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Contents

Monthly Volume 16 Number 7 July 15, 2024

## EDITORIAL



## REVIEW



## MINIREVIEWS



## ORIGINAL ARTICLE

### Case Control Study



### Retrospective Study

*World Journal of Gastrointestinal Oncology*

**Monthly Volume 16 Number 7 July 15, 2024**

## Contents

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**Monthly Volume 16 Number 7 July 15, 2024**

## Contents

*World Journal of Gastrointestinal Oncology*

**Monthly Volume 16 Number 7 July 15, 2024**

## Contents

## ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Meysam Ebrahimifar, MSc, PhD, Research Assistant Professor, Department of Toxicology, Islamic Azad University, Isfahan 1477893855, Iran. ebrahimifar67@gmail.com

## AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

*WJGO* mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

## INDEXING/ABSTRACTING

The *WJGO* is now abstracted and indexed in PubMed, PubMed Central, Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2024 edition of Journal Citation Reports® cites the 2023 journal impact factor (JIF) for *WJGO* as 2.5; JIF without journal self cites: 2.5; 5-year JIF: 2.8; JIF Rank: 71/143 in gastroenterology and hepatology; JIF Quartile: Q2; and 5-year JIF Quartile: Q2. The *WJGO*'s CiteScore for 2023 is 4.2 and Scopus CiteScore rank 2023: Gastroenterology is 80/167; Oncology is 196/404.

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ORIGINAL ARTICLE

Basic Study

# RBM5 suppresses proliferation, metastasis and glycolysis of colorectal cancer cells *via* stabilizing phosphatase and tensin homolog mRNA

Chu-Xiang Wang, Feng Liu, Yi Wang

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**Chu-Xiang Wang,** Department of Anorectal Surgery, The Affiliated Hospital of Dalian Medical University, Dalian 116000, Liaoning Province, China

**Feng Liu,** Department of Scientific Research, Beijing Fresta Technologies Co, Ltd., Beijing 100176, China

**Yi Wang,** Department of VIP Medical Services, National Cancer Center, National Clinical Research Center for Cancer, Cancer Hospital Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China

**Corresponding author:** Yi Wang, MD, Associate Chief Physician, Department of VIP Medical Services, National Cancer Center, National Clinical Research Center for Cancer, Cancer Hospital Chinese Academy of Medical Sciences and Peking Union Medical College, No. 17 Panjiayuan Nanli, Chaoyang District, Beijing 100021, China. dr_wangyi1018@163.com

## Abstract

### *BACKGROUND*

RNA binding motif 5 (RBM5) has emerged as crucial regulators in many cancers.

### *AIM*

To explore more functional and mechanistic exploration of RBM5 since the lack of research on RBM5 in colorectal cancer (CRC) dictates that is essential.

### *METHODS*

Through Gene Expression Profiling Interactive Analysis, we analyzed RBM5 expression in colon adenocarcinoma and rectum adenocarcinoma tissues. For detecting the mRNA expression of RBM5, quantitative real time-polymerase chain reaction was performed. Protein expression levels of RBM5, hexokinase 2, lactate dehydrogenase A, phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K), phosphorylated-protein kinase B (p-AKT), and AKT were determined *via* Western blot. Functionally, cell counting kit-8 and 5-ethynyl-2′-deoxyuridine (EDU) assay were performed to evaluate proliferation of CRC cells. Invasiveness and migration of CRC cells were evaluated through conducting transwell assays. Glucose consumption, lactate production and adenosine-triphosphate (ATP) production were measured through a glucose assay kit, a lactate assay kit and an ATP production assay kit, respectively. Besides, RNA

immunoprecipitation assay, half-life RT-PCR and dual-luciferase reporter assay were applied to detect interaction between RBM5 and PTEN. To establish a xenotypic tumor mice, CRC cells were subcutaneously injected into the right flank of each mouse. Protein expression of RBM5, Ki67, and PTEN in tumor tissues was examined using immunohistochemistry staining. Haematoxylin and eosin staining was used to evaluate tumor liver metastasis in mice.

***RESULTS***

We discovered down-regulation of RBM5 expression in CRC tissues and cells. RBM5 overexpression repressed proliferation, migration and invasion of CRC cells. Meantime, RBM5 impaired glycolysis in CRC cells, presenting as decreased glucose consumption, decreased lactate production and decreased ATP production. Besides, RBM5 bound to PTEN mRNA to stabilize its expression. PTEN expression was positively regulated by RBM5 in CRC cells. The protein levels of PI3K and p-AKT were significantly decreased after RBM5 overexpression. The suppressive influences of RBM5 on glycolysis, proliferation and metastasis of CRC cells were partially counteracted by PTEN knockdown. RBM5 suppressed tumor growth and liver metastasis *in vivo*.

***CONCLUSION***

This investigation provided new evidence that RBM5 was involved in CRC by binding to PTEN, expanding the importance of RBM5 in the treatment of CRC.

**Key Words:** Colorectal cancer; RNA binding motif 5; Phosphatase and tensin homolo; Phosphoinositide 3-kinase/anti-protein kinase; Glycolysis



**Core Tip:** RNA binding motif 5 (RBM5) exhibited low expression in colorectal cancer (CRC) tissues and cells. RBM5 retarded tumorigenesis and metastasis of CRC *in vitro* and *in vivo*. In terms of mechanism, RBM5 overexpression-induced tumor inhibition of CRC was partially mediated by the phosphatase and tensin homolog/phosphoinositide 3-kinase/anti-protein kinase pathway. These findings extend our knowledge of the link between RBM5 and CRC, shedding light on the mechanisms how RBM5 impair CRC tumorigenesis.

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## INTRODUCTION

Colorectal cancer (CRC) ranks the third most common malignancies, which has risen to the second major cause of tumor-associated death around the world[1-3]. Main manifestations of CRC include blood in the stool, iron deficiency, abdominal pain, anemia, loss of appetite and weight loss[4]. According to the statistics in 2018, approximately two million people are diagnosed with CRC and about 50% of them died annually[5,6]. Early diagnosis has a better prognosis, with 5-year survival rates of more than 90% in early stage CRC patients and less than 10% in late stage CRC patients[7]. Despite of much progress in treatment tactics, including surgery therapy, adjuvant therapy and targeted therapy, the prognosis of CRC patients at advanced stage remains challenging[8-10]. Therefore, exploring underlying mechanisms of CRC and seeking new therapeutic targets are indispensable.

RNA-binding proteins (RBPs) are recognized as a type of proteins containing the mRNA-binding domain[11,12]. Through the binding domain, RBPs can interact with intracellular mRNA to modulate mRNA maturation, translation and localization[13]. As a member of the RBPs family, RNA binding motif 5 (RBM5) is demonstrated to implicate in pathological processes of many cancers[14,15]. For instance, RBM5 up-regulation impairs proliferation of lung cancer cells [16,17]. RBM5 up-regulation suppresses invasion and growth of prostate cancer cells[18]. Long noncoding RNA AFAP1-AS1 facilitates cell proliferation and metastasis by decreasing RBM5 expression in prostate cancer[19]. Notably, Gene Expression Profilling Interactive Analysis (GEPIA) of The Cancer Genome Atlas database shows low expression of RBM5 in CRC tissues. However, few studies have clarified the function of RBM5 in CRC.

Phosphatase and tensin homolog (PTEN) is identified as a tumor suppressor gene that participates in development of diverse tumors[20]. PTEN is demonstrated to repress invasion and metastasis of breast cancer cells[21]. PTEN is demonstrated to prevent oncogenesis in liver cancer, prostate cancer and brain cancer[22,23]. Notably, existing evidence has indicated that several members of RBPs play important roles in cancers *via* interacting with PTEN. For example, RBM24 functions as a tumor suppressor *via* stabilizing PTEN mRNA[24]. RBM38 retards tumor development in breast cancer and CRC, which binds to PTEN and maintains PTEN stability[25,26]. However, whether RBM5 can bind to PTEN in CRC deserves further exploration.

Hereon, we explored the expression and role of RBM5 in CRC. At the same time, we probed the underlying mechanism of the RBM5/PTEN link in CRC. This study implicates RBM5 as a promising target for management of CRC and enriches investigation of CRC regulation mechanisms.

## MATERIALS AND METHODS

### Cell culture and treatment

Human five CRC cell lines (HT29, HCT116, SW480, LOVO, and SW620) and human normal colon epithelial cell line [fetal human cell (FHC)] were bought by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modification of Eagle's Medium (DMEM; KeyGen Biotechn, Nanjing, Jiangsu Province, China) containing 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin was used to culture these cells. All cells were incubated in a humidified atmosphere (37 °C) with 5% $CO_2$.

