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Knockdown of Rap2B Inhibits the Proliferation and Invasion in Hepatocellular Carcinoma Cells

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Rap2B, a member of the Ras family of small GTP-binding proteins, was found to be highly expressed in various human tumors and plays an important role in the development of tumors. However, the function of Rap2B in hepatocellular carcinoma (HCC) remains unclear. Therefore, in this study, we investigated the biological functions of Rap2B in HCC and the potential underlying mechanisms. Our results indicated that Rap2B was highly expressed in HCC tissues and cell lines. Rap2B silencing obviously inhibited the proliferation, migration, and invasion of HCC cells, as well as attenuated xenografted tumor growth in vivo. Furthermore, Rap2B silencing greatly reduced the expression levels of phosphorylated focal adhesion kinase (p-FAK), matrix metalloproteinase-2 (MMP-2), and MMP-9 in HCC cells. In conclusion, our data suggest that Rap2B silencing inhibits the proliferation and invasion in HCC cells. Thus, Rap2B may have potential as a treatment for HCC.

Key words: Rap2B; Hepatocellular carcinoma (HCC); Proliferation; Invasion; FAK pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. It is characterized by high recurrence, high metastasis, and a poor prognosis (1). There are about 600,000 new-onset patients with this disease every year, and its incidence has a rising trend (2,3). Although surgical approaches and adjuvant chemotherapy have been improved, the clinical outcome of patients remains unsatisfactory (4–6). Thus, it is urgent to investigate the molecular mechanisms of HCC in order to improve therapeutic approaches and patient outcomes.

Rap2B, a member of the Ras family of small GTP-binding proteins, was first discovered from a platelet cDNA library in the early 1990s (7). The Rap2B gene is located at 3q25.2 of the human chromosome, and its cDNA contains an open reading frame of 552 bp. It was reported to play an important role in regulating cellular processes including cytoskeletal organization, cell growth, and proliferation (8–10). Furthermore, a growing body of evidence suggests a critical role for Rap2B in the tumorigenesis of human cancers (11–14). A recent study has reported that overexpression of Rap2B promoted proliferation, migration, and invasion abilities in breast cancer cells (15). The expression of Rap2B was also significantly upregulated in human suprarenal epithelioma, and overexpression of Rap2B promoted suprarenal epithelioma cell migration via increasing matrix metalloproteinase-2 (MMP-2) protein expression (16). However, the expression and function of Rap2B in human HCC have not been fully elucidated. The aim of our study was to discover the role of Rap2B in the development of human HCC. We found that Rap2B was highly expressed in human HCC tissues and cell lines. Rap2B silencing inhibited proliferation and invasion in human HCC cells.

MATERIALS AND METHODS

Tissue Specimens

A total of nine HCC and nine noncancerous liver tissue samples were obtained from the Department of Gastroenterology and Hepatology, Chinese PLA General Hospital (China) between 2013 and 2015. Dissected samples were frozen immediately after surgery and stored at −80°C until needed. Every participant signed a consent form. A protocol for the use of patient samples was approved by the Medical Ethics Committee of the Chinese PLA General Hospital (China).

Cell Culture

Three human HCC lines (HepG2, 97H, and HCCLM3) and a hepatocyte cell line (HL-7702) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were grown in Dulbecco's modified

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Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 U/ml streptomycin and penicillin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2.

RNA Interference

The short hairpin RNA against Rap2B (sh-Rap2B) or nontargeting control shRNA (sh-NC) (GenePharma, Shanghai) was transfected into human HCC cells in six-well plates using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were assayed 48 h after transfection.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HCC tissues and cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using a PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Dalian, China). qRT-PCR was performed with the SYBR Green Detection System (Bio SYBR Green Master Mix, Takara, Japan). The primer sequences used for PCR amplification were Rap2B, 5′-CTGCCCCTTCATGGAGACA-3′ (forward) and 5′-TG CGAATAGCTCATCCACTGA-3′ (reverse); β-actin was used as an internal standard, and the primers were as follows: 5′-GTCCACCGCAAATGCTTCTA-3′ (forward) and 5′-TGCTGTCACCTTCACCGTTC-3′ (reverse). The band intensities of amplification products were measured by a densitometer, and the results were normalized with β-actin.

