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Overexpression of Hepatocyte Cell Adhesion Molecule (hepaCAM) Inhibits the Proliferation, Migration, and Invasion in Colorectal Cancer Cells

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Hepatocyte cell adhesion molecule (hepaCAM), a new type of CAM, belongs to the immunoglobulin superfamily. Recently, hepaCAM was reported to be implicated in cancer development, and many researchers investigated its biological function in the tumorigenesis of various cancers. However, what kind of role hepaCAM plays in colorectal cancer (CRC) remains unknown. In this study, we found that hepaCAM was downregulated in CRC tissues and cell lines. Overexpression of hepaCAM inhibited CRC cell proliferation, migration, and invasion in vitro. Furthermore, the tumorigenesis assay showed that increased expression of hepaCAM suppressed CRC tumor growth and metastasis in vivo. We also demonstrated that overexpression of hepaCAM reduced the protein expression levels of β-catenin, cyclin D1, and c-Myc, indicating its inhibitory effect on the Wnt/β-catenin signaling pathway. In conclusion, our study results suggest hepaCAM as a promising therapeutic target for CRC and provide a better understanding for the molecular basis of CRC progression.

Key words: Hepatocyte cell adhesion molecule (hepaCAM); Proliferation; Migration; Invasion; Colorectal cancer (CRC)

INTRODUCTION

Colorectal cancer (CRC), a common malignancy, ranks among the top leading causes of cancer-related deaths[1,2]. CRC is initiated due to changes in genomes and epigenomes, which is a complex and long-term biological process[3]. With more than 100,000 new cases reported annually in the world, CRC has become a globally prevalent disease[4]. CRC has a high morbidity and mortality, mainly because of metastasis in patients[5]. Currently, therapeutic approaches for CRC have advanced considerably, and CRC mortality has declined correspondingly, but the rate of overall survival is still unsatisfactory[6–9]. Therefore, identifying novel molecules and a better understanding of molecular mechanisms underlying CRC metastasis and progression may make CRC treatment more effective.

Cell adhesion molecules (CAMs), a group of cell surface receptors, function as a link between cells or a mediator of the extracellular matrix[10]. With tightly regulated expression, CAMs play an essential role in developing and maintaining tissue architecture[11]. Based on their structures and functions, CAMs are generally divided into four categories: cadherins, selectins, integrins, and immunoglobulins[12,13]. CAMs are capable of modulating signal transduction, which is necessary for the regulation of some important biological processes such as cellular adhesion, proliferation, migration, apoptosis, and differentiation[14,15]. Studies found that aberrant expression of CAMs might cause deregulation of these biological processes in tumors, indicating a significant role of CAMs in cancer progression[16,17]. Recently, a new type of CAMs was identified, which is hepatocyte cell adhesion molecule (hepaCAM)[18]. This new molecule belongs to the immunoglobulin superfamily[18]. Some researchers observed ubiquitous expression of hepaCAM in normal liver tissues[19]. Others found downregulation of hepaCAM in hepatocellular cancer and suggested it as a tumor-suppressor gene[20]. However, what kind of role hepaCAM plays in CRC remains unknown.

The aim of this study was to investigate the functional significance of hepaCAM in CRC. We found that hepaCAM had a low expression in CRC tissues and cell lines. Overexpression of hepaCAM inhibited CRC cell proliferation, migration, and invasion in vitro and suppressed CRC cell growth and metastasis in vivo. In addition, we demonstrated that hepaCAM overexpression reduced the protein expression levels of β-catenin,

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cyclin D1, and c-Myc, indicating an inhibitory effect of hepaCAM on the Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS

Patients and Tissue Samples

Twenty-eight patients from the Department of Oncology, Binzhou Medical University Hospital (P.R. China) took part in the study and provided written consent. No patients received adjuvant therapies before collection of CRC tissues and adjacent normal tissues. All tissue specimens were frozen in liquid nitrogen and kept at −80°C until use. The study was approved by the ethics committee of Binzhou Medical University Hospital.