### Cell transfection

The pcDNA3.1-RBM5, short hairpin (sh)-RBM5, sh-PTEN, sh-negative control (NC) and pcDNA3.1-NC were bought from RiboBio (Guangzhou, Guangdong Province, China). HCT116 and SW480 cells were seeded into 6-well plates and cultured for 24 hours until they reached a confluency of 60%-70%. Then Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States) was used to transfect above plasmids (2 μg) into HCT116 and SW480 cells for 48 hours.

### Quantitative real time-polymerase chain reaction

Total RNAs were extracted following the guidance of a GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States). The RNA concentration and purity were measured using NanoDrop 2000 (Thermo Fisher Scientific). Based on instructions of EasyScript® First-Strand complementary DNA (cDNA) Synthesis SuperMix bought from Beijing TransGen (Beijing, China), cDNA was synthesized. The reaction conditions were as follows: 37 °C for 15 minutes and 85 °C for 5 seconds. The cDNA product was immediately used as a template for the PCR reaction. The ransScript® One-Step RT-PCR SuperMix (Beijing TransGen Biotech) was used to conduct quantitative real time-polymerase chain reaction (qRT-PCR). Amplification procedures were exhibited as follows: 5 minutes at 95 °C, 30 cycles of 20 seconds at 95 °C, 30 seconds at 55 °C and 2 minutes at 72 °C. Primer sequences from Yilaibo (Shanghai, China) were exhibited as follows: RBM5-F, 5'-GCACGACTATAGGCATGACAT-3'; RBM5-R, 5'-AGTCAAACTTGTCTGCTCCA-3'; PTEN-F, 5'-CCAGGACCAGAGGAAACCT-3'; PTEN-R, 5'-GCTAGCCTCTGGATTTGA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F, 5'-CCATCTTCCAGGAGCGAGAT-3'; GAPDH-R, 5'-TGCTGATGATCTTGAGGCTG-3'. Relative mRNA expression of RBM5 and PTEN was calculated through the $2^{-\Delta\Delta ct}$, which was normalized to GAPDH.

### Cell counting kit-8 assay

The cell viability was evaluated *via* a cell counting kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Shanghai, China). In detail, SW480 and HCT116 cells ($5 \times 10^3$ cells/well) were seeded into 96-well plates. These cells were incubated for 0 hour, 24 hours, 48 hours, 72 hours, and 96 hours, after which CCK-8 reagent (10 μL/well) was added to incubate at 37 °C for 3 hours. At last, the optical density at a wavelength of 450 nm was measured through a microplate reader (MG LABTECH, Durham, NC, United States).

### 5-ethynyl-2'-deoxyuridine assay

The cell proliferation was assessed *via* a 5-ethynyl-2'-deoxyuridine (EDU) assay kit (KeyGen Biotech., Nanjing, China). In brief, SW480 and HCT116 cells were put into 96-well plates, incubating two days. Then 50 μM EDU was added to treat these cells for 2 hours. After fixation through 4% polyformaldehyde, 4,6-diamino-2-phenyl indole (DAPI) was added to label cell nuclei. A fluorescence microscopy (Ribobio) was utilized for visualizing EDU-positive cells.

### Transwell assay

Transwell chambers (Millipore, Billerica, MA, United States) without Matrigel or with Matrigel (diluted to a final concentration of 5 μg/μL) were used to evaluate migration and invasion of SW480 and HCT116 cells, respectively. Firstly, SW480 and HCT116 cells ($5 \times 10^4$ cells/well), which were re-suspended in serum-free medium, were put on upper transwell chambers (pore size, 8 μm). And 600 μL complete medium containing 10% FBS was added to lower transwell chambers and small bubbles were avoided in the lower chamber. Following incubation for 48 hours at 37 °C in a 5% humidified atmosphere, the chambers were taken out and residual cells were removed. Cells were unable to migrate or invade to the surface of the lower chamber were removed with cotton swabs. Meantime, migrating or invading cells were fixed *via* paraformaldehyde (4.0%) for 20 minutes and stained *via* crystal violet (0.1%) for 5 min. An optical microscope from Olympus (Tokyo, Japan) was utilized for observing stained cells.

### Western blot analysis

Protein extraction was carried out through radio immunoprecipitation assay (RIPA) buffer (CWBio, Beijing, China). The lysates were centrifuged at 12000 r/minute for 10 minutes at 4 °C. The supernatant was mixed with 5 × loading buffer and denatured at 100 °C for 5 minutes. A BCA Kit (Beyotime, Shanghai, China) was applied to detect protein concentration. A sodium dodecyl sulphate-polyacrylamide gel with 10% separation gel and 5% stacking gel were prepared. The protein samples (30 μg) were separated by electrophoresis in 1 × buffer (1.51 g Tris-base, 9.4 g glycine, 0.5 g SDS, 500 mL

dd$H_2O$) at 80 V for 30 minutes and at 120 V for 1 hour, followed by moving onto polyvinylidene difluoride membranes (Cytiva, Shanghai, China). Following being blocked with 5% skim milk for 2 hours, the primary antibodies including anti-RBM5 (1:2000, ab245646, Abcam, Cambridge, CA, United States), anti-PTEN (1:1000, ab267787, Abcam), anti-hexokinase 2 (HK2; 1:1000, ab209847, Abcam), anti-lactate dehydrogenase A (LDHA; 1:5000, ab52488, Abcam), anti-protein kinase B (AKT; 1:2000, ab185633, Abcam), anti-phosphorylated-protein kinase B (p-AKT; 1:500, ab38449, Abcam), anti-phosphoinositide 3-kinase (PI3K; 1:1000, ab302958, Abcam) and anti-β-actin (1:200, ab115777, Abcam) were appended to membranes. Next day, the secondary antibody (1:2000, ab6721, Abcam) was appended. At last, protein blots were visualized through the ECL chemiluminescent system, which were quantified using Image J software (NIH, United States).

### RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) assay was conducted using a Thermo Fisher RIP kit from Thermo Fisher Scientific. HCT116 and SW480 cells were dissolved with RIP lysis buffer (25 mmol/L Tris-HCl, pH = 7.5, 150 mmol/L KCl, 2 mmol/L EDTA, 0.5% NP-40, 1 mmol/L DTT, 100 U/mL RNasin). Afterwards, RIP buffer containing magnetic beads with anti-RBM5 or anti-IgG was incubated with cell lysates. Following incubation at 4 °C for 2 hours, magnetic beads were washed and 0.5 mg/mL Proteinase K was used to digest the proteins. Then immunoprecipitated RNAs were eluted from the beads, after which purified RNAs were utilized for detecting PTEN.

### Half-life qRT-PCR of RNA

Firstly, actinomycin D (Sigma, MO, United States) was appended to incubate transfected HCT116 and SW480 cells. After being treated with actinomycin D for 0 hour, 2 hours, 4 hours, and 6 hours, RNAs in HCT116 and SW480 cells were collected *via* TRIzol RNA Purification (Life Technologies, CA, United States) and then used for qRT-PCR. The RNA half-life was calculated using the time when percent remaining of PTEN reduced to 50%.

### Determination of glucose consumption, lactate and adenosine-triphosphate production

After collecting the culture medium of HCT116 and SW480 cells, glucose consumption, lactate production and adenosine-triphosphate (ATP) production were measured through a glucose assay kit (Sigma-Aldrich, St. Louis, MO, United States), a lactate assay kit (Sigma-Aldrich) and an ATP production assay kit (Solarbio), respectively.

### Dual-luciferase reporter assay

Dual-luciferase reporter (DLR) assay was performed to confirm the binding of RBM5 to 3'UTR of PTEN. SW480 and HCT116 cells transfected with pcDNA3.1-RBM5/pcDNA3.1-NC were plated in a 24-well plate ($1 \times 10^5$ cells/well). Next day, a pGL3 reporter (Promega, Madison, WI, United States) containing several regions of PTEN 3'UTR was co-transfected into HCT116 and SW480 cells using Lipofectamine 3000 (Invitrogen). After 48 hours of transfection, cells were lysed by Lysis Buffer. The lysate was incubated with Luciferase Assay Regent, and the fireny luciferase was measured by the dual-Luciferase reporter assay system (Promega). Stop Reagent was then added, and renilla luciferase was detected. The ratio of Firefly luciferase and Renilla luciferase indicated relative luciferase activity.

