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Overexpression of RAS-Association Domain Family 6 (RASSF6) Inhibits Proliferation and Tumorigenesis in Hepatocellular Carcinoma Cells

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Ras-association domain family 6 (RASSF6), a member of the RASSF family, is frequently downregulated in various types of cancer. However, the roles of RASSF6 in human hepatocellular carcinoma (HCC) are still unclear. In this study, we investigated the biological functions and related molecular mechanisms in HCC. Our results found that RASSF6 is expressed in low amounts in HCC tissues and cell lines. Overexpression of RASSF6 obviously inhibited the proliferation, invasion, and EMT process in HCC cells. Furthermore, overexpression of RASSF6 greatly downregulated the protein levels of phosphorylated focal adhesion kinase (FAK), MMP-2, and MMP-9 in HepG2 cells. Last, overexpression of RASSF6 significantly attenuated tumor growth in Balb/c nude mice. In conclusion, the present study revealed that RASSF6 can inhibit the proliferation, invasion, and migration of HCC cells both *in vivo* and *in vitro*. These inhibitory effects are through suppressing FAK phosphorylation, leading to decreased MMP-2/9 expression. RASSF6 is therefore a potential therapeutic target for treating HCC.

Key words: Ras-association domain family 6 (RASSF6); Hepatocellular carcinoma (HCC); Invasion; Focal adhesion kinase (FAK) pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world¹. Of the approximately 782,500 new HCC cases annually, China accounts for nearly half². Despite advances in surgery and new chemotherapy regimens over the last few decades, the prognosis is extremely poor for advanced and metastatic HCC. Therefore, it is of paramount importance to understand the molecular mechanisms underlying the tumorigenesis of HCC.

The Ras-association domain family (RASSF) was first described in 2000 by the characterization of the first family member RASSF1A³. It comprises six members (RASSF1–6) that each contains a RalGDS/AF-6 (RA) and Sav/RASSF/Hippo (SARAH) domain. Increasing evidence has reported that the RASSF family of proteins plays an important role in regulating microtubule network, cell proliferation, invasion, and apoptosis^{4,5}. RASSF6, a member of the RASSF family, is frequently downregulated in various types of cancer, including gastric cardia adenocarcinoma, pancreatic ductal adenocarcinoma, clear cell renal cell carcinoma, etc^{6–8}. Chen et al. reported

that RASSF6 is expressed in low amounts in sporadic colorectal cancer tissues; restoration of RASSF6 obviously inhibited cell proliferation, migration, invasion, and induced apoptosis *in vitro* as well as tumor growth *in vivo*⁹. However, the roles of RASSF6 in human HCC are still unclear. In this study, we investigated the biological functions and related molecular mechanisms in HCC. Our findings show that RASSF6 is downregulated and exhibits tumor suppressor activities in HCC and may be a potential therapeutic target for HCC treatment.

MATERIALS AND METHODS

Patient Samples

A total of 12 patients with HCC who underwent surgery between 2014 and 2015 at the Hepatic Surgery Department, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University (P.R. China) were enrolled in the present study. Paired tumor and adjacent nontumor tissues were collected. All fresh samples were immediately frozen after resection and stored at -80°C until use. Written informed consent was obtained from the patients before surgery. This study was approved by

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the ethics committee of the Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University.

Cell Culture

Four human HCC cell lines (HepG2, SMMC-7721, and MHCC-97H) and a hepatocyte cell line (HL-7702) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All cell lines were grown in a humidified chamber supplemented with 5% CO₂ at 37°C.

