot (Figure 5B), MTDH overexpression in HCC cells resulted in an increase in PRMT5 and a decrease in β-Catenin in the cytoplasm, with a corresponding decrease in PRMT5 and increase in β-catenin in the nucleus. In addition, the inhibition of PRMT5 led to increased cytoplasmic β-catenin and decreased nuclear β-catenin (Figure 5C). These results confirmed that MTDH overexpression led to the translocation of PRMT5 from the nucleus to the membrane, and of β-catenin from the membrane to the nucleus.

The earlier results suggested that PRMT5 and β-catenin competitively bind to the same domain on MTDH. To confirm our hypothesis, we co-expressed full-length HA-β-catenin and either Myc-MTDH or Myc-49-69-MTDH in 293 T cells, and co-IP assays were carried out with an HA antibody, and the co-eluted proteins were visualized by western blot assay with a Myc antibody. The results showed that both the full-length protein and the 49–69 domain of MTDH bound to β-catenin (Figure 5D).

To determine whether MTDH, PRMT5 and β-catenin formed functional ternary complexes, we co-expressed Myc-MTDH and HA-β-catenin together with an increasing amount of Flag-PRMT5 in 293 T cells, and performed co-IP assays with Flag antibody. The co-eluted proteins were visualized by western blot with either Flag or HA antibody (Figure 5E). MTDH readily co-immunoprecipitated both PRMT5 and β-catenin. Strikingly, increasing the amount of PRMT5 caused MTDH to immuoprecipitate more PRMT5 and less β-catenin, suggesting that PRMT5 decreased the assembly of the MTDH and β-catenin complex. These results suggest that PRMT5 and β-catenin may competitively bind to the same domain on MTDH.

Discussion

Herein, we identified the MTDH–PRMT5 complex as an enhancer of HCC metastasis via the WNT–β-catenin signaling pathway. We demonstrated that PRMT5 and β-catenin may competitively bind to the same domain on MTDH. When MTDH is overexpressed, MTDH preferentially recruits PRMT5, and the redundant β-catenin translocates to the nucleus, and participates in the WNT–β-catenin signaling pathway. PRMT5 knockdown hampers the effect of MTDH on the proliferation and motility of the HCC cells, and inhibition of β-catenin disrupts the effect of both MTDH and PRMT5.

PRMT5 is an important modulator of tumor cell function, and PRMT5 dysregulation is observed in multiple malignancies (26–28). Powers *et al.* (26) revealed that high levels of PRMT5, with corresponding arginine methylation of the tumor suppressor programmed cell death 4, correlate with a worse outcome in patients with breast cancer by Chen *et al.* (29) demonstrated that PRMT5 plays a key role in a TGFβ-PRMT5-MEP50-axis-mediated EMT in lung cancer and breast cancer. Recently, PRMT5 was confirmed to have an oncogenic function in the context of leukemia/lymphoma (28) and has been recognized as a promising target for leukemia therapy (28,30). Through co-IP and MS, we demonstrated that PRMT5 is closely associated with MTDH. Functional assays revealed that the overexpression of PRMT5 promoted HCC cell proliferation and motility, an association that we previously observed with high MTDH levels (6). We also found that when PRMT5 was inhibited, MTDH overexpression promoted neither the proliferation nor motility of HCC cells, implying that MTDH functioned only in the presence of PRMT5. These results highlight the significance of PRMT5 in MTDH mediated regulation.

PRMT5 has been linked to the remodeling of chromatin structure via arginine methylation of histone proteins H4 and H3, thereby modifying gene transcription, DNA repair and RNA processing (28,31,32). For instance, several studies have demonstrated that PRMT5 is required in the regulation of the P53 gene via arginine methylation (28,33–35). Pal *et al.* (18) found that PRMT5 inhibited tumor suppressor gene tumorigenicity 7 and non-metastatic 23 through H3R8 methylation and H3K9 deacetylation. PRMT5 also forms a complex with the ajuba LIM protein and Snail proteins and suppressed the expression of E-cadherin (16).

In our study, we provide considerable evidence showing that in addition to epigenetic regulation of histone methylation, PRMT5 effects the location of β-catenin in HCC through spatial interactions. Using co-IP and IHC analysis of serial sections of HCC samples, we confirmed that PRMT5 and β-catenin competitively bind to the same domain on MTDH. Furthermore, using reciprocal co-IP, we revealed that PRMT5 and β-catenin share the same binding site in the 49–69 region of MTDH. The translocation of β-catenin from the cytoplasm to the nucleus after MTDH overexpression was also confirmed and was consistent with the observations of our previous report (6). In addition, the competitive binding and translocation of β-catenin depended on the expression levels of MTDH and PRMT5 in the cells (Figure 5F);