### Xenograft assay

BALB/c nude mice (6-week-old, female, weighing 20-30 g) were bought from Shanghai Model Organisms Center (Shanghai, China). Animal experiments were executed in accordance with the Animal Care and Use Committee of Beijing Viewsolid Biotechnology Co. LTD (VS2126A00176). For establishing xenograft tumor models, $2 \times 10^6$ HCT116 cells (suspended in 0.2 mL PBS) transfected with pcDNA3.1-NC/pcDNA3.1-RBM5 were subcutaneously injected into the right flank of each mouse ($n$ = 5/group). The short diameter (W) and long diameter (L) of tumors were monitored every week and calculated *via* the formula: Volume = $0.5 \times L \times W^2$. Following injection for four weeks, mice anesthetized through pentobarbital sodium were sacrificed. Isolated tumors were weighed and subjected to further experiments.

For establishing a mouse model of liver metastasis, the spleens of anaesthetized mice were exposed by making an incision through the skin and peritoneum. Then $1 \times 10^6$ HCT116 cells transfected with pcDNA3.1-NC/pcDNA3.1-RBM5 were introduced into the spleen of each mouse ($n$ = 5/group). These mice were housed until the end of this investigation (6 weeks). To evaluate the metastatic lesions, mouse livers were dissected for haematoxylin and eosin (H&E) staining.

### Immunohistochemistry staining

Protein expression levels of RBM5, Ki67 and PTEN were examined *via* immunohistochemistry (IHC) staining. In brief, mouse tumor tissues were fixed through formalin, embedded through paraffin, cut into sections (4 μm), dewaxed *via* xylene and rehydrated by gradient alcohol. After washing with PBS, tissue sections were repaired with an antigen retrieval solution at 100 °C for 3 minutes and then cooled to room temperature for 30 minutes. Endogenous peroxidases were then blocked with 3 % $H_2O_2$ for 15 minutes at room temperature. Afterwards, these tissue sections were incubated with primary antibodies including the RBM5 antibody (1:500, ab69770, Abcam), Ki67 antibody (1:200, ab16667, Abcam) and PTEN antibody (1:2000, ab267787, Abcam) at 4 °C. Next day, the anti-rabbit secondary antibody bought from Abcam (1:500, ab6112) was appended to incubate for 30 minutes at room temperature, after which 3,3'-diaminobenzidine substrate solution was appended. Eventually, hematoxylin was applied to stain tissue sections and a microscope (Olympus) was employed to observe staining areas.

### H&E staining

Tumor tissues were fixed with 4% paraformaldehydeat 4 °C overnight. After dehydrated by ethanol, these tissues were paraffin-embedded, cut into 5 μm slices and deparaffinized. Then a H&E staining kit (Beyotime) was used to stain these sections, followed by dehydration *via* xylene and graded ethanol. Finally, stained sections were observed under an Eclipse Ti-S microscope (Nikon, Tokyo, Japan).

### Statistical analysis

GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, United States) was used to conduct statistical analysis. Data from at least three separate experiments were exhibited as mean ± SD. Comparisons among multiple groups were assessed through one-way analysis of variance and Tukey's post hoc analysis. For comparisons between two groups, Student's *t*-test was used. Any difference with $P < 0.05$ was considered as statistical significance.

## RESULTS

### Low expression of RBM5 is found in CRC tissues

Through GEPIA, we discovered that RBM5 expression was dramatically down-regulated in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) tissues as opposed to corresponding normal tissues (Figure 1A, $P < 0.05$). Then qRT-PCR and Western blot analysis were carried out for detecting expression of RBM5. Compared to FHC cells, mRNA and protein expression levels of RBM5 in CRC cells (SW620, SW480, HT29, LOVO, and HCT116) were distinctly decreased (Figure 1B and C, $P < 0.01$), especially SW480 and HCT116 cells.

### Overexpression of RBM5 represses proliferation, metastasis and glycolysis of CRC cells

For unraveling the influence of RBM5 on CRC cells, we overexpressed RBM5 through transfection of pcDNA3.1-RBM5. As expected, RBM5 displayed higher protein expression in the pcDNA3.1-RBM5 group than the pcDNA3.1-NC group (Figure 2A, $P < 0.01$). Subsequently, we performed functional experiments on RBM5. It was found that overexpression of RBM5 evidently attenuated the proliferation ability of SW480 and HCT116 cells, reflected by decreasing cell viability (Figure 2B, $P < 0.001$) and EDU positive cells (Figure 2C). Overexpression of RBM5 dampened migration and invasiveness of SW480 and HCT116 cells (Figure 2D-E, $P < 0.01$).

Furthermore, the effect of RBM5 on glycolysis was explored *via* measuring parameters of glycolysis. As exhibited in Figure 2F, glucose consumption, lactate production and ATP production were evidently reduced after RBM5 up-regulation in SW480 and HCT116 cells ($P < 0.01$). Inhibited glycolysis was also affirmed by detecting glycolysis-related proteins. It came out that protein levels of LDHA and HK2 in SW480 and HCT116 cells were all diminished in response to RBM5 overexpression (Figure 2G, $P < 0.01$).

### RBM5 binds to PTEN to stabilize its expression

We next investigated whether RBM5 could regulate PTEN expression in CRC. Firstly, we discovered that PTEN protein expression in HCT116 and SW480 cells was boosted after RBM5 overexpression and diminished after RBM5 knockdown (Figure 3A and B, $P < 0.01$), suggesting that PTEN expression was positively regulated by RBM5 in CRC cells. Then correlation analysis (Figure 3C) exhibited an evident positive correlation between RBM5 and PTEN in COAD ($P = 0.0082$) and READ ($P = 0.0093$).

Afterwards, whether RBM5 could directly bind to PTEN and stabilize its expression in CRC cells was confirmed. Data from RIP assay displayed that the PTEN mRNA was dramatically enriched in RBM5 precipitate, but not in control IgG precipitate (Figure 3D, $P < 0.001$). Besides, data from qRT-PCR demonstrated that RBM5 overexpression led to an increase of PTEN half-life in HCT116 and SW480 cells (Figure 3E, $P < 0.05$). Then catRAPID (http://service.tartaglialab.com/page/catrapid_group) predicted a series of binding regions between RBM5 protein and PTEN 3'UTR (Figure 3F). DLR assay indicated that RBM5 overexpression markedly increased relative luciferase activity for reporters carrying 3'-UTR-d of PTEN, whereas failed to change 3'-UTR-a, b, and c of PTEN (Figure 3G, $P < 0.001$). Above data implied that 3'-UTR-d of PTEN was responsible for RBM5 to facilitate expression of PTEN.

### RBM5 regulates the PTEN/PI3K/AKT pathway

Previously, PTEN is proved to negatively modulate the PI3K/AKT signal pathway that participates in progression of diverse cancers[27,28]. We next delved into whether RBM5 made impact on the PTEN/PI3K/AKT pathway. We observed decreasing protein levels of PI3K and p-AKT as well as increasing PTEN after RBM5 overexpression in HCT116 and SW480 cells (Figure 4, $P < 0.001$). These findings suggested that RBM5 could regulate the PTEN/PI3K/AKT pathway in CRC cells.

### PTEN knockdown partially reversed suppressive impacts of RBM5 on glycolysis, proliferation, and metastasis of CRC cells

For investigating whether PTEN overexpression participated in RBM5-mediated regulation in proliferation, migration, invasion and glycolysis, following experiments were conducted. Western blot analysis showed that PTEN expression was dramatically reduced after transfection of sh-PTEN in HCT116 and SW480 cells, suggesting successful PTEN silencing (Figure 5A, $P < 0.001$). PTEN expression was raised by RBM5 overexpression, which was reversed by PTEN silencing