Construction of Plasmids and Transfection

The full-length RASSF6 complementary DNA (cDNA) was cloned into the pcDNA3.1 vector (GeneChem, Shanghai, P.R. China). HCC cells were transfected with pcDNA3.1-RASSF6 using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The expression level of RASSF6 was determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot assays. Empty vector-transfected cells (mock) were used as control.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from HCC tissues or cells using the TRIzol reagent (Invitrogen) according to the supplier's instructions. The cDNA was synthesized from 5 µg of total RNA using M-MLV reverse transcriptase (Sigma-Aldrich). qRT-PCR was performed using an ABI Prism 7500 System (Applied Biosystems, Foster City, CA, USA) with the SYBR Green Supermix (Invitrogen). The primers for qRT-PCR were as follows: RASSF6, 5'-A GCTGCCAGTTCTTGGAAATG-3' (sense) and 5'-AGGC CAGACAGCTCTGATGT-3' (antisense); GAPDH, 5'-GT CCACCACCCTGTTGCTGTA-3' (sense) and 5'-CTTC AACAGCGACACCCACTC-3' (antisense). The relative expression levels were calculated using the 2^{-ΔΔCt} method, and the target gene was normalized to the internal reference gene.

Protein Extraction and Western Blot

HCC tissues or cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with a 1% protease inhibitor cocktail (Roche, Basel, Switzerland). The protein extracts were size fractionated using 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking, the membranes were incubated with specific primary antibodies at 4°C overnight. The anti-RASSF6, anti-E-cadherin, anti-N-cadherin, anti-p-focal adhesion kinase (FAK), anti-matrix metalloproteinase 2 (MMP-2), anti-MMP-9, and anti-GAPDH were purchased from Santa

Cruz Biotechnology (Santa Cruz, CA, USA). Subsequently, the membrane was washed and incubated with peroxidase-conjugated IgGs (Santa Cruz Biotechnology) for 1 h. Then the bands were visualized using enhanced chemiluminescence reagents (Bio-Rad). The density of the bands was analyzed by the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell Proliferation Assay

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8; Sigma-Aldrich). In brief, the infected cells at a density of 1 × 10⁵ cells per well were seeded into 96-well plates and then incubated at 37°C. At the indicated time points, 10 µl of CCK-8 solution was added into each well and incubated for 2 h. Absorbance at 570 nm was measured using a microplate reader (Bio-Rad).

Cell Migration and Invasion Assays

Cell migration and invasion were analyzed using Transwell chamber assays. For the migration assay, infected cells (1 × 10⁵ cells per well) were suspended in serum-free medium and plated on the upper chamber (BD Biosciences, Eugene, OR, USA). For the invasion assay, infected cells were seeded into the upper chamber that was precoated with Matrigel. For both assays, the lower chamber was filled with DMEM containing 10% FBS. After 24 h of incubation, cells adhering to the lower membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution for 15 min. Cells were enumerated by counting six random fields per Transwell chamber under a light microscope (magnification: 100×).

Xenograft Model in Nude Mice

The experimental protocols for the animal studies were evaluated and approved by the Animal Care and Use Committee of the Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University. Female Balb/c nude mice (4–6 weeks old, weighing 18–22 g; n=5) were purchased from the Academy of the Second Military Medical University and were housed in cages and given food and water ad libitum. Briefly, 5 × 10⁶ pcDNA3.1-RASSF6- or mock-infected Hep-G2 cells in 100 µl of PBS were injected subcutaneously into the right and left flanks of nude mice, respectively. Tumor volumes were measured with calipers every 5 days after injection. The tumor volume (V) was determined according to the following equation: V=length×width²/2. The mice were sacrificed at day 25 when the tumors were dissected and weighed.

Statistical Analysis

The results were presented as the average from triplicate experiments and expressed as the mean ± SD. One-way ANOVA was performed to determine the statistical

significance among the groups. A value of $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS

RASSF6 Is Lowly Expressed in HCC Specimens and Cell Lines

We initially analyzed RASSF6 expression in human HCC specimens and their corresponding adjacent normal tissues. A significant decrease in RASSF6 expression at both the mRNA and protein levels was observed in HCC tissues compared with that in patient-matched adjacent normal tissues (Fig. 1A and B). Furthermore,

the expression of RASSF6 in human HCC cell lines was examined. The results showed that the expression of RASSF6 was also obviously decreased in HCC cell lines compared with that in the HL-7702 cell line (Fig. 1C and D). We chose the HepG2 cell line for stable transfection with RASSF6 expression vector.