Cell Lines and Cell Culture

Human CRC cell lines (RKO and SW480) and normal colorectal mucosa cell line (FHC) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Quantitative Real-Time RT-PCR

Total RNA was isolated from tissues or cells with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of RNA was performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). RT-PCR was carried out with the ABI 7500 System (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min. The primers were as follows: hepaCAM, 5′-TACTGTA GATGTGCCCATTTC-3′ (forward) and 5′-CTTCTGGT TTCAGGCGGTC-3′ (reverse); GAPDH, 5′-TGACTTC AACAGCGACACCCA-3′ (forward) and 5′-CACCCTG TTGCTGTAGCCAAA-3′ (reverse). GAPDH was used as an internal control. The comparative Ct method was adopted to calculate fold changes.

Western Blot Analysis

Tissues or cells were lysed in ice-cold RIPA buffer (Thermo Fisher Scientific). Protein was separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with skim milk and incubated overnight at 4°C with primary antibodies against hepaCAM, β-catenin, cyclin D1, c-Myc, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Protein was visualized using the ECL detection reagent (Cell Signaling Technology, Danvers, MA, USA) and quantified by Quantity One software (Bio-Rad).

Cell Transfection

The hepaCAM expression vector was purchased from Applied Biosystems (Foster City, CA, USA). An empty vector was used as the control. RKO and SW480 cells were transfected with hepaCAM expression vector or empty vector using the Lipofectamine 2000 reagent (Invitrogen). After 48 h, the transfection efficiency was confirmed via Western blot.

Cell Proliferation Assay

Cell proliferation was evaluated by the MTT assay. Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h under normal conditions. After MTT solution (Sigma-Aldrich) was added to each well at a concentration of 5 mg/ml, cell incubation was continued for 4 h. Subsequently, culturing medium was removed and DMSO (Sigma-Aldrich) was added. Absorbance was measured at 490 nm with a spectrophotometer.

Cell Migration and Invasion Assays

Transwell chambers (pore size: 8 μm) were used to evaluate cell migration and invasion. For the cell migration assay, cells (5×10^4) were seeded in the upper chamber containing 100 μl of serum-free medium. Normal medium with 10% FBS was added to the lower chamber. Twenty-four hours later, migrating cells were fixed with methanol and stained with 0.1% crystal violet. Four random fields were selected to calculate the number of migrating cells under a microscope. For the cell invasion assay, the aforementioned procedure was repeated with Matrigel-coated Transwell chambers.

In Vivo Xenograft Tumor Assay

Male BALB/c nude mice (4 weeks old) were purchased from SLAC Laboratory Animals Co. Ltd. (Shanghai, China) and used to establish xenograft models. All animal experiments were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University Hospital. RKO cells (1×10^6) stably expressing hepaCAM or empty vector were suspended in 100 μl of PBS and subcutaneously injected into the left flank of nude mice ($n=6$). Tumor volume was measured every 5 days and calculated using the following formula: tumor volume = (length × width^2)/2. After 30 days, tumors were dissected and weighed. For the tumor metastasis assay, 1×10^6 transfected RKO cells were intravenously injected into the tail vein of nude mice ($n=6$). Thirty days later, mice were sacrificed to check for lung metastasis.

Statistical Analysis

SPSS software (Chicago, IL, USA) was used for statistical analysis. All data were represented as means ± standard deviation (SD). Significant differences between different groups of data were evaluated via Student's *t*-tests. A value of $p<0.05$ was considered statistically significant.

RESULTS

Expression of hepaCAM Is Decreased in CRC Tissues and Cell Lines

The expression of hepaCAM was measured in 28 pairs of CRC tissues and corresponding normal tissues via both RT-PCR and Western blot analysis. We found that hepaCAM was markedly reduced in CRC tissues compared with the normal tissues (Fig. 1A and B). To further investigate the expression pattern of hepaCAM, we detected its expression levels in two CRC cell lines (RKO and SW480) and a normal colorectal mucosa cell line (FHC). hepaCAM was also significantly decreased in CRC cell lines compared with the normal colorectal mucosa cell line (Fig. 1C and D).