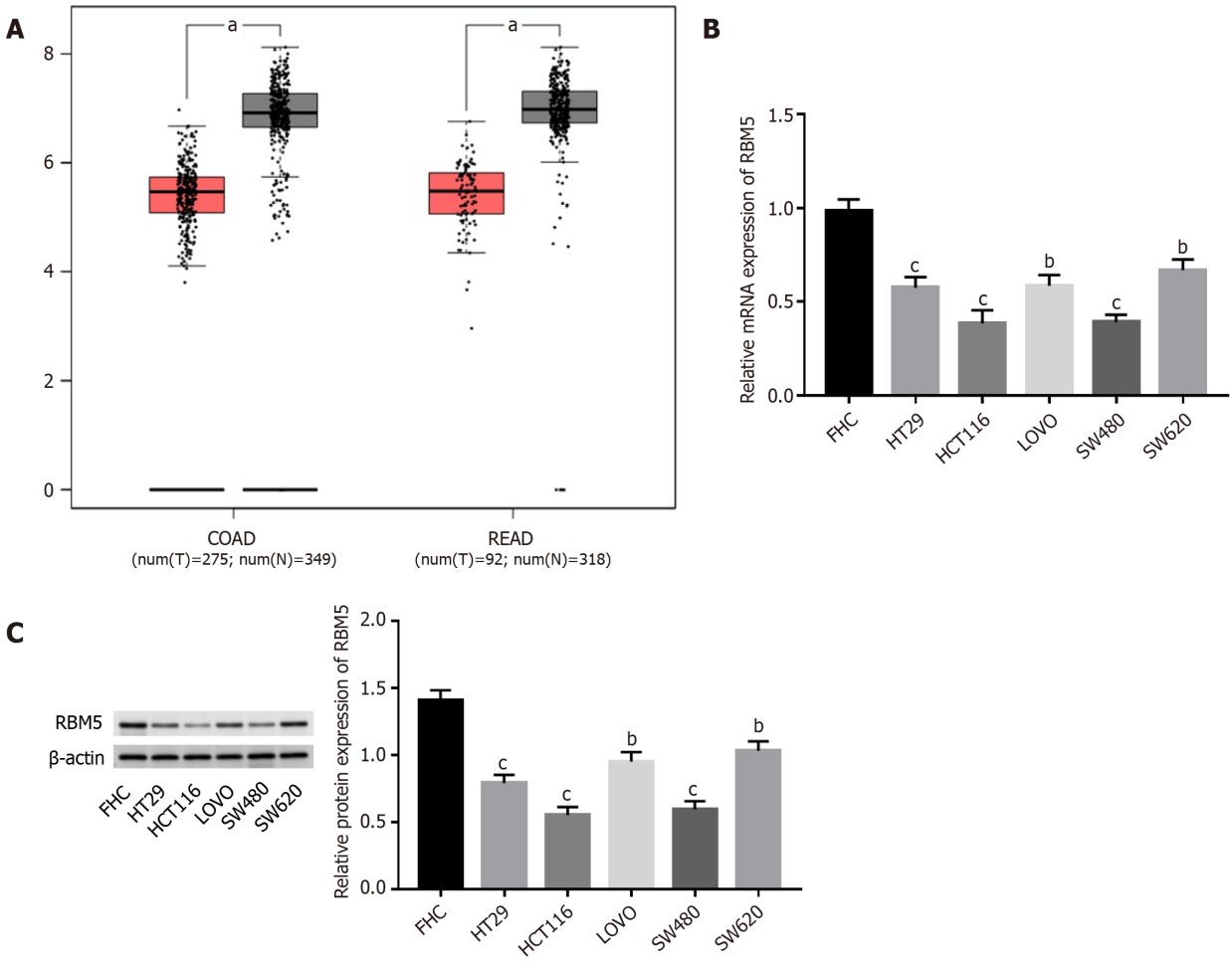



**Figure 1 RNA binding motif 5 is lowly expressed in colorectal cancer tissues and cells.** A: The mRNA expression of RNA binding motif 5 (RBM5) in tumor tissues and normal tissues was analyzed *via* the Gene Expression Profiling Interactive Analysis; B: Relative mRNA expression of RBM5 in fetal human cell (FHC), HT29, HCT116, LOVO, SW480 and SW620 cells was detected by quantitative real time-polymerase chain reaction; C: Relative protein expression of RBM5 in FHC, HT29, HCT116, LOVO, SW480, and SW620 cells was detected by Western blot. RBM5: RNA binding motif 5. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

(Figure 5B, $P < 0.01$). Then rescue experiments were performed in HCT116 and SW480 cells. It was demonstrated that RBM5 overexpression-mediated inhibition of cell proliferation, migration and invasion was reversed by PTEN silencing (Figure 5C-F, $P < 0.01$). Besides, RBM5 overexpression-mediated suppression of glycolysis was abrogated by PTEN silencing in HCT116 and SW480 cells (Figure 5G, $P < 0.05$). Collectively, these data indicated that RBM5 may impair cell malignant behaviors in CRC by up-regulating PTEN expression.

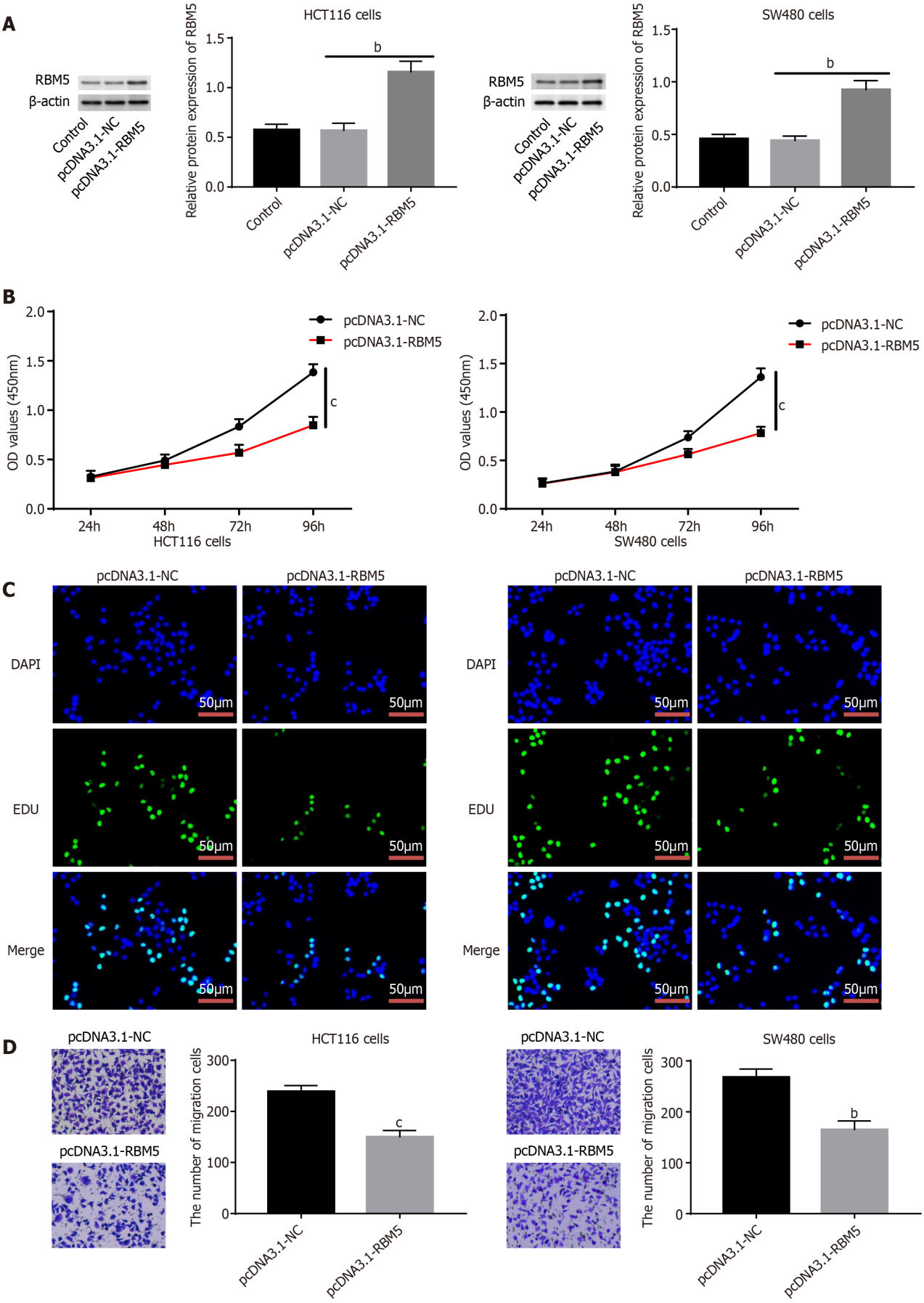
### *Overexpression of RBM5 suppresses tumor growth and liver metastasis in vivo*

Finally, we explored the role of RBM5 in CRC *in vivo*. As displayed in Figure 6A-C, the mouse tumor volume ($P < 0.01$) and tumor weight ($P < 0.01$) were significantly reduced after RBM5 overexpression. Increasing PTEN expression and decreasing Ki-67 expression was found after RBM5 overexpression in xenograft mouse tumors according to IHC staining data (Figure 6D). Decreasing levels of PI3K and p-AKT as well as increasing PTEN expression were observed after RBM5 overexpression in xenograft mouse tumors (Figure 6E, $P < 0.001$). In addition, we discovered that less liver metastatic nodules were observed in the pcDNA3.1-RBM5 group than the pcDNA3.1-NC group (Figure 6F, $P < 0.05$).