RASSF6 Inhibits the Proliferation of HCC Cells

To investigate the effect of RASSF6 on cell proliferation, we treated HepG2 cells with pcDNA3.1-RASSF6. The transfection efficiency was confirmed by qRT-PCR (Fig. 2A) and Western blot (Fig. 2B). Cell proliferation was then evaluated using the CCK-8 assay. Compared

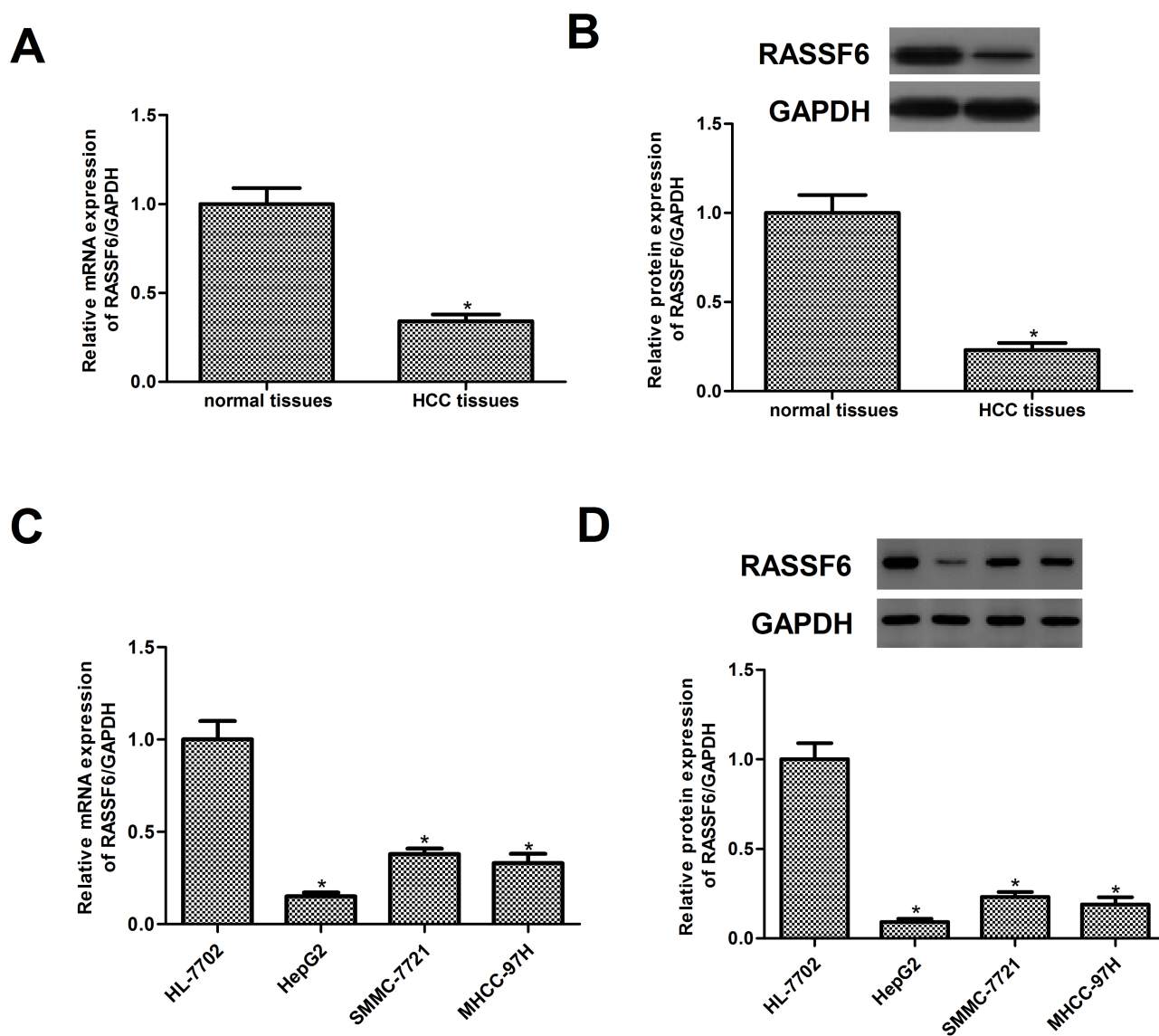


Figure 1. RASSF6 is expressed in low amounts in HCC specimens and cell lines. (A, C) The mRNA expression levels of RASSF6 in human HCC tissues and HCC cell lines were detected by qRT-PCR. (B, D) The protein expression levels of RASSF6 in human HCC tissues and HCC cell lines were detected by Western blot. These data are from three independent experiments and presented as the mean \pm SD. * $p < 0.05$.