Overexpression of hepaCAM Inhibits the Proliferation of CRC Cells

We first increased the expression of hepaCAM in RKO and SW480 cells by transfecting these cells with

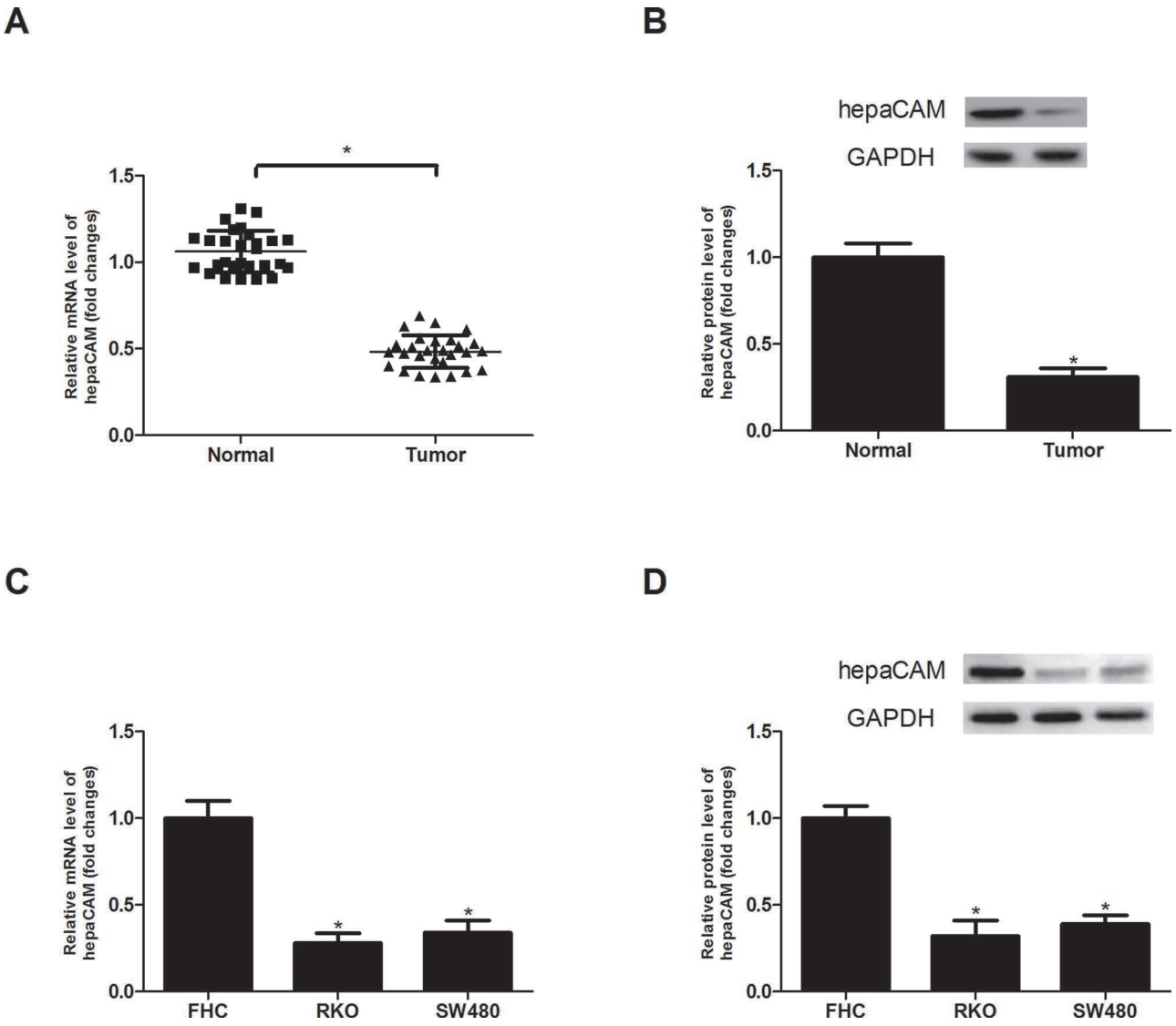

Figure 1. Expression of hepatocyte cell adhesion molecule (hepaCAM) is decreased in colorectal cancer (CRC) tissues and cell lines. (A, B) Relative hepaCAM expression in CRC tissues and adjacent normal tissues ($n=28$). (C, D) Relative hepaCAM expression in CRC cell lines RKO and SW480 and normal colorectal mucosa cell line FHC. *$p<0.05$.