## DISCUSSION

Even if the CRC incidence among the elderly has declined, the incidence among younger patients is rising on account of unhealthy lifestyle[29,30]. As a result, CRC continues to be a serious health problem around the world[31,32]. In recent years, growing evidence has connected RBM5 to diverse cancers, which demonstrates that RBM5 expression is down-regulated in bladder cancer[33], non-small cell lung cancer[34], breast cancer[35] and vestibular schwannoma[36]. In our study, we also observed the decrease of RBM5 expression in CRC tissues and cells, which was in line with above mentioned studies. These results implied that RBM5 was closely related to CRC.

With the deepening research of RBM5, many studies have proved that RBM5 exerts crucial roles in occurrence and development of varied cancers. For instance, up-regulated RBM5 represses cell proliferation in lung cancer[16]. Up-


A
RBM5
β-actin
Control
pcDNA3.1-NC
pcDNA3.1-RBM5
HCT116 cells
Relative protein expression of RBM5
b
SW480 cells
B
OD values (450nm)
pcDNA3.1-NC
pcDNA3.1-RBM5
c
24h
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72h
96h
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DAPI
EDU
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50μm
D
The number of migration cells

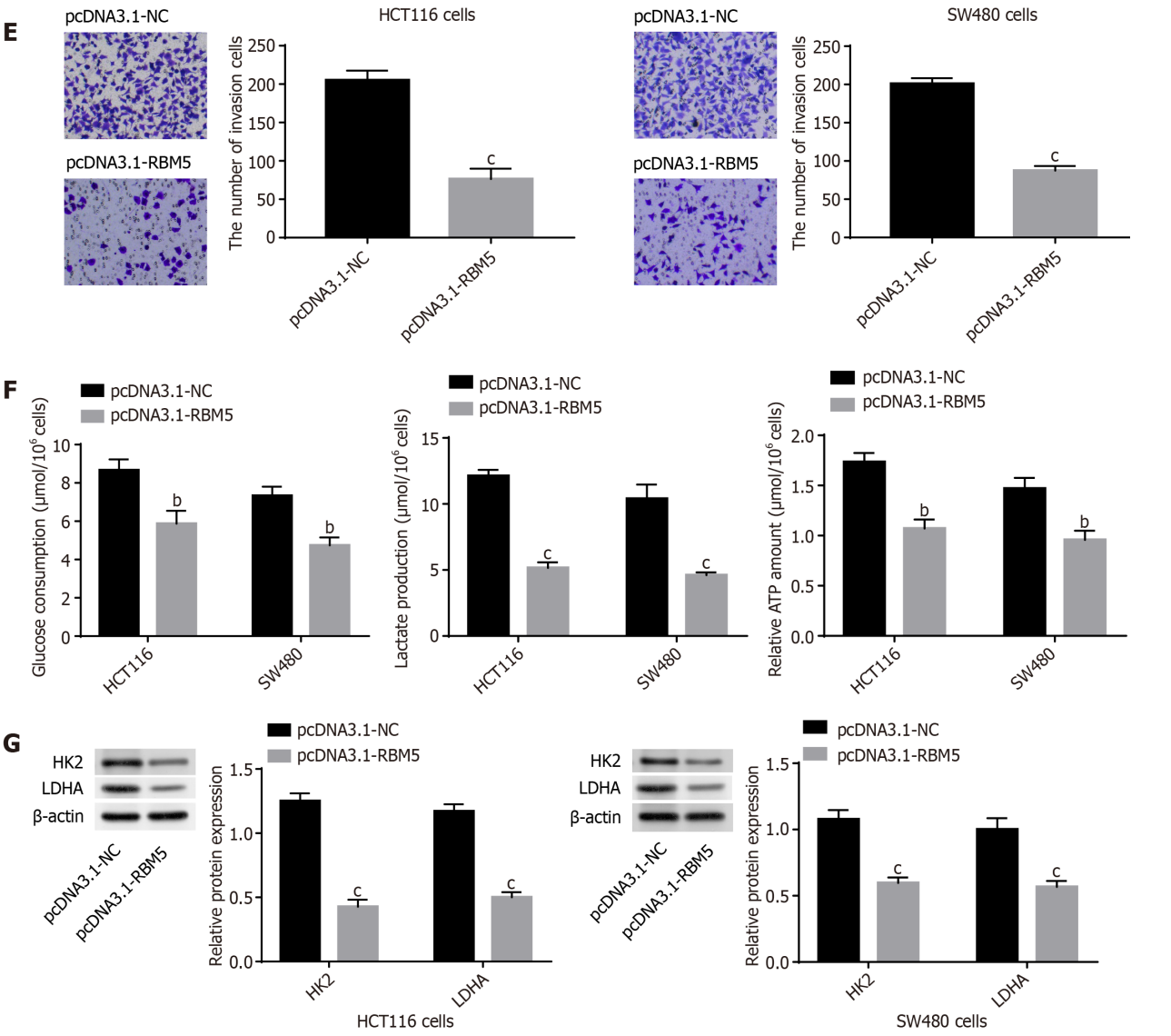
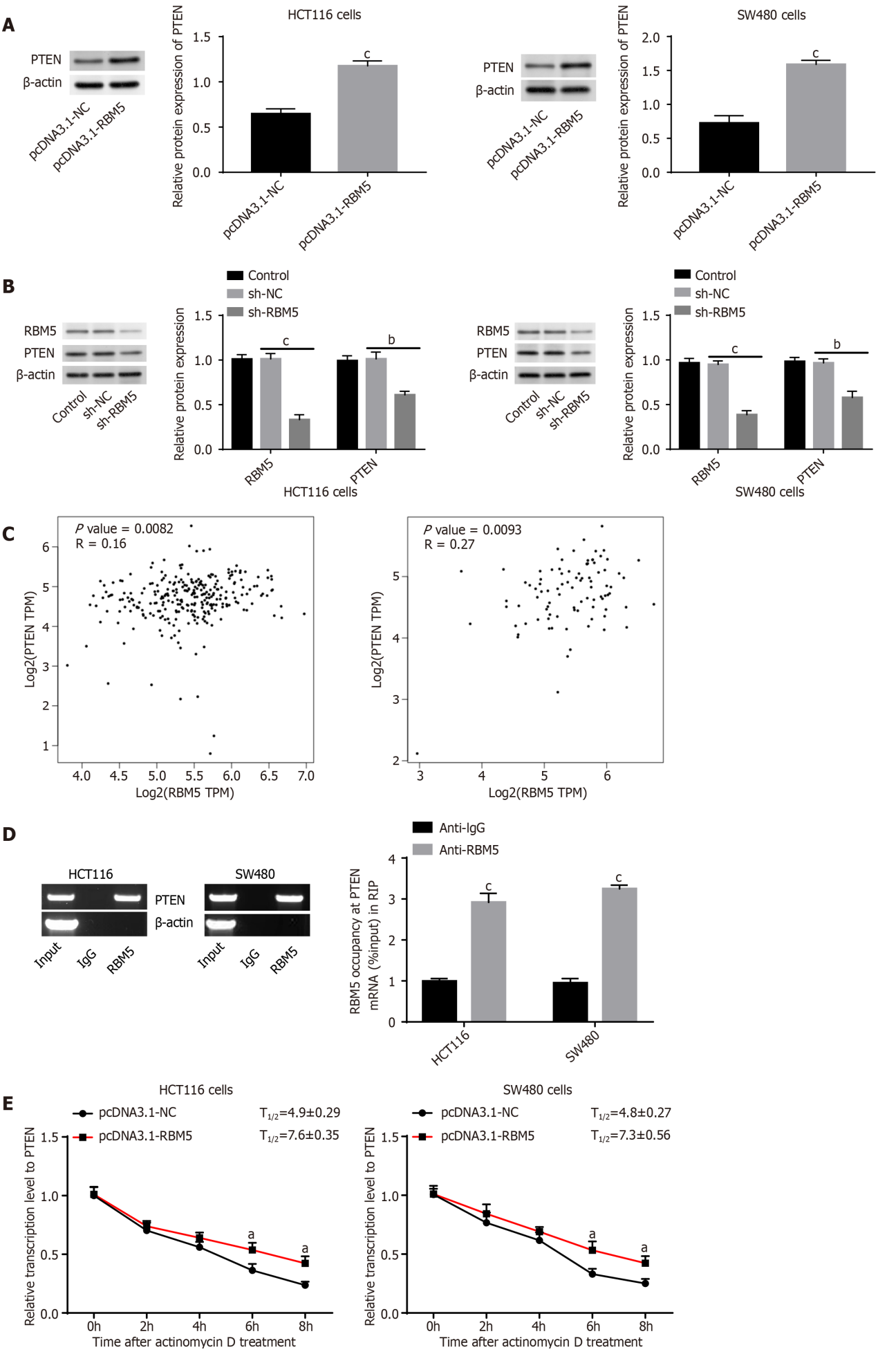