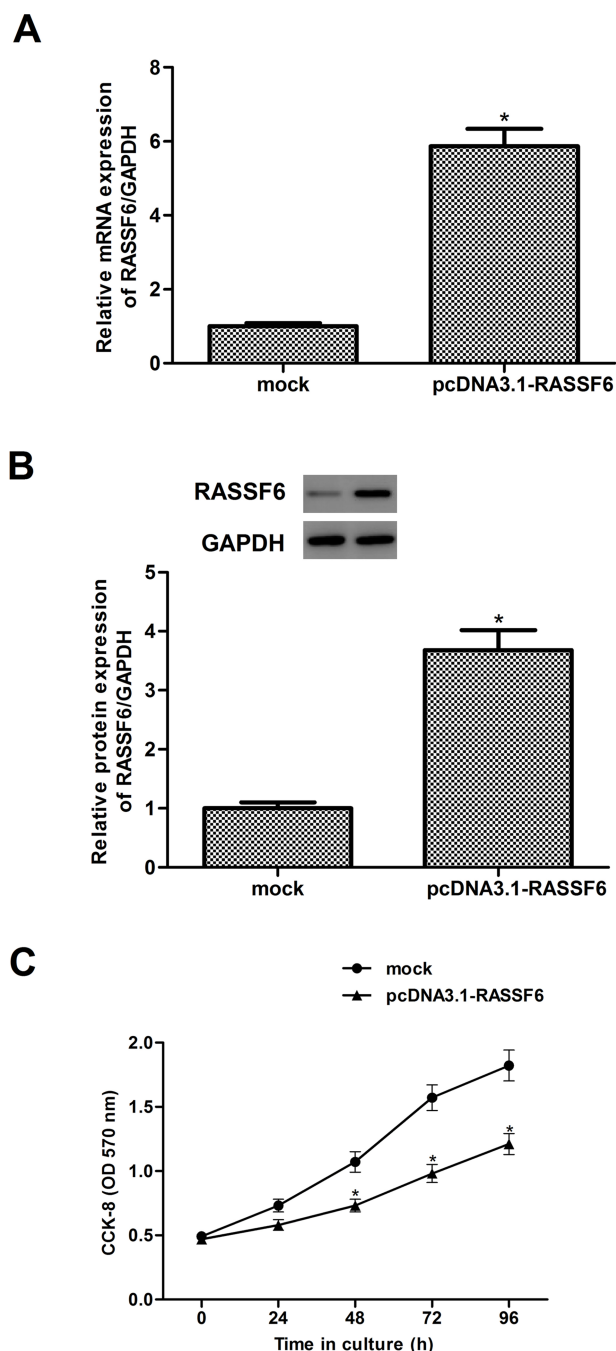


Figure 2. RASSF6 inhibits the proliferation of HCC cells. HepG2 cells were transfected with pcDNA3.1-RASSF6 or mock for 48 h. (A) Relative mRNA expression of RASSF6 was detected by qRT-PCR. (B) Relative protein expression of RASSF6 was detected by Western blot. GAPDH was used as a loading control. (C) Cell proliferation was evaluated by the CCK-8 assay. These data are from three independent experiments and presented as the mean \pm SD. * $p < 0.05$.

with the mock group, the proliferation of HepG2 cells was significantly inhibited by pcDNA3.1-RASSF6 in a time-dependent manner (Fig. 2C).

RASSF6 Inhibits HCC Cell Migration and Invasion by Suppressing the EMT Phenotype

We next examined the effect of RASSF6 on HCC cell migration and invasion. The results of the Transwell migration assay showed that the overexpression of RASSF6 sharply inhibited the migration of HepG2 cells (Fig. 3A). The Matrigel invasion assay demonstrated that the number of invading cells was also significantly suppressed by RASSF6 overexpression (Fig. 3B). In addition, we examined the effect of RASSF6 on the expression of EMT-related markers by Western blotting. Ectopic expression of RASSF6 led to an increase in E-cadherin expression and a decrease in N-cadherin expression in HepG2 cells (Fig. 3C).