Western Blot Analysis

Proteins were extracted from HCC tissues and cells using a cell lysis buffer. A total of 30 μg of protein was separated by 10% SDS-PAGE electrophoresis and transferred onto PVDF membranes (Amersham, Little Chalfont, UK). After blocking with Tween–Tris-buffered saline (T-TBS) containing 5% nonfat milk powder, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies were utilized for Western blot: mouse anti-Rap2B, mouse anti-p-FAK, mouse anti-MMP-2, mouse anti-MMP-9, and mouse anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the membranes were washed with TBST three times and incubated with peroxidase-conjugated goat anti-mouse IgG (1:5,000; Santa Cruz Biotechnology) for 3 h at room temperature. The target protein was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Cell Proliferation Assay

Cellular proliferation was evaluated using Cell Counting Kit-8 (CCK-8) (Beyotime, Nantong, China). Briefly, cells transfected with sh-Rap2B or sh-NC were seeded into 96-well plates in a density of 1×10^4 cells/well in 200 μl of medium. At each time point, 10 μl of CCK-8 was added to each well, followed by incubation at 37 °C for 1 h. The absorbance at 450 nm was measured on an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell Migration and Invasion Assays

Cell migration was detected using the Transwell system (8.0 μm; Millipore, BD Biosciences, San Jose, CA, USA). Briefly, cells transfected with sh-Rap2B or sh-NC were seeded into the upper chamber, and 500 μl of DMEM medium containing 10% FBS was added to the lower chamber. Then the Transwell chambers (Corning Costar, Corning, NY, USA) were incubated in a 37°C, 5% CO_2, humidified incubator for 24 h. Cells in the upper chamber were removed with a cotton swab, and the cells that had traversed the membrane were fixed, stained, and counted under a microscope. The invasion assay was performed by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier.

In Vivo Xenograft Tumor Assay

BALB/c mice (6 to 8 weeks old) were purchased from the Center of Medical Experimental Animals of the Chinese PLA General Hospital. The mice were maintained in the accredited animal facility of the Chinese PLA General Hospital and used for studies approved by the Animal Care and Use Committee of the Chinese PLA General Hospital. To evaluate the long-lasting effect of Rap2B on tumor cells, HCC cells were untreated or treated with sh-Rap2B in vitro for 48 h, and then were subcutaneously injected into the flanks of nude mice. Tumor growth was monitored every 5 days. The length (L) and width (W) of tumors were measured, and the volume of tumors (V) was determined by the formula: $V = (L \times W^2)/2$.

Statistical Analysis

Statistical analysis was conducted using SPSS version 13.0 (Chicago, IL, USA). All the results were presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance or paired t-tests. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Rap2B Is Upregulated in HCC Tissues and Cell Lines

We first evaluated the expression of Rap2B in human HCC tissues by Western blot. The results of the Western blot analysis showed that Rap2B protein expression in human HCC tissues was higher than that in normal liver tissues (Fig. 1A). We further investigated the mRNA expression of Rap2B in human HCC cell lines and found that the mRNA expression of Rap2B was significantly