**Figure 2 RNA binding motif 5 overexpression represses proliferation, migration, invasion and glycolysis of colorectal cancer cells.** A: Relative protein expression of RNA binding motif 5 in HCT116 and SW480 cells was detected by Western blot; B: Cell counting kit-8 (CCK-8) assay was used to assess the viability of HCT116 and SW480 cells; C: 5-ethynyl-2'-deoxyuridine assay was used to assess proliferation of HCT116 and SW480 cells; D and E: Migration and invasion of HCT116 and SW480 cells were evaluated by transwell assays; F: Glucose consumption, lactic acid production, and adenosine-triphosphate production were detected by corresponding kits; G: Protein levels of hexokinase 2 and lactate dehydrogenase A were detected by Western blot in HCT116 and SW480 cells. RBM5: RNA binding motif 5; EDU: 5-ethynyl-2'-deoxyuridine; HK2: Hexokinase 2; LDHA: Lactate dehydrogenase A. $^{b}P < 0.01$, $^{c}P < 0.001$.

regulated RBM5 suppresses cell growth and invasion in prostate cancer[18]. Down-regulated RBM5 prevents cell apoptosis in bladder cancer[33]. RBM5 overexpression inhibits cell proliferation and migration in medulloblastoma[37]. Here, we discovered that RBM5 overexpression distinctly attenuated CRC cell proliferative, migratory and invasive abilities *in vitro*, suggesting that RBM5 acted as a protective factor in CRC. We subsequently validated the ability of RBM5 to inhibit tumorigenesis and liver metastasis *in vivo*. Our findings were similar to previous studies which show that RBM5 functions as a tumor suppressor in CRC[16,18,33], and we inferred that RBM5 may be a potential target against CRC. It is uncovered that dependence on glycolysis is a sub-optimal way for cancer cells, which affects tumor microenvironment in favor of cancer malignancy and development[38-40]. Many genes are reported to inhibit glycolysis in CRC. For instance, STK25 overexpression represses aerobic glycolysis *via* reducing expression levels of LDHA, HK2 and GLUT1[42]. Similarly, we found that RBM5 repressed glycolysis of CRC cells, presenting as decreased glucose consumption, lactate production and ATP production as well as decreased expression levels of LDHA and HK2. Collectively, we deduced that targeting RBM5 may be a feasible method to treat CRC.

Given the fact that RBPs such as RBM24 and RBM38 play anti-tumor role in CRC through stabilizing PTEN[24,25], it is reasonable for us to assume that RBM5 may affect malignant behaviors of CRC cells *via* binding to PTEN. We then validated our assumption, and found that RBM5 stabilized transcript of PTEN and up-regulated PTEN *via* binding to 3'UTR of PTEN. Moreover, we performed rescue assays to confirm whether PTEN was involved in RBM5-mediated inhibition of cell proliferation, invasion, glycolysis and migration in CRC. We discovered that the suppressive influences of RBM5 on proliferation, invasiveness, glycolysis and migration of CRC cells were reversed by silencing of PTEN. Taken together, our findings offered evidence that RBM5 served as an anti-tumor gene *via* up-regulating PTEN expression in


A
PTEN
β-actin
pcDNA3.1-NC
pcDNA3.1-RBM5
HCT116 cells
Relative protein expression of PTEN
SW480 cells
B
RBM5
PTEN
β-actin
Control
sh-NC
sh-RBM5
Relative protein expression
C
P value = 0.0082
R = 0.16
Log2(PTEN TPM)
Log2(RBM5 TPM)
P value = 0.0093
R = 0.27
D
HCT116
SW480
Input
IgG
RBM5
Anti-IgG
Anti-RBM5
RBM5 occupancy at PTEN mRNA (%input) in RIP
E
pcDNA3.1-NC
pcDNA3.1-RBM5
T1/2=4.9±0.29
T1/2=7.6±0.35
T1/2=4.8±0.27
T1/2=7.3±0.56
Relative transcription level to PTEN
Time after actinomycin D treatment
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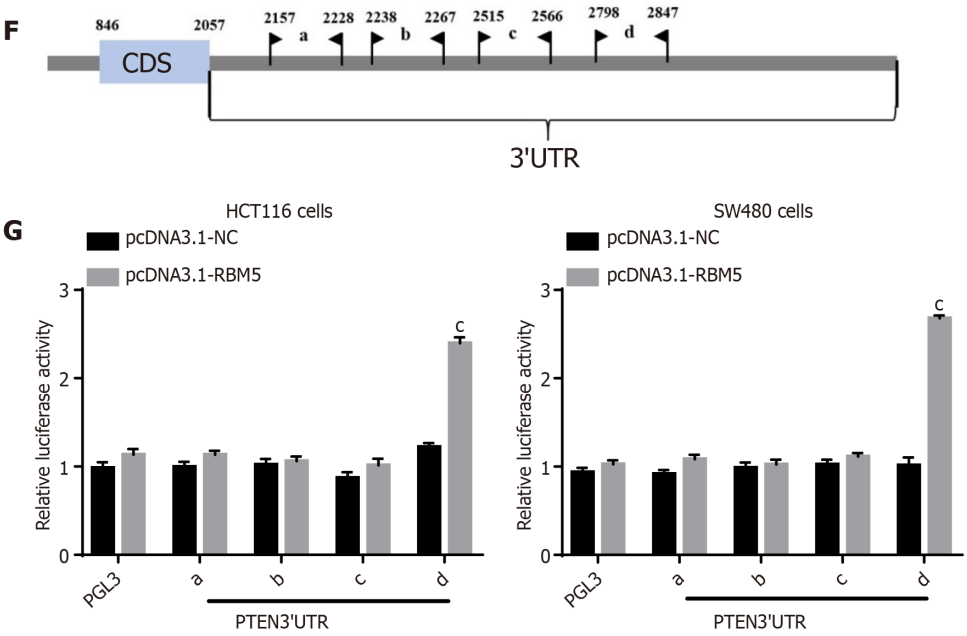



**Figure 3 RNA binding motif 5 binds to phosphatase and tensin homolog mRNA to stabilize its expression.** A: Relative protein expression of phosphatase and tensin homolog (PTEN) in HCT116 and SW480 cells was detected by Western blot after RNA binding motif 5 (RBM5) overexpression; B: Relative protein expression of PTEN and RBM5 in HCT116 and SW480 cells was detected by Western blot; C: The correlation between the expression of RBM5 and PTEN in colon adenocarcinoma and rectum adenocarcinoma; D: The binding of RBM5 and PTEN in HCT116 and SW480 cells was confirmed by RNA immunoprecipitation assay; E: Relative mRNA expression of PTEN in HCT116 and SW480 cells was detected by quantitative real time-polymerase chain reaction after treatment with 5 μg/mL actinomycin D for 0-2-4-6-8 hours; F: CatRAPID (http://service.tartaglialab.com/page/catrapid_group) was used to predict the binding regions between RBM5 and PTEN 3 'UTR; G: Dual-luciferase reporter assay was used to confirm the interaction between RBM5 and PTEN 3'UTR. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. RBM5: RNA binding motif 5; PTEN: Phosphatase and tensin homolog.