RASSF6 Inhibits the Activation of the FAK Pathway in HCC Cells

To further explore the molecular mechanisms responsible for RASSF6-mediated HCC proliferation and invasion, we examined the effect of RASSF6 on the activation of the FAK pathway. The results of the Western blot analysis showed that, compared with control cells, the level of phosphorylated FAK was decreased in RASSF6-overexpressing cells, while the total protein level was unaffected (Fig. 4A). In addition, we observed that overexpression of RASSF6 greatly downregulated the protein expression levels of MMP-2 and MMP-9 in HepG2 cells. Furthermore, we examined the effects of the FAK inhibitor (PF-562271) on RASSF6-inhibited HCC cell proliferation and invasion. We found that PF-562271 markedly enhanced the inhibitory effects of RASSF6 on HepG2 cell proliferation (Fig. 4D) and invasion (Fig. 4E).

RASSF6 Inhibits HCC Cell Growth In Vivo

To investigate the effect of RASSF6 on tumor formation in vivo, HepG2 cells infected with pcDNA3.1-RASSF6 or mock were injected subcutaneously into the right and left flanks of nude mice, respectively. The results showed that the mean volumes of the tumors that were obtained from the Ad-RASSF6 group were obviously lower than those from the vector group (Fig. 5A). In addition, the weight of tumors from the RASSF6-overexpressing cells was dramatically smaller than that of the controls when the tumors were removed from the sacrificed mice on day 25 after injection (Fig. 5B).

DISCUSSION

To the best of our knowledge, this is the first study indicating that RASSF6 is expressed in low amounts in