the hepaCAM expression vector. The Western blot analysis showed successful transfection in RKO and SW480 cells (Fig. 2A and B). We then measured the effect of hepaCAM overexpression on cell proliferation by MTT assay. The results indicated that the proliferation of RKO and SW480 was significantly inhibited in hepaCAM-transfected cells compared with the control cells (Fig. 2C and D).

Overexpression of hepaCAM Inhibits the Migration and Invasion of CRC Cells

The effect of hepaCAM overexpression on the migration and invasion of CRC cells was examined by Transwell assay. Our results showed that overexpression of hepaCAM remarkably suppressed the migration in RKO and SW480 cells (Fig. 3A and B). Moreover, hepaCAM overexpression similarly decreased the invasion of RKO and SW480 cells (Fig. 3C and D).

Overexpression of hepaCAM Inhibits the Activity of Wnt/β-Catenin Signaling Pathway

There have been studies demonstrating the close association of hepaCAM with the Wnt/β-catenin signaling pathway[21]. In addition, increasing evidence has shown the crucial role of Wnt/β-catenin signaling in CRC development[22,23]. Therefore, we hypothesized that Wnt/β-catenin signaling took part in hepaCAM-mediated CRC progression. We examined the protein levels of β-catenin and its downstream targets cyclin D1 and c-Myc. hepaCAM overexpression decreased the protein expression levels of