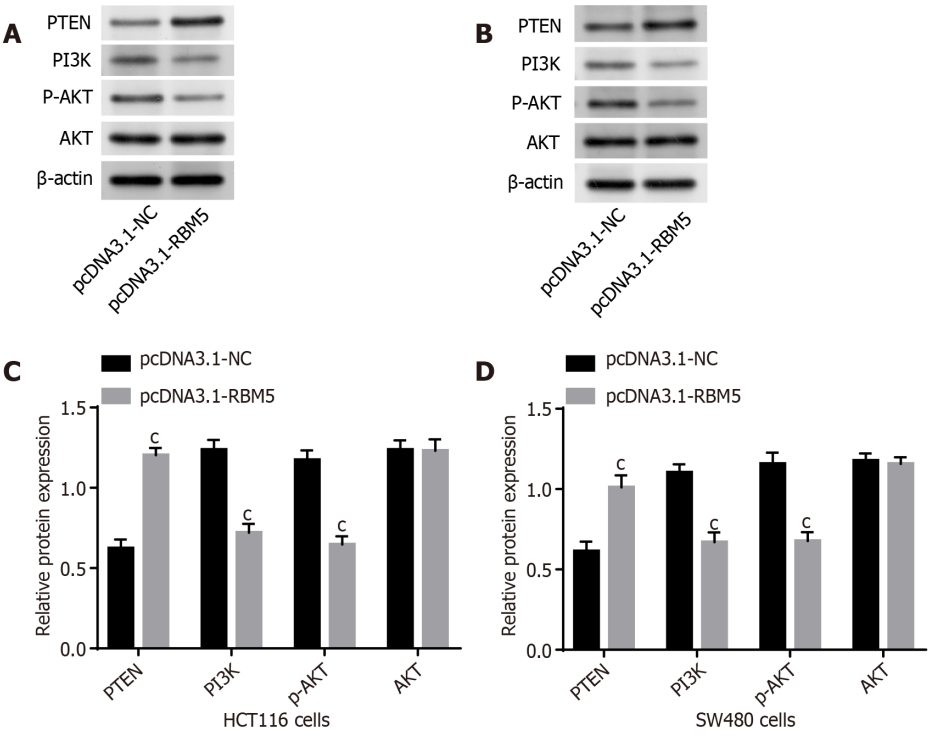
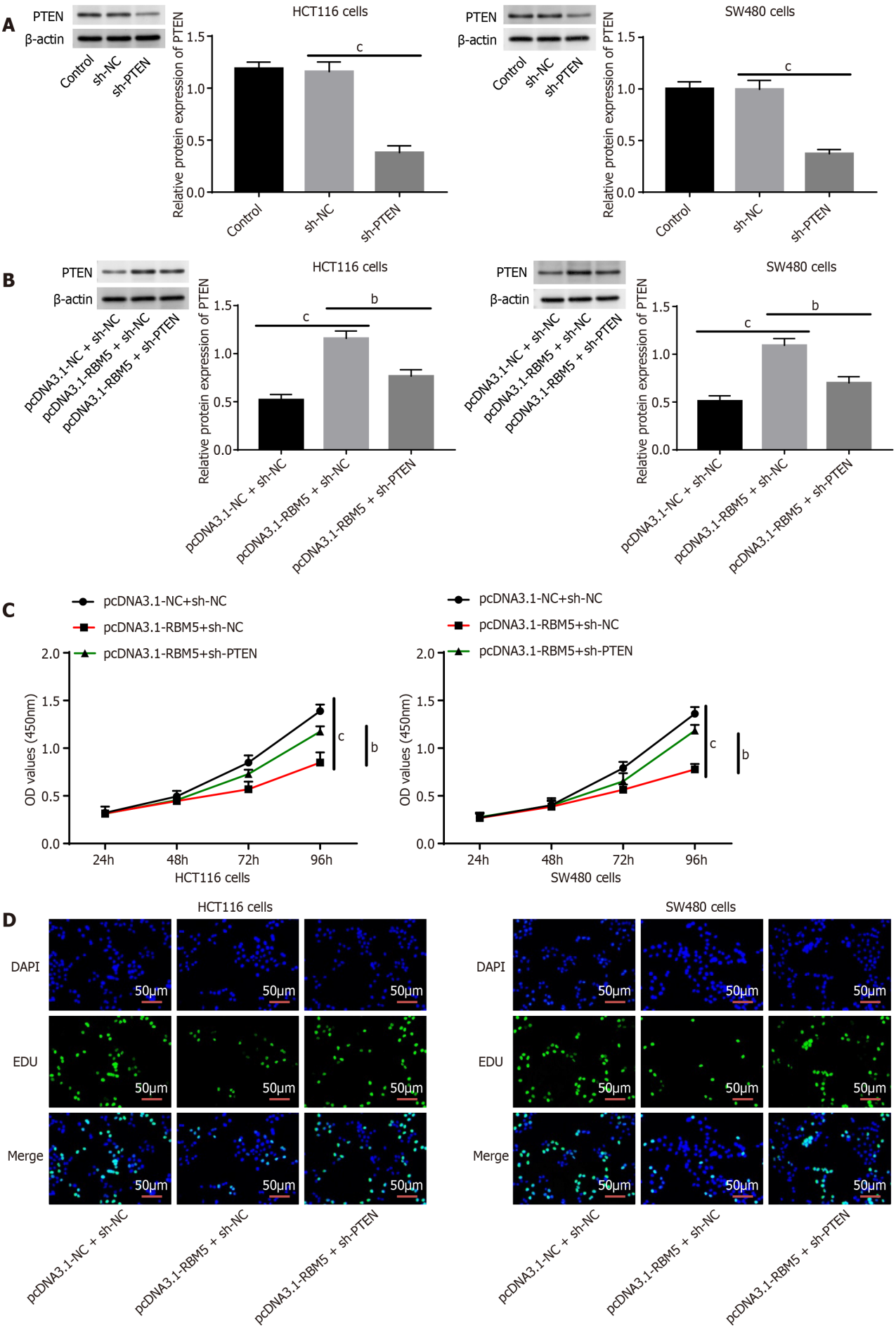



**Figure 4 RNA binding motif 5 regulates the phosphatase and tensin homolog/PI3K/AKT pathway in colorectal cancer cells.** A-D: Protein levels of phosphatase and tensin homolog, phosphoinositide 3-kinase, phosphorylated-protein kinase B (p-AKT) and protein kinase B (AKT) were determined by Western blot in HCT116 and SW480 cells. $^{c}P < 0.001$. RBM5: RNA binding motif 5; PTEN: Phosphatase and tensin homolog; PI3K: Phosphoinositide 3-kinase; p-AKT: Phosphorylated-protein kinase B.


A
PTEN
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Control
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HCT116 cells
SW480 cells
Relative protein expression of PTEN
c
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pcDNA3.1-NC + sh-NC
pcDNA3.1-RBM5 + sh-NC
pcDNA3.1-RBM5 + sh-PTEN
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pcDNA3.1-RBM5+sh-NC
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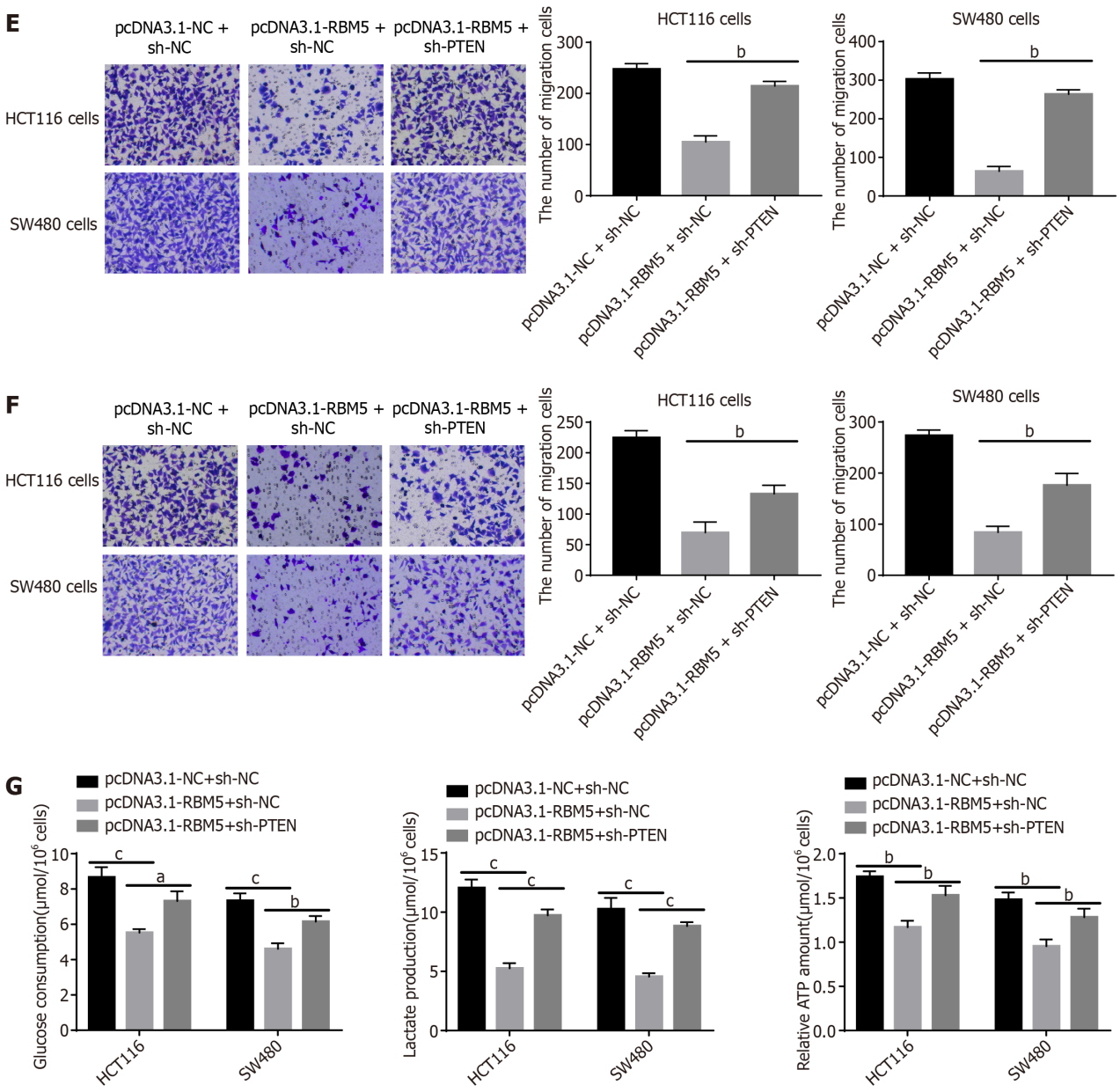