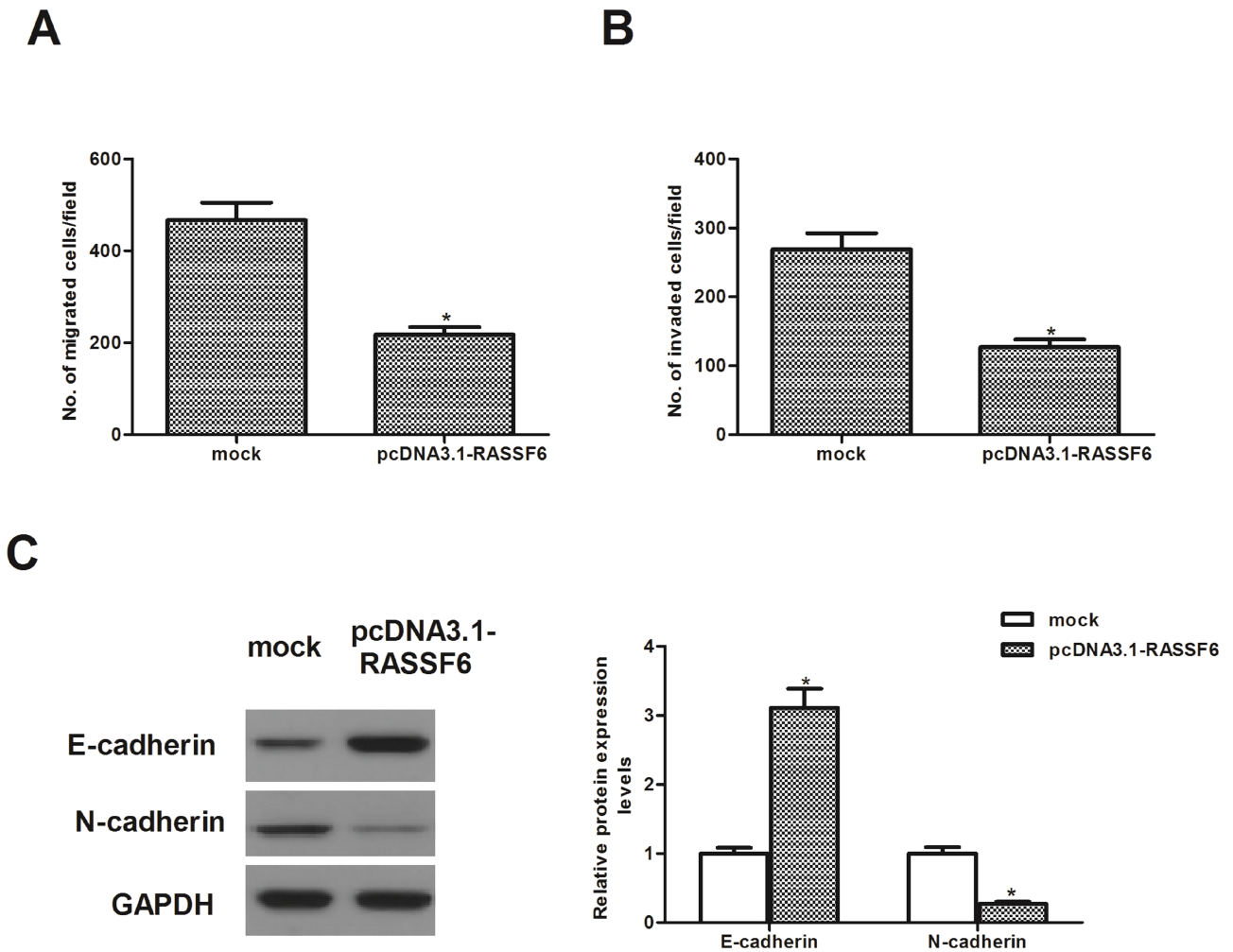


Figure 3. RASSF6 inhibits HCC cell migration and invasion by suppressing the EMT phenotype. HepG2 cells were transfected with pcDNA3.1-RASSF6 or mock for 48 h. (A) Cell migration was determined by the Transwell assay. (B) Cell invasion was determined using Matrigel-coated Transwell invasion chamber. (C) The expression levels of E-cadherin and N-cadherin were detected by Western blot. These data are from three independent experiments and presented as the mean \pm SD. * $p < 0.05$.

HCC tissues and cell lines. Overexpression of RASSF6 obviously inhibited the proliferation, invasion, and EMT process in HCC cells. Furthermore, overexpression of RASSF6 greatly downregulated the protein levels of phosphorylated FAK, MMP-2, and MMP-9 in HepG2 cells. Last, overexpression of RASSF6 significantly attenuated the tumor growth in Balb/c nude mice.

RASSF6 has been implicated in various types of human malignancies¹⁰⁻¹³. Liang et al. confirmed that the expression of RASSF6 is downregulated in clear cell renal cell carcinoma tissues, and ectopic expression of RASSF6 inhibited cell proliferation, clonogenicity, and tumor growth in mice⁸. Similarly, herein we observed that RASSF6 is expressed in low amounts in HCC tissues and cell lines. Overexpression of RASSF6 obviously inhibited

the proliferation in vitro and xenografted tumor growth in vivo. These results suggest that RASSF6 may be a tumor suppressor in the development and progression of HCC.

Metastasis is not only a complex process but also the major cause of cancer-related deaths¹⁴. Previous studies have shown that the EMT process plays a critical role in regulating cancer metastasis¹⁵⁻¹⁷. During EMT, the actin cytoskeleton is reorganized, and cells acquire increased cell-matrix contacts, leading to dissociation from surrounding cells and enhanced migratory and invasive capabilities¹⁸. Attenuated expression of E-cadherin is recognized as an important determinant and biomarker of HCC progression¹⁹. The current study demonstrated that overexpression of RASSF6 obviously inhibited HCC cell migration and invasion. In addition, RASSF6 promoted