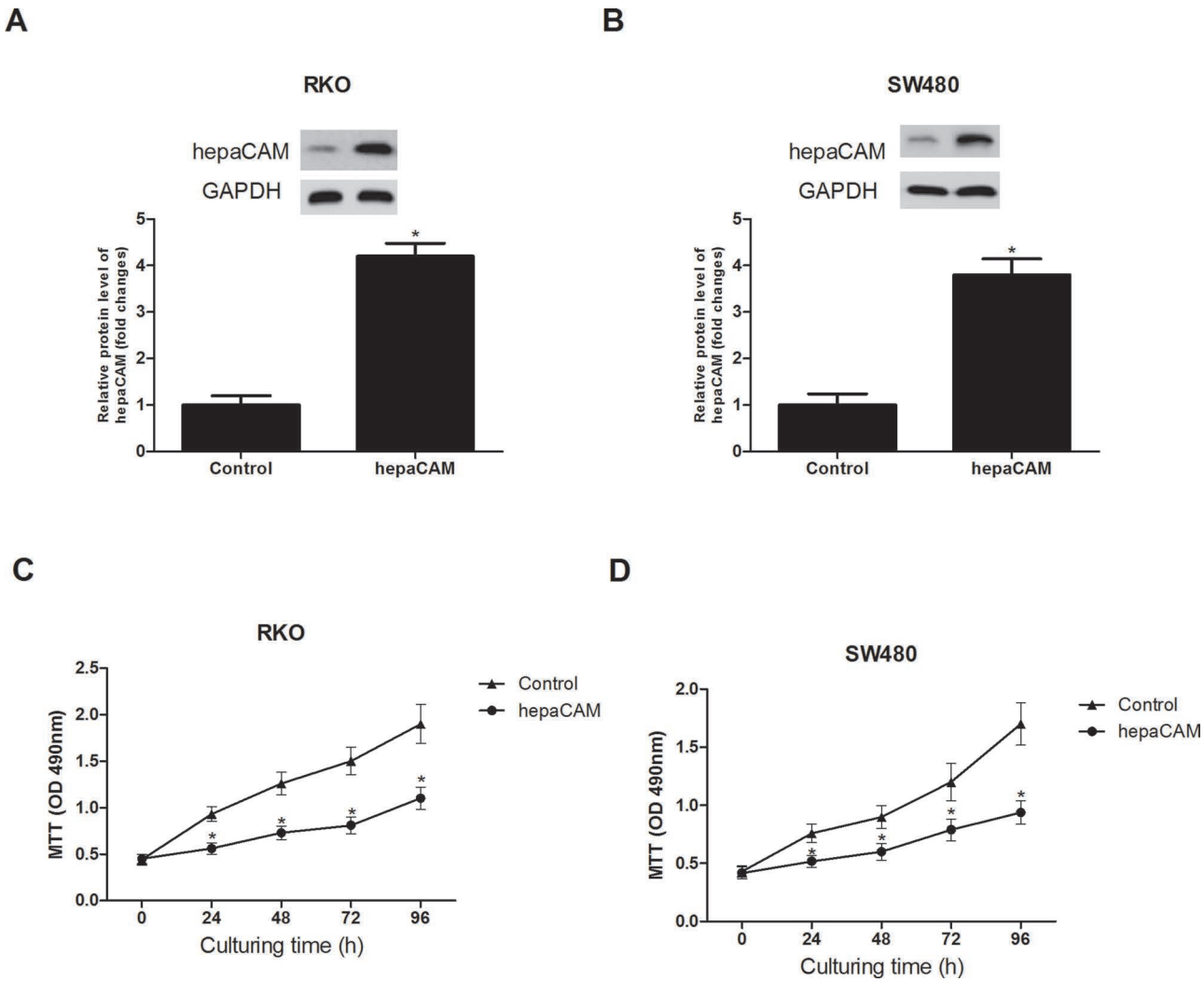

Figure 2. Overexpression of hepaCAM inhibits the proliferation of CRC cells. (A, B) Transfection of RKO and SW480 cells with the hepaCAM expression vector greatly increased the expression of hepaCAM in comparison with the control cells. (C, D) The proliferation of RKO and SW480 cells after hepaCAM transfection. *$p < 0.05$.

β-catenin, cyclin D1, and c-Myc, indicating the inhibitory effect of hepaCAM overexpression on the Wnt/β-catenin signaling pathway (Fig. 4).

Overexpression of hepaCAM Inhibits CRC Tumor Growth and Metastasis In Vivo

To assess the effect of hepaCAM overexpression on tumor growth in vivo, hepaCAM-transfected RKO cells or control cells were subcutaneously injected into nude mice. Tumor volume was measured every 5 days. Tumors derived from hepaCAM-overexpressing cells grew slower than those derived from the control cells (Fig. 5A). After a growth period of 30 days, tumors were stripped and weighed. Tumors derived from hepaCAM-overexpressing cells were remarkably lighter than those derived from the control cells (Fig. 5B). We also determined the effect of hepaCAM overexpression on tumor metastasis in vivo. RKO cells transfected with hepaCAM expression vector or control vector were intravenously injected into the tail vein of nude mice. Thirty days later, mice were sacrificed, and lung metastasis was checked. We found that the RKO/hepaCAM group showed less metastatic foci than the control group (Fig. 5C).

DISCUSSION

hepaCAM, a newly identified immunoglobulin-like CAM, was recently reported to be implicated in cancer development and thus aroused the intense interest of researchers. Many studies have found a downregulated