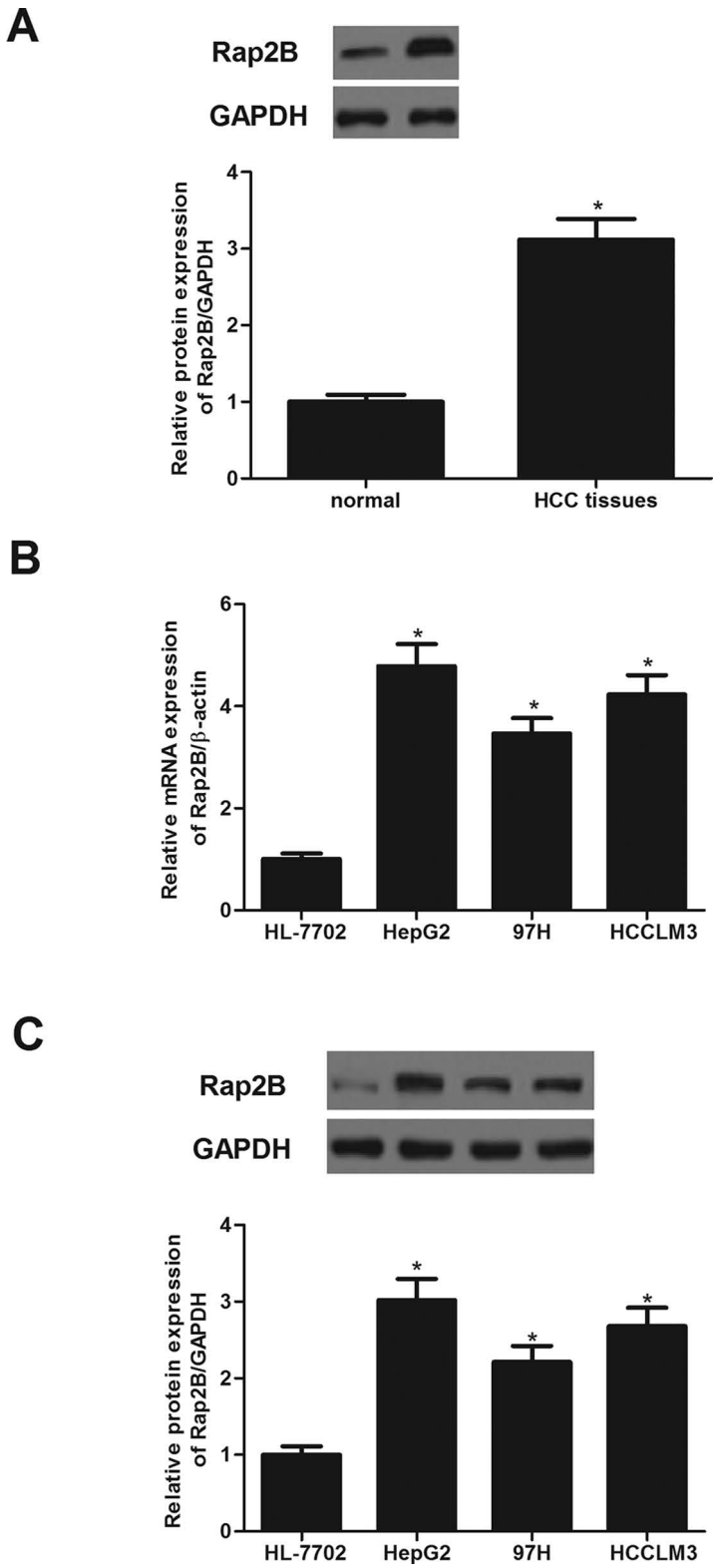

Figure 1. Rap2B is upregulated in HCC tissues and cell lines. (A) Western blot was used to measure the expression levels of Rap2B in normal liver tissues (normal) and HCC tissues. *$p<0.05$ versus normal group. (B) The mRNA levels of Rap2B in the human HCC cell lines (HepG2, 97H, and HCCLM3), as well as the hepatocyte cell line (HL-7702), were examined using qRT-PCR. (C) The protein levels of Rap2B in the human HCC cell lines (HepG2, 97H, and HCCLM3), as well as the hepatocyte cell line (HL-7702), were examined using Western blot. *$p<0.05$ versus HL-7702 group.

increased in human HCC cell lines (HepG2, 97H, and HCCLM3), compared with the hepatocyte cell line (HL-7702) (Fig. 1B). The results of the Western blot analysis showed that the Rap2B protein expression levels were highly expressed in HCC cell lines compared with hepatocyte cells (Fig. 1C).