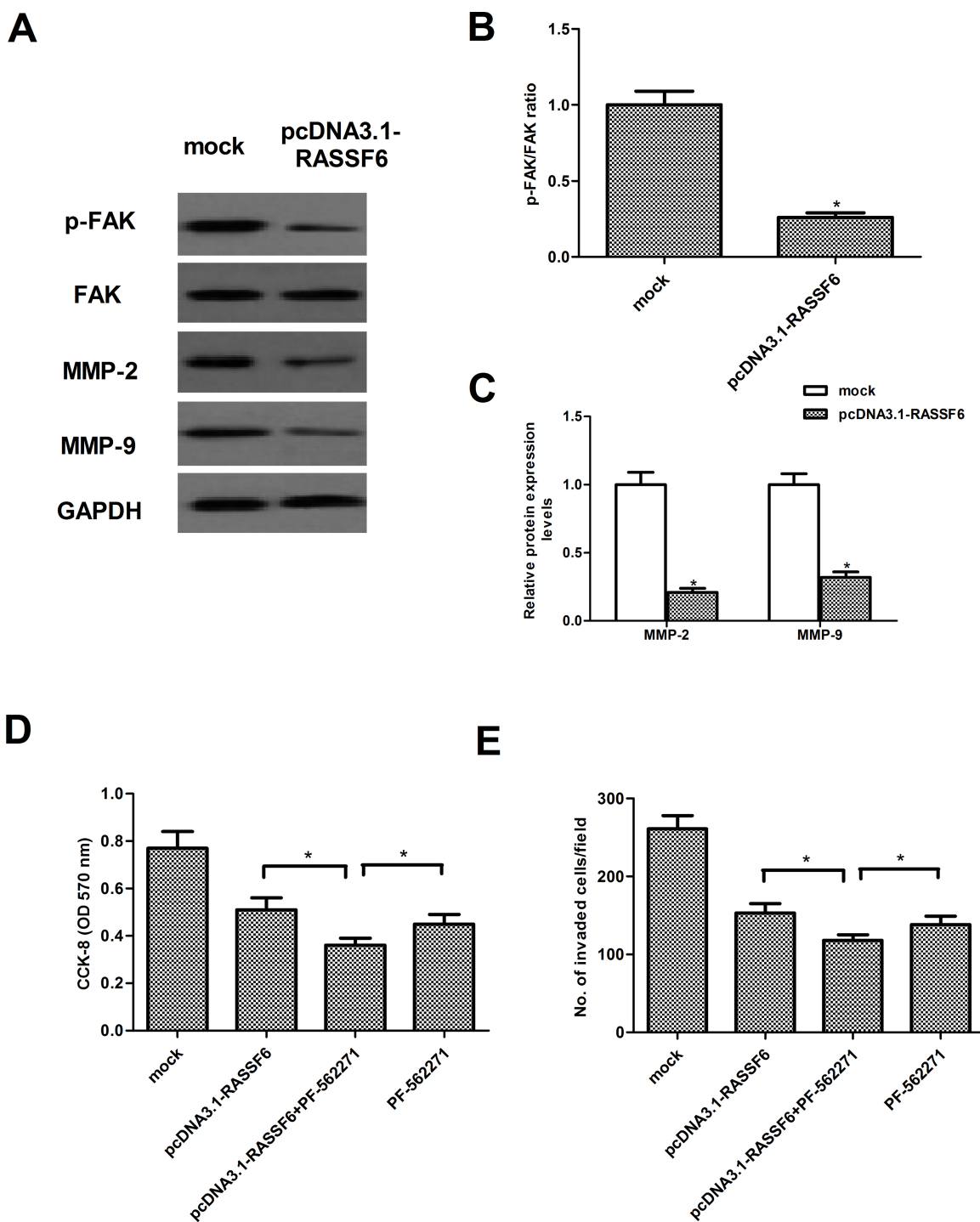


Figure 4. RASSF6 inhibits the activation of the FAK pathway in HCC cells. HepG2 cells were transfected with pcDNA3.1-RASSF6 or mock for 48 h. (A) The expression levels of p-FAK, FAK, MMP-2, and MMP-9 were detected by Western blotting. Quantification of (B) p-FAK/FAK and (C) MMP-2 and MMP-9. HepG2 cells were transfected with pcDNA3.1-RASSF6 or mock in the presence or absence of the FAK inhibitor (PF-562271; 1 μ M) for 24 h. (D) Cell proliferation was determined by the CCK-8 assay. (E) Cell invasion was evaluated by the Matrigel invasion assay. These data are from three independent experiments and presented as the mean \pm SD. * p < 0.05.