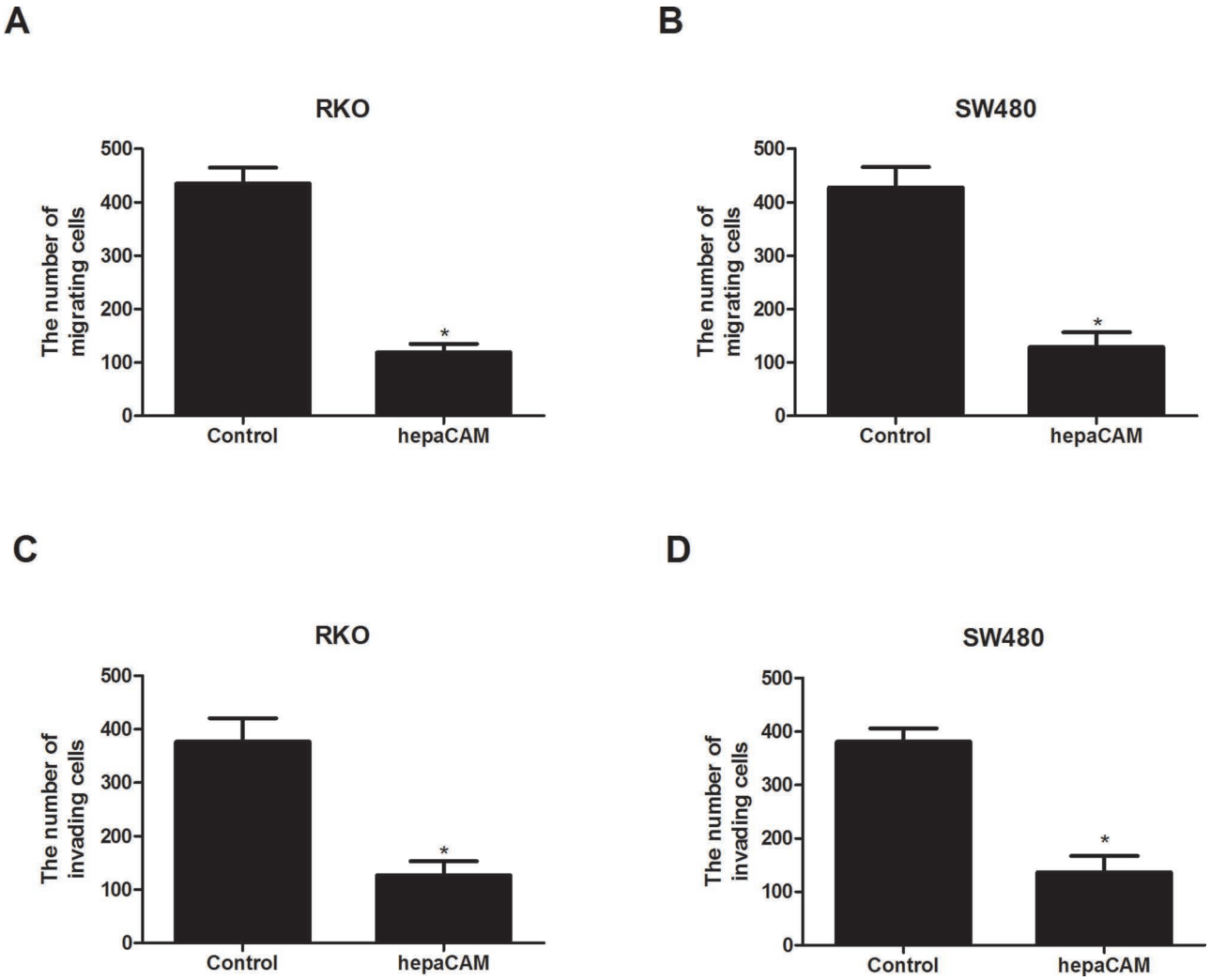

Figure 3. Overexpression of hepaCAM inhibits the migration and invasion of CRC cells. (A, B) The migratory abilities of RKO and SW480 cells were significantly reduced after hepaCAM transfection. (C, D) The invasive abilities of RKO and SW480 cells were markedly decreased after hepaCAM transfection. *$p<0.05$.