Rap2B Silencing Inhibits the Proliferation of HCC Cells

To investigate the potential role of Rap2B in the development of HCC, we used shRNA to knock down Rap2B in HepG2 and HCCLM3 cells, respectively, which resulted in a low level of endogenous EphB2 expression, as confirmed by Western blotting (Fig. 2A and B). The CCK-8 proliferation assay was then used to detect the effect of Rap2B on HCC cell proliferation. As shown in Figure 2C, knockdown of Rap2B significantly inhibited HepG2 cell proliferation compared with sh-NC-transfected cells. Similar results were observed in HCCLM3 cells (Fig. 2D).

Rap2B Silencing Inhibits the Migration/Invasion of HCC Cells

To determine whether the downregulation of Rap2B inhibits cell migration, we performed cell migration assay using the Transwell system. We found that knockdown of Rap2B inhibited the invasiveness of HepG2 (Fig. 3A) and HCCLM3 cells (Fig. 3B), compared with sh-NC-transfected cells, as indicated by a marked decrease in the number of

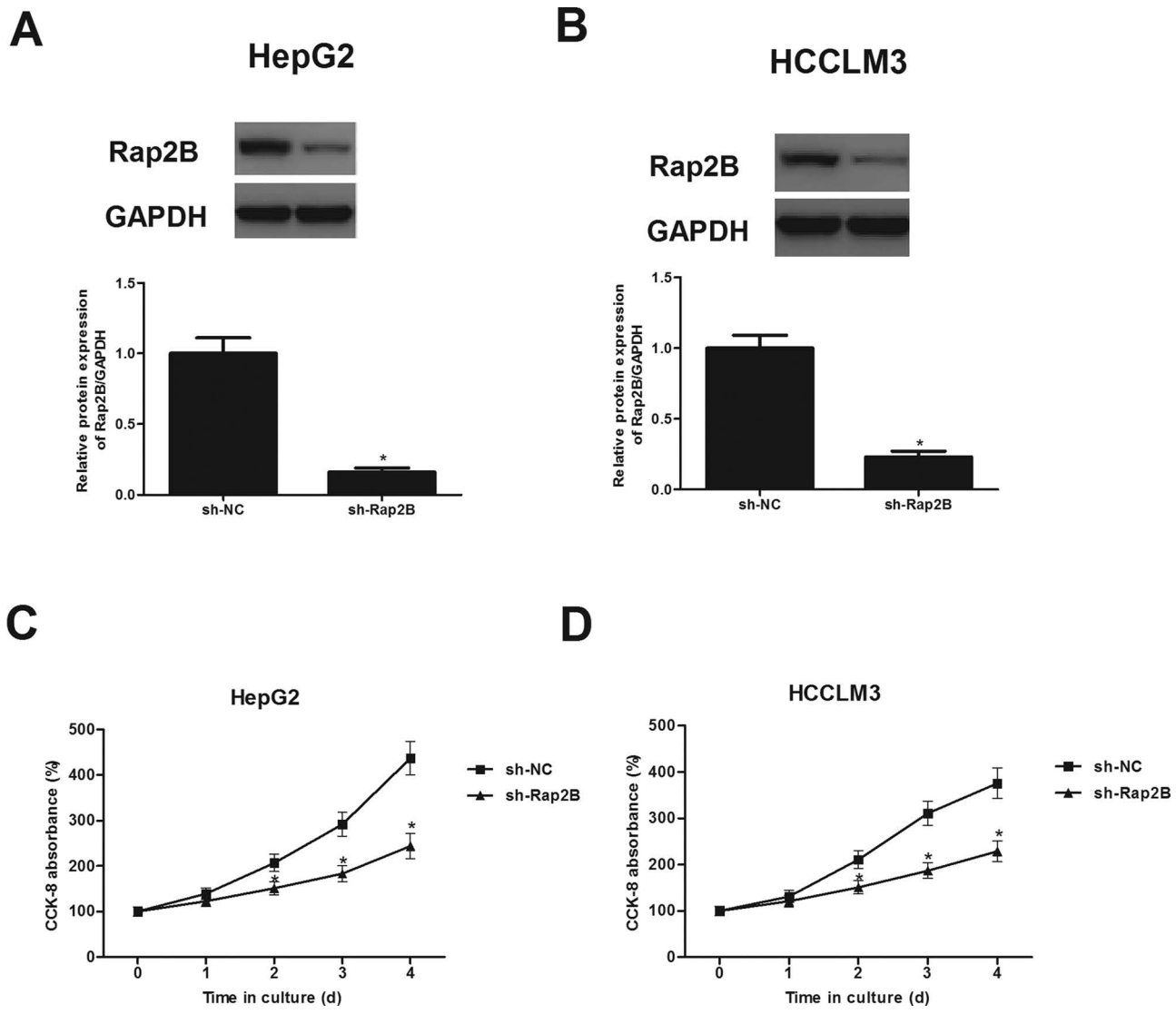