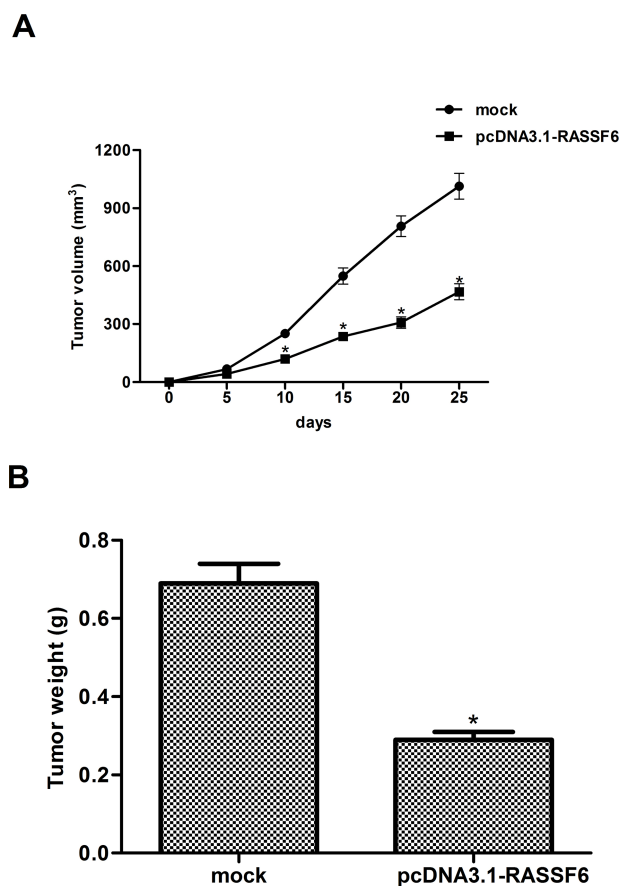


Figure 5. RASSF6 inhibits HCC cell growth in vivo. HepG2 cells infected with pcDNA3.1-RASSF6 or mock were injected subcutaneously into the right and left flanks of nude mice, respectively. (A) The volumes of the tumors were monitored every 5 days. (B) After 25 days, the mice were sacrificed, and the tumors were dissected out and weighed. These data are from three independent experiments and presented as the mean \pm SD. * $p < 0.05$.

E-cadherin expression and downregulated N-cadherin expression in Hep-G2 cells. These data suggest that RASSF6 significantly suppressed HCC migration and invasion by inhibiting the EMT phenotype.

Previous studies have shown that the FAK signaling pathway is activated in several human tumors, including HCC, and plays an important role in tumor progression^{20–22}. It was reported that phospho-FAK is overexpressed in HCC samples and HCC cell lines. The activated phospho-FAK was closely associated with tumor stage and vascular invasion in HCC²³. Furthermore, MMPs are a family of zinc-dependent endopeptidase that plays a critical role in the matrix degradation required for tumor growth and invasion^{24–26}. MMP-2 and MMP-9 are crucial downstream molecules in the FAK signaling pathway and are closely associated with migration and

invasion in HCC²⁷. Chen et al. confirmed that knock-down of FAK induced a significant reduction in expressions and activities of both MMP-2 and MMP-9 in HCC cells²³. Most recently, Liu et al. reported that overexpression of RASSF10 inhibited HCC invasion partially mediated by FAK or p38 MAPK to decrease the accumulation of MMP-2²⁸. Similarly, in this study we found that overexpression of RASSF6 greatly downregulated the protein levels of phosphorylated FAK, MMP-2, and MMP-9 in HepG2 cells. These data suggest that RASSF6 inhibits the proliferation and invasion through suppressing the FAK signaling pathway in HCC cells.

In conclusion, the present study revealed that RASSF6 could inhibit the proliferation, invasion, and migration of HCC cells both in vivo and in vitro. These inhibitory effects are through suppressing FAK phosphorylation, leading to decreased MMP-2/9 expression. RASSF6 is therefore a potential therapeutic target for treating HCC.

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