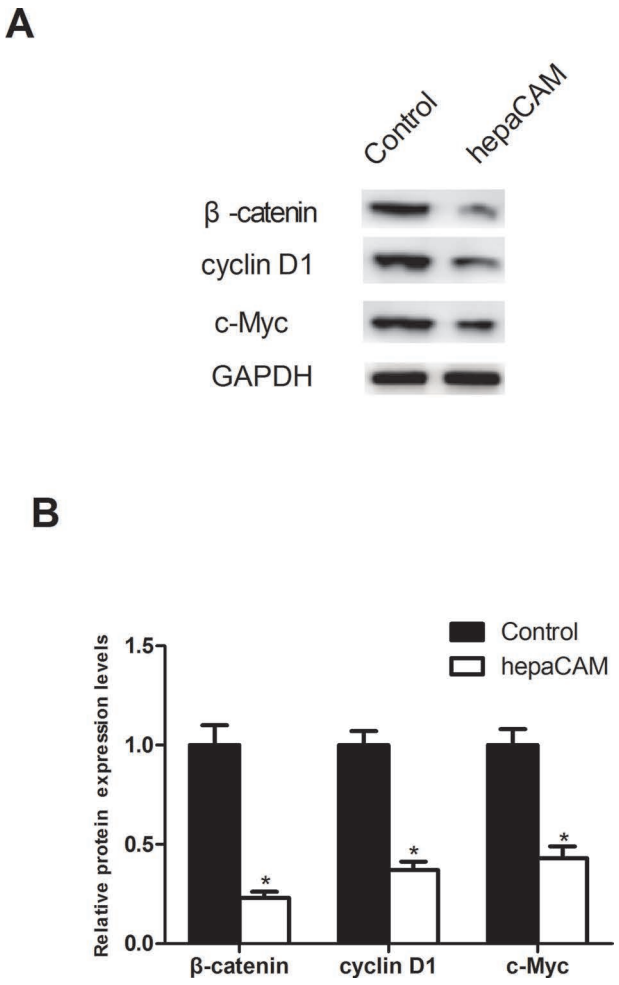

Figure 4. Overexpression of hepaCAM inhibits the activity of the Wnt/β-catenin signaling pathway. (A) The protein expression levels of β-catenin, cyclin D1, and c-Myc in RKO cells were detected via Western blot analysis. (B) The relative protein expression levels of β-catenin, cyclin D1, and c-Myc in RKO cells were quantified by Quantity One software. *$p<0.05$.