Figure 2. Rap2B silencing inhibits the proliferation of HCC cells. HepG2 and HCCLM3 cells were transfected with sh-Rap2B or nontargeting control siRNA (sh-NC) for 48 h. The expression of Rap2B was measured using Western blot in HepG2 (A) and HCCLM3 (B) cells, respectively. (C, D) The cell proliferation was tested by CCK-8 assay. *$p < 0.05$ versus sh-NC group.

cells that migrated to the bottom well ($p<0.05$) (Fig. 3A). In addition, we measured cell invasion in Rap2B-silenced and its control cells. The results indicated that knockdown of Rap2B significantly increased cell invasiveness in HepG2 (Fig. 3C) and HCCLM3 cells (Fig. 3D).

Rap2B Silencing Inhibits Xenografted Tumor Growth In Vivo

To evaluate the long-lasting effect of Rap2B on tumor cells, HepG2 cells were untreated or treated with sh-Rap2B in vitro for 48 h and then subcutaneously injected into the flanks of nude mice. As shown in Figure 4A, knockdown of Rap2B significantly suppressed the tumor size of BALB/c nude mice compared with the control group. In addition, knockdown of Rap2B obviously reduced the weight of the xenografted tumor (Fig. 4B).

Rap2B Silencing Inhibits the Activation of FAK Signaling Pathway in HCC Cells

To further explore the molecular mechanisms responsible for Rap2B-mediated HCC migration and invasion, we examined the expression levels of phosphorylated