In this study, we also demonstrated that PRMT5 may promote the proliferation and motility of HCC cells by activating the canonical WNT pathway. Nuclear β-catenin can bind to high-mobility group box proteins, which contains the T-cell factor/lymphoid enhancer factor (TCF/LEF), and thereby activate the target genes of the WNT signaling pathway (23). Accumulating evidence shows that the activation of WNT/β-catenin-mediated signaling plays a key role in the development of HCC (6,24,36,37). Yoo *et al.* (24) showed that the ERK42/44-mediated activation of WNT/β-catenin signaling upregulates TCF/LEF, which is the ultimate initiator of the WNT pathway. Tsao *et al.* (37) revealed that WNT/β-catenin signaling plays a significant role in the SOX1-mediated development of HCC. These findings show that WNT/β-catenin signaling is pivotal in the progression of HCC. LEF1, Cyclin D1 and TCF1/TCF7 are central mediators of the WNT–β-catenin pathway (23,38,39). Notably, we have shown that these proteins are upregulated and activated when PRMT5 is overexpressed, indicating that a high level of PRMT5 may activate the WNT–β-catenin signaling pathway (Figure 5F). β-Catenin disrupted the effect of both MTDH and PRMT5, which suggests that β-catenin plays a pivotal role in MTDH/PRMT5-mediated HCC metastasis.

WNT/β-catenin signaling also induces EMT via the translocation of β-catenin from the cytoplasm to the nucleus, where β-catenin contributes to enhancing the expression of several EMT-inducing transcription factors (40,41). This outcome might explain our former findings whereby MTDH may promote the EMT of HCC cells via the WNT–β-catenin signaling pathway (6).

Nevertheless, as MTDH is a transmembrane protein that interacts with multiple proteins (2,10,42,43) β-catenin is unlikely to be the only factor affected by the MTDH–PRMT5 complex. The spatial characterizations of the binding motifs in MTDH and PRMT5/β-catenin need further investigation.

In summary, our findings indicate that PRMT5 and β-catenin may competitively bind to the same domain on MTDH. Thus, the MTDH–PRMT5 complex may promote HCC metastasis by regulating the WNT–β-catenin signaling pathway. This dynamic may provide new targets for therapy and markers for HCC diagnosis.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

National Natural Science Funds of China (nos. 81402376, 81572296, 81572823, 81502028, 31601114); Shanghai Hospital Development Center (SHDC12015104); National Key Research and Development Program (2016YFC0902400).
Conflict of Interest Statement: The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