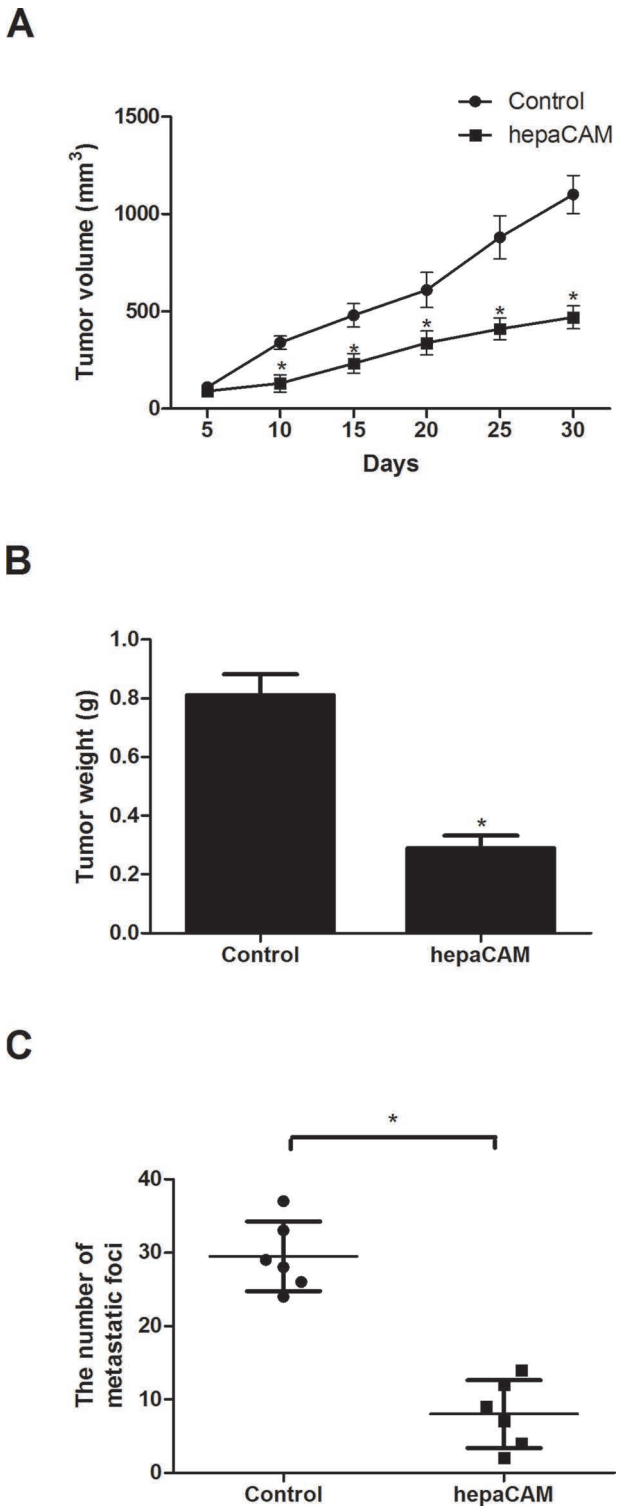

Figure 5. Overexpression of hepaCAM inhibits CRC tumor growth and metastasis in vivo. (A) Quantitative analysis of tumor volume at the indicated days. (B) The weight of tumors was measured 30 days after injecting nude mice with hepaCAM-transfected RKO cells or the control cells. (C) Quantification of metastatic foci in each mouse ($n=6$). *$p<0.05$.

expression of hepaCAM in a variety of human cancers such as kidney and breast cancers[11]. In addition, a growing body of evidence has demonstrated the tumor-suppressive role of hepaCAM. However, it remains unclear whether hepaCAM exerts any effect on CRC.

We investigated the expression pattern of hepaCAM in CRC tissues and cells. Consistent with the previous studies, our results showed that hepaCAM was significantly decreased in CRC tissues and cells. We then examined the effect of hepaCAM overexpression on several cellular processes. In tumor formation, cell proliferation plays an essential role and is crucial for cell survival[24,25]. In this study, we found that hepaCAM overexpression remarkably suppressed CRC cell proliferation. Mei et al. achieved similar results, in that hepaCAM exerted

an inhibitory effect on breast cancer cell growth[11]. Cell migration and invasion are of great importance for many physiological processes such as embryonic development, tissue repair, angiogenesis, and immune response, all of which are frequently deregulated in cancer cells[26,27]. Therefore, we also detected the effect of hepaCAM overexpression on cell migration and invasion. As expected, hepaCAM overexpression greatly weakened the migratory and invasive capabilities of CRC cells. These findings matched those obtained by Shao et al., who reported that forced expression of hepaCAM suppressed the migration of non-small cell lung cancer cells[28]. Moreover, we performed xenograft tumor assays and found that overexpression of hepaCAM inhibited CRC tumor growth and metastasis in vivo, showing a good agreement with our in vitro results. Based on these observations in our study, we suggested that hepaCAM exerted a suppressive function in CRC progression.

The Wnt/β-catenin signaling pathway plays a significant role in cancer development via modulating biological processes such as cell proliferation, invasion, apoptosis, and differentiation[29–32]. In addition, researchers have demonstrated the involvement of Wnt/β-catenin signaling in CRC tumorigenesis. More importantly, a close relation between hepaCAM and the Wnt/β-catenin signaling pathway has been proven in many studies. Therefore, we reasonably inferred that the Wnt/β-catenin signaling pathway might account for hepaCAM-mediated CRC progression. The Western blot analysis showed that hepaCAM overexpression decreased the protein expression levels of β-catenin and its downstream targets cyclin D1 and c-Myc, revealing a suppressive effect of hepaCAM on the Wnt/β-catenin signaling pathway. These results were consistent with our assumption and indicated that the Wnt/β-catenin signaling pathway participated in, at least in part, hepaCAM-regulated CRC progression. In consideration of the complication and changeability in the biological behavior of CRC, other mechanisms might be involved, and further investigations are required.

In conclusion, this study revealed that hepaCAM was downregulated in CRC tissues and cell lines. Overexpression of hepaCAM inhibited CRC cell proliferation, migration, and invasion in vitro. Furthermore, the tumorigenesis assay showed that increased expression of hepaCAM suppressed CRC tumor growth and metastasis in vivo. We also found that hepaCAM overexpression negatively regulated the Wnt/β-catenin signaling pathway in CRC. Taken together, our study results suggested hepaCAM as a promising therapeutic target for CRC and provided a better understanding for the molecular basis of CRC progression.

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