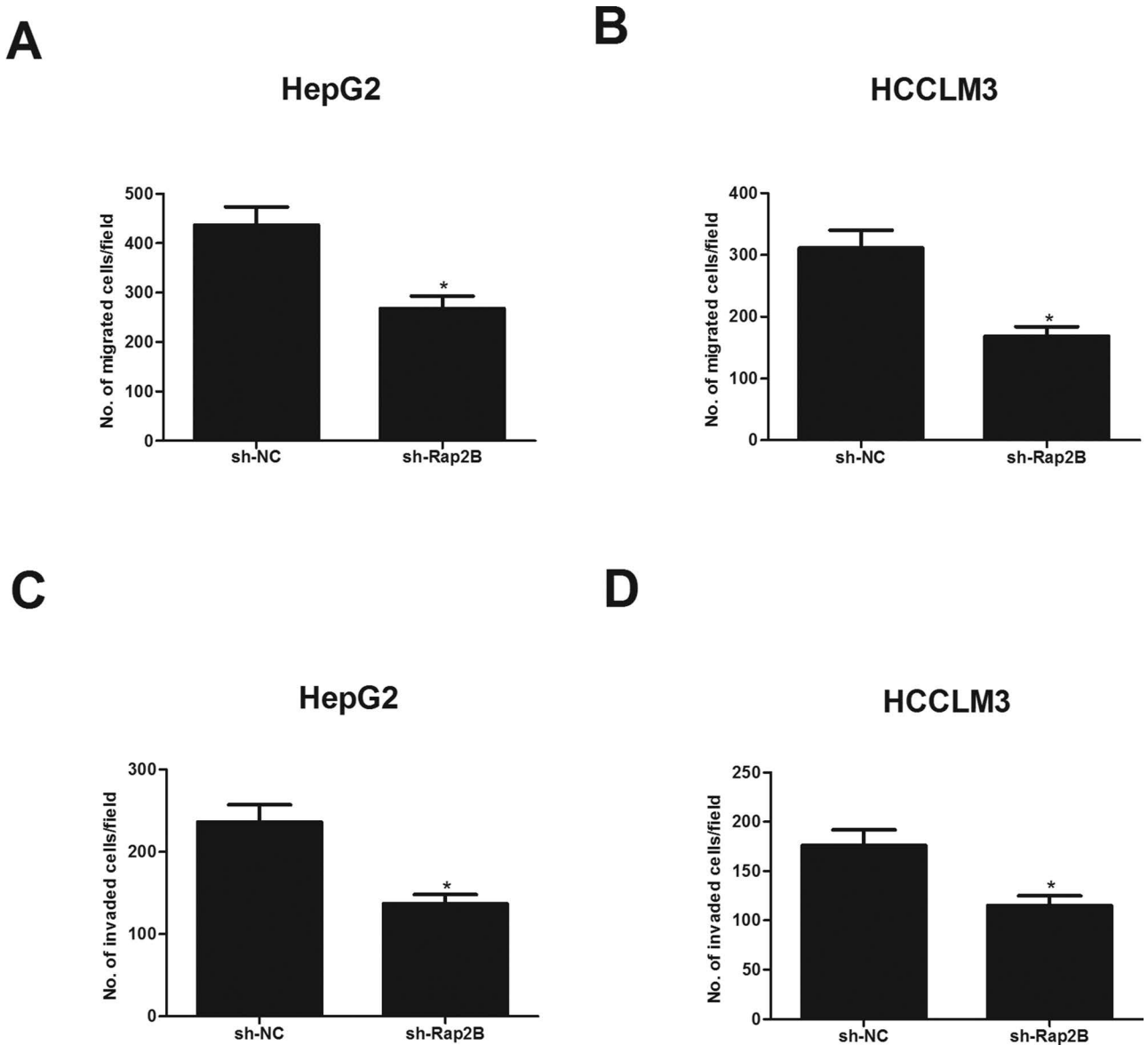

Figure 3. Rap2B silencing inhibits the migration/invasion of HCC cells. HepG2 and HCCLM3 cells were transfected with sh-Rap2B or nontargeting control siRNA (sh-NC) for 48 h. Transwell assay showing that knockdown of Rap2B inhibited cell migration in HepG2 (A) and HCCLM3 (B) cells compared to control cells. Matrigel invasion assay showing that knockdown of Rap2B suppressed cell invasion in HepG2 (C) and HCCLM3 (D) cells compared to control cells. *$p<0.05$ versus sh-NC group.