**Figure 5 Phosphatase and tensin homolog knockdown partially reversed inhibitory effects of RNA binding motif 5 on the proliferation, metastasis and glycolysis of colorectal cancer cells.** A: The knockdown efficiency of phosphatase and tensin homolog (PTEN) was detected using Western blot in HCT116 and SW480 cells; B: The expression of PTEN in HCT116 and SW480 cells was detected using Western blot; C: Cell counting kit-8 assay was used to assess the viability of HCT116 and SW480 cells; D: 5-ethynyl-2'-deoxyuridine assay was used to assess proliferation of HCT116 and SW480 cells; E and F: Migration and invasion of HCT116 and SW480 cells were evaluated by transwell assays; G: Glucose consumption, lactic acid production and adenosine-triphosphate production were detected by corresponding kits. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. PTEN: Phosphatase and tensin homolog.

CRC, identifing a novel mechanism in which RBM5 enhanced the PTEN transcript by directly binding the 3'-UTR of PTEN mRNA. Moreover, PTEN is considered to an important negative regulator of the PI3K/Akt signal pathway that can facilitate cell proliferation, growth and migration[43,44]. This led us to focus on whether RBM5 regulated the PTEN/PI3K/Akt axis *via* interacting with PTEN in CRC cells. It turned out that RBM5 overexpression resulted in increasing PTEN expression as well as decreasing protein levels of PI3K and p-AKT in CRC cells and mice. This evidence strongly suggested that RBM5 overexpression regulated PTEN mRNA abundancy and leads to the inactivation of the PTEN/PI3K/Akt pathway, thereby inhibiting tumorigenesis in CRC.

## CONCLUSION

In summary, RBM5 was lowly expressed in CRC tissues and cells. RBM5 overexpression-induced tumor inhibition was partially mediated by the PTEN/PI3K/Akt pathway. These findings extend our knowledge of RBM5 function in CRC, providing a potential target for treatment of CRC.

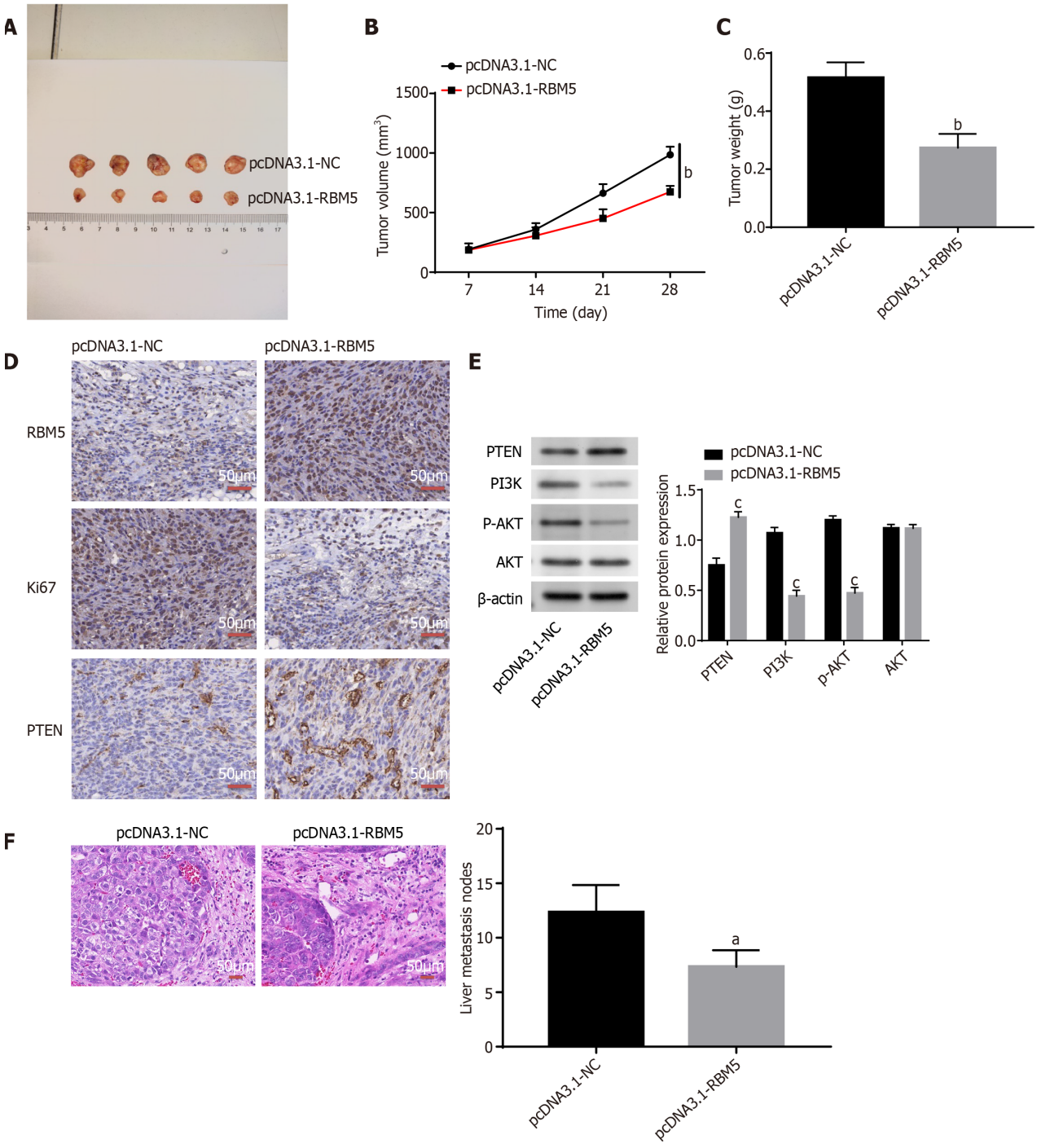


**Figure 6 Overexpression of RNA binding motif 5 inhibits the tumor formation and metastasis of colorectal cancer *in vivo*.** A: Images of a tumor in xenograft mice, $n = 5$; B: The tumor volume in every 7 days; the construction of tumor growth curves of mice, $n = 5$; C: Xenograft mice' average tumor weight, $n = 5$; D: Protein expression levels of RNA binding motif 5, phosphatase and tensin homolog (PTEN) and Ki67 in xenograft mice' tumor tissues were detected by immunohistochemical staining, $n = 5$; E: Protein levels of PTEN, phosphoinositide 3-kinase, phosphorylated-protein kinase B, and AKT were determined by Western blot; F: Tumor liver metastasis was assessed by haematoxylin and eosin staining, $n = 5$. $^{a}P < 0.05$,$^{b}P < 0.01$,$^{c}P < 0.001$. RBM5: RNA binding motif 5; PTEN: Phosphatase and tensin homolog; PI3K: Phosphoinositide 3-kinase; p-AKT: Phosphorylated-protein kinase B.

## FOOTNOTES

**Author contributions:** Wang CX and Wang Y designed the study; Wang CX, Liu F, and Wang Y carried out experiments; Liu F and Wang Y analyzed experimental results; Wang CX was a major contributor in writing the manuscript; and all authors reviewed and approved the manuscript.

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**Conflict-of-interest statement:** All authors have nothing to disclose.

**Data sharing statement:** The authors confirm that the data supporting the findings of this study are available within the article.

**ARRIVE guidelines statement:** The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.



**Country of origin:** China

**ORCID number:** Yi Wang 0009-0002-8535-2461.

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