References

1. El-Serag, H. B. *et al.* (2008) Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology*, 134, 1752–1763.
2. Hu, G. *et al.* (2009) MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell*, 15, 9–20.
3. Garnis, C. *et al.* (2004) Overexpression of LRP12, a gene contained within an 8q22 amplicon identified by high-resolution array CGH analysis of oral squamous cell carcinomas. *Oncogene*, 23, 2582–2586.
4. Lee, S. G. *et al.* (2009) Astrocyte elevated gene-1 contributes to the pathogenesis of neuroblastoma. *Oncogene*, 28, 2476–2484.
5. Liu, L. *et al.* (2010) Astrocyte elevated gene-1 upregulates matrix metalloproteinase-9 and induces human glioma invasion. *Cancer Res.*, 70, 3750–3759.
6. Zhu, K. *et al.* (2011) Metadherin promotes hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. *Clin. Cancer Res.*, 17, 7294–7302.
7. Bhutia, S. K. *et al.* (2010) Astrocyte elevated gene-1 induces protective autophagy. *Proc. Natl. Acad. Sci. USA.*, 107, 22243–22248.
8. Lee, S. G. *et al.* (2006) Astrocyte elevated gene-1 (AEG-1) is a target gene of oncogenic Ha-ras requiring phosphatidylinositol 3-kinase and c-Myc. *Proc. Natl. Acad. Sci. USA.*, 103, 17390–17395.
9. Emdad, L. *et al.* (2009) Astrocyte elevated gene-1 (AEG-1) functions as an oncogene and regulates angiogenesis. *Proc. Natl. Acad. Sci. USA.*, 106, 21300–21305.
10. Brown, D. M. *et al.* (2004) Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. *Cancer Cell*, 5, 365–374.
11. Yoo, B. K. *et al.* (2009) Identification of genes conferring resistance to 5-fluorouracil. *Proc. Natl. Acad. Sci. USA.*, 106, 12938–12943.
12. Yoo, B. K. *et al.* (2010) Molecular mechanism of chemoresistance by astrocyte elevated gene-1. *Cancer Res.*, 70, 3249–3258.
13. Lee, S. G. *et al.* (2008) Astrocyte elevated gene-1 activates cell survival pathways through PI3K-Akt signaling. *Oncogene*, 27, 1114–1121.
14. Zhu, K. *et al.* (2014) MiR-302c inhibits tumor growth of hepatocellular carcinoma by suppressing the endothelial-mesenchymal transition of endothelial cells. *Sci. Rep.*, 4, 5524.
15. Laban, S. *et al.* (2014) Simultaneous cytoplasmic and nuclear protein expression of melanoma antigen-A family and NY-ESO-1 cancer-testis antigens represents an independent marker for poor survival in head and neck cancer. *Int. J. Cancer*, 135, 1142–1152.
16. Hou, Z. *et al.* (2008) The LIM protein AJUBA recruits protein arginine methyltransferase 5 to mediate SNAIL-dependent transcriptional repression. *Mol. Cell. Biol.*, 28, 3198–3207.
17. Bedford, M.T. *et al.* (2005) Arginine methylation an emerging regulator of protein function. *Mol. Cell*, 18, 263–272.
18. Pal, S. *et al.* (2004) Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. *Mol. Cell. Biol.*, 24, 9630–9645.
19. Pal, S. *et al.* (2007) Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3 methylation in mantle cell lymphoma. *EMBO J.*, 26, 3558–3569.
20. Kim, J. M. *et al.* (2005) Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. *Clin. Cancer Res.*, 11(2 Pt 1), 473–482.
21. Kryukov, G. V. *et al.* (2016) MTAP deletion confers enhanced dependency on the PRMT5 arginine methyltransferase in cancer cells. *Science*, 351, 1214–1218.
22. Kang, D. C. *et al.* (2005) Cloning and characterization of HIV-1-inducible astrocyte elevated gene-1, AEG-1. *Gene*, 353, 8–15.
23. Orsulic, S. *et al.* (1999) E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J. Cell Sci.*, 112 (Pt 8), 1237–1245.
24. Yoo, B. K. *et al.* (2009) Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. *J. Clin. Invest.*, 119, 465–477.
25. Damsky, W. E. *et al.* (2011) β-catenin signaling controls metastasis in Braf-activated Pten-deficient melanomas. *Cancer Cell*, 20, 741–754.
26. Powers, M. A. *et al.* (2011) Protein arginine methyltransferase 5 accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death 4. *Cancer Res.*, 71, 5579–5587.
27. Gu, Z. *et al.* (2012) Protein arginine methyltransferase 5 is essential for growth of lung cancer cells. *Biochem. J.*, 446, 235–241.
28. Li, Y. *et al.* (2015) PRMT5 is required for lymphomagenesis triggered by multiple oncogenic drivers. *Cancer Discov.*, 5, 288–303.
29. Chen, H. *et al.* (2016) A TGFβ-PRMT5-MEP50 axis regulates cancer cell invasion through histone H3 and H4 arginine methylation coupled transcriptional activation and repression. *Oncogene*, 36, 373–386.
30. Jin, Y. *et al.* (2016) Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia. *J. Clin. Invest.*, 126, 3961–3980
31. Wolf, S. S. (2009) The protein arginine methyltransferase family: an update about function, new perspectives and the physiological role in humans. *Cell. Mol. Life Sci.*, 66, 2109–2121.
32. Krause, C. D. *et al.* (2007) Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential. *Pharmacol. Ther.*, 113, 50–87.
33. Bezzi, M. *et al.* (2013) Regulation of constitutive and alternative splicing by PRMT5 reveals a role for Mdm4 pre-mRNA in sensing defects in the spliceosomal machinery. *Genes Dev.*, 27, 1903–1916.
34. Yang, M. *et al.* (2009) Caenorhabditis elegans protein arginine methyltransferase PRMT-5 negatively regulates DNA damage-induced apoptosis. *PLoS Genet.*, 5, e1000514.
35. Durant, S.T. *et al.* (2009) p53 methylation—the Arg-ument is clear. *Cell Cycle*, 8, 801–802.
36. Bengochea, A. *et al.* (2008) Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. *Br. J. Cancer*, 99, 143–150.
37. Tsao, C. M. *et al.* (2012) SOX1 functions as a tumor suppressor by antagonizing the WNT/β-catenin signaling pathway in hepatocellular carcinoma. *Hepatology*, 56, 2277–2287.
38. Ortega, P. *et al.* (2008) Differential Wnt pathway gene expression and E-cadherin truncation in sporadic colorectal cancers with and without microsatellite instability. *Clin. Cancer Res.*, 14, 995–1001.
39. Lu, Z. *et al.* (2003) Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. *Cancer Cell*, 4, 499–515.
40. Polyak, K. *et al.* (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat. Rev. Cancer*, 9, 265–273.
41. Thiery, J. P. *et al.* (2009) Epithelial-mesenchymal transitions in development and disease. *Cell*, 139, 871–890.
42. Thirkettle, H. J. *et al.* (2009) Nuclear LYRIC/AEG-1 interacts with PLZF and relieves PLZF-mediated repression. *Oncogene*, 28, 3663–3670.
43. Sarkar, D. *et al.* (2008) Molecular basis of nuclear factor-kappaB activation by astrocyte elevated gene-1. *Cancer Res.*, 68, 1478–1484.