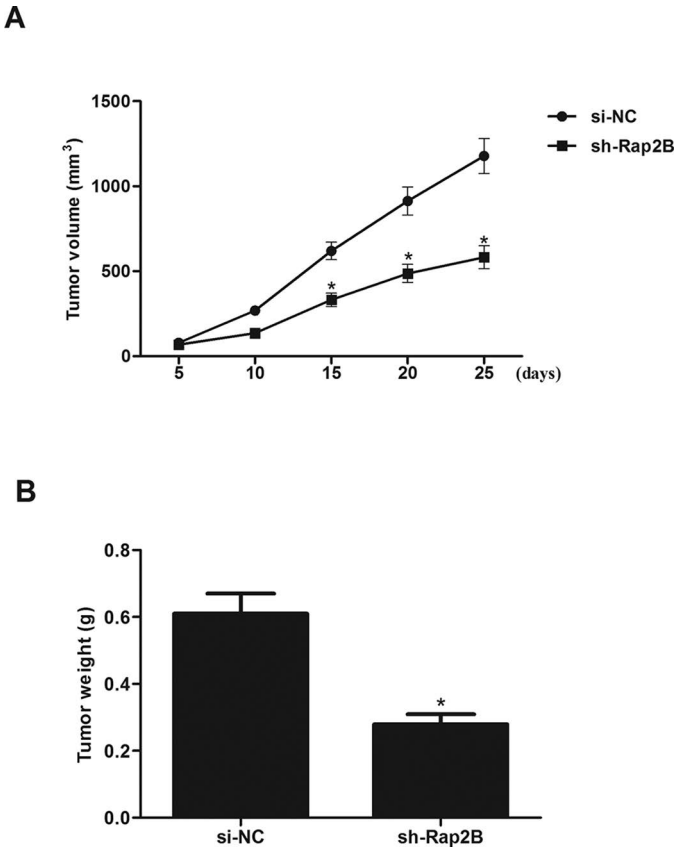

Figure 4. Rap2B silencing inhibits xenografted tumor growth in vivo. HepG2 cells were untreated or treated with sh-Rap2B in vitro for 48 h and then were subcutaneously injected to the flanks of nude mice. (A) The tumor volumes were monitored beginning on the fifth day. The tumors were measured every 5 days. (B) At 25 days after injection, the animals were euthanized, and the tumors were weighed. *$p < 0.05$ versus sh-NC group.

focal adhesion kinase (p-FAK) by Western blot analysis. As shown in Figure 5A, knockdown of Rap2B dramatically decreased p-FAK expression in HepG2 cells compared with sh-NC-transfected cells. Quantification of p-FAK/FAK is shown in Figure 5B. In addition, we found that the protein expression levels of MMP-2 and MMP-9 were significantly decreased in HepG2-sh-Rap2B cells compared with the control cells (Fig. 5C).

DISCUSSION

In the current study, we found that Rap2B was highly expressed in HCC tissues and cell lines. Rap2B silencing obviously inhibited the proliferation, migration, and invasion of HCC cells, as well as attenuated xenografted tumor growth in vivo. Furthermore, Rap2B silencing greatly reduced the expression levels of p-FAK, MMP-2, and MMP-9 in HCC cells.

Several lines of evidence indicate that Rap2B plays an important role in the development of tumors. Peng et al. reported that Rap2b expression was obviously increased in lung cancer, and Rap2b overexpression promoted the abilities of lung cancer cell wound healing, migration, and invasion (17). Another study reported that miR-342-3p targets Rap2B to suppress cell proliferation, migration, and invasion of non-small cell lung cancer (18). In accordance with previous studies, we found that Rap2B was highly expressed in HCC tissues and cell lines. Rap2B silencing obviously inhibited the proliferation, migration, and invasion of HCC cells, as well as attenuated xenografted tumor growth in vivo. The results obtained from both in vivo and in vitro experiments strongly suggest that Rap2B functions as an oncogene in the development and progression of HCC.

Previous research has demonstrated that the FAK signaling pathway plays an important role in the development of HCC (19–21). Both FAK and p-FAK Tyr397 have been shown to be overexpressed in HCC samples and HCC cell lines (22). Activated FAK forms a complex with Src and p130Cas, which leads to tumor growth and metastasis by promoting cell survival, cell cycle progression, migration, and invasion. Therefore, inactivation of the FAK pathway may be a good way to inhibit the

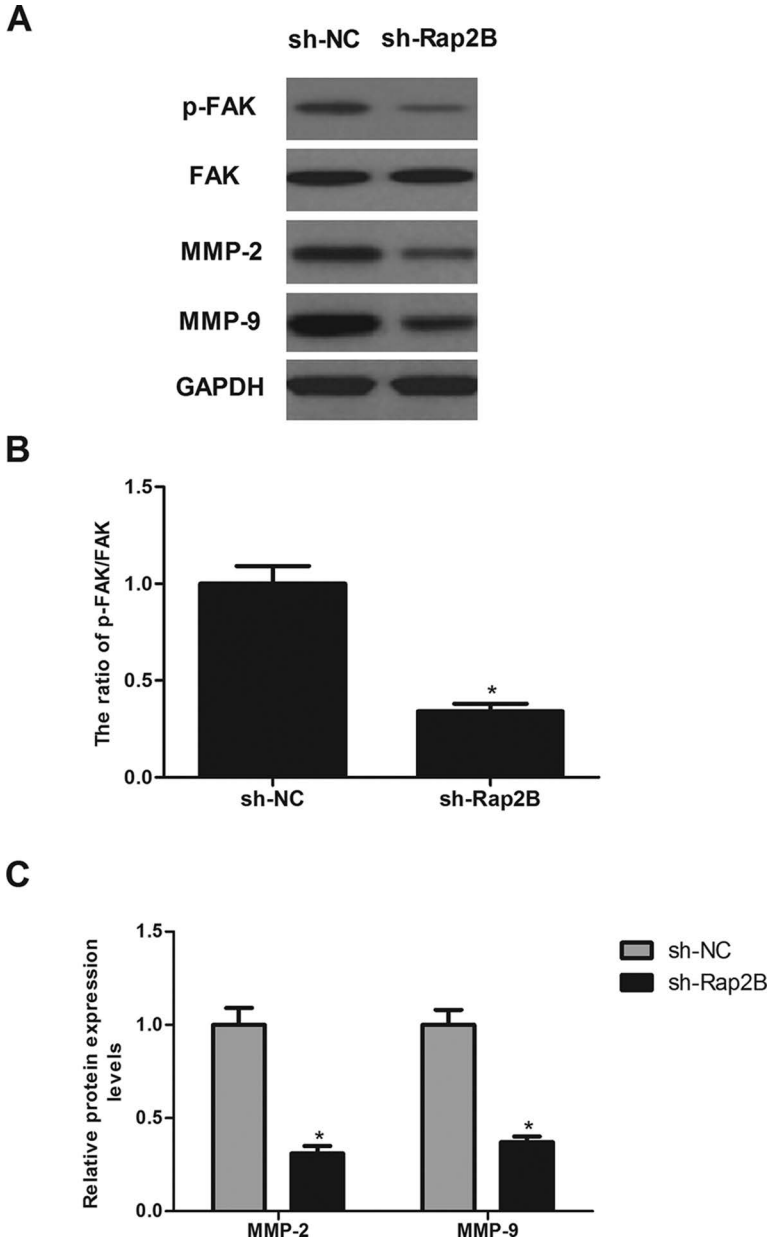

Figure 5. Rap2B silencing inhibits the activation of FAK signaling pathway in HCC cells. HepG2 cells were transfected with sh-Rap2B or nontargeting control siRNA (sh-NC) for 48 h. (A) The expression levels of p-FAK, FAK, MMP-2, and MMP-9 were determined by Western blot. (B, C) Quantification analysis was performed using the Gel-Pro Analyzer version 4.0 software. *$p<0.05$ versus sh-NC group.

metastasis of human HCC (23,24). For example, Bai et al. showed that RNA interference targeting FAK suppressed prostaglandin E2-mediated adhesion and migration in HCC cells (25). Shang et al. confirmed that deletion of FAK after tumors form greatly repressed c-Met (MET)/β-catenin (CAT)-induced tumor progression and that ectopic FAK expression restored HCC formation in hepatocyte-specific FAK-deficient mice (26). Most recently, one study reported that Rap2B positively regulated prostate cancer growth and metastasis and that expression of p-FAK and FAK-specific inhibitor (PF-573228) can abort Rap2B-induced FAK phosphorylation in prostate cancer cells (27). In this study, we found that Rap2B silencing greatly reduced the expression levels of phosphorylation of FAK in HCC cells.

MMPs are a family of zinc-dependent endopeptidase that plays an important role in the matrix degradation required for tumor growth and invasion (28). It was reported that increasing the expression of MMP-2 is related to enhanced tumor invasion and metastasis (29).

Furthermore, MMP-2 and MMP-9 are crucial downstream molecules in the FAK signaling pathway (30). In this study, we observed that Rap2B silencing greatly reduced the expression levels of MMP-2 and MMP-9 in HCC cells. These data suggest that knockdown of Rap2B inhibits proliferation and invasion via downregulation of p-FAK and downstream molecules in HCC cells.

In summary, our studies have found that knockdown of Rap2B inhibits the proliferation and invasion in HCC cells. These findings reveal that Rap2B plays an important role in the regulation of HCC progression; thus, Rap2B may be a potential therapeutic target for the treatment of HCC.

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