

# World Journal of *Gastroenterology*

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**ABOUT COVER**

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## Retrospective Study

# RBBP4 promotes colon cancer malignant progression via regulating Wnt/ $\beta$ -catenin pathway

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**Author contributions:** Li YD designed, supervised the study and provided consultation during the entire study; Lv Z performed the research; Zhu WF analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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## Abstract

### BACKGROUND

Our previous study demonstrated that RBBP4 was upregulated in colon cancer and correlated with poor prognosis of colon cancer and hepatic metastasis. However, the potential biological function of RBBP4 in colon cancer is still unknown.

### AIM

To investigate the biological role and the potential mechanisms of RBBP4 in colon cancer progression.

### METHODS

Real-time polymerase chain reaction and western blot analysis were used to detect the expression of RBBP4 in colon cancer cell lines. The cell proliferation and viability of SW620 and HCT116 cells with RBBP4 knockdown was detected by Cell Counting Kit-8 and 5-ethynyl-2'-deoxyuridine staining. The transwell assay was used to detect the invasion and migration capabilities of colon cancer cells with RBBP4 knockdown. Flow cytometry apoptosis assay was used to detect the apoptosis of colon cancer cells. Western blotting analysis was used to detect the expression of epithelial-mesenchymal transition and apoptosis related markers in colon cancer. The nuclear translocation of  $\beta$ -catenin was examined by Western blotting analysis in colon cancer cells with RBBP4 knockdown. The TOPFlash luciferase assay was used to detect the effect of RBBP4 on Wnt/ $\beta$ -catenin activation. The rescue experiments were performed in colon cancer cells treated

Wfzhu@163.com. Participants gave informed consent for data sharing.

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with Wnt/ $\beta$ -catenin activator LiCl and RBBP4 knockdown.

## RESULTS

We found that RBBP4 was highly expressed in colon cancer cell lines. The 5-ethynyl-2'-deoxyuridine assay showed that knockdown of RBBP4 significantly inhibited cell proliferation. RBBP4 inhibition reduced cell invasion and migration *via* regulating proteins related to epithelial-mesenchymal transition. Knockdown of RBBP4 significantly inhibited survivin-mediated apoptosis. Mechanistically, the TOPFlash assay showed that RBBP4 knockdown increased activity of the Wnt/ $\beta$ -catenin pathway. Meanwhile, RBBP4 knockdown suppressed nuclear translocation of  $\beta$ -catenin. With Wnt/ $\beta$ -catenin activator, rescue experiments suggested that the role of RBBP4 in colon cancer progression was dependent on Wnt/ $\beta$ -catenin pathway.

## CONCLUSION

RBBP4 promotes colon cancer development *via* increasing activity of the Wnt/ $\beta$ -catenin pathway. RBBP4 may serve as a novel therapeutic target in colon cancer.

**Key Words:** Colon cancer; Wnt/ $\beta$ -catenin; RBBP4; Epithelial-mesenchymal transition; Apoptosis; Invasion

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**Core Tip:** Our previous study demonstrated upregulation of RBBP4 in colon cancer and correlation of poor prognosis with colon cancer and hepatic metastasis. This study explored the potential biological function of RBBP4 in colon cancer. We found that RBBP4 was highly expressed in colon cancer cell lines. Knockdown of RBBP4 significantly inhibited cell proliferation and survivin-mediated apoptosis and suppressed nuclear translocation of  $\beta$ -catenin. RBBP4 inhibition reduced cell invasion and migration *via* regulating proteins related to epithelial-mesenchymal transition. Mechanistically, RBBP4 knockdown increased activity of the Wnt/ $\beta$ -catenin pathway. RBBP4 may serve as a novel therapeutic target in colon cancer.

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## INTRODUCTION

Colon cancer is one of the most common malignancies in developed countries. There are more than one million new cases of colon cancer worldwide and 608000 deaths every year<sup>[1]</sup>. Although the treatment of colon cancer has made great progress, including surgery or radiotherapy and chemotherapy, the prognosis of patients with colon cancer has shown no marked progress in recent years<sup>[2]</sup>. The 5-year relative survival rate of patients with stage IV disease is slightly higher than 10%<sup>[3,4]</sup>. Although considerable efforts have been made in the past few years to clarify the mechanisms underlying the development and progression of colon cancer, it is still far from completely understood. Hence, it is necessary to explore the further mechanisms involved in the pathogenesis of colon cancer and to develop new therapeutic targets.

RBBP4 is a new, 48-kD tumor-specific protein found in HeLa cell lysates<sup>[5,6]</sup>. RBBP4 belongs to a highly conserved subfamily of nucleoproteins with four WD repeat sequences. RBBP4 binds to retinoblastoma protein *in vivo* and *in vitro*, hence the name<sup>[7]</sup>. The *RBBP4* gene encodes a protein that is part of several chromatin-modified protein complexes, such as nucleosome remodeling and deacetylation complex<sup>[8]</sup>, polycomb repressor complex 2<sup>[9]</sup>, and SIN3-chromatin modulating complexes<sup>[10]</sup>, which influence gene transcription, and regulates cell cycle and proliferation<sup>[11]</sup>. In recent decades, accumulated research has demonstrated that RBBP4 plays a key role in the pathogenesis of cancers, such as liver<sup>[12]</sup>, breast<sup>[13]</sup>, and gastric<sup>[14,15]</sup> cancers. In our previous study, we proved that RBBP4 is upregulated in colon cancer and may serve

as a novel predictor for poor prognosis of colon cancer and liver metastasis<sup>[16]</sup>. However, its potential role and mechanisms in colon cancer have not been reported.

Therefore, the present study aimed to explore the potential role of RBBP4 in colon cancer aggravation and the underlying molecular mechanisms. We detected expression of RBBP4 in colon cancer cell lines, then investigated the role of RBBP4 in colon cancer cell proliferation, migration, invasion, and apoptosis and finally explored the molecular mechanisms of RBBP4 in colon cancer malignancy characteristics. This study clarifies the role of RBBP4 in colon cancer development through its effect on the Wnt/ $\beta$ -catenin signaling pathway.

## MATERIALS AND METHODS

### Cell culture

A normal human colon cell line (NCM640) and colon cancer cell lines (SW620, HT29, LoVo, SW480, and HCT-116) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cell lines originated from the American Type Culture Collection (ATCC, Manassas, VA, United States). All the cell lines were cultured in the corresponding medium according to the suggestion of ATCC with 10% fetal bovine serum (FBS). All the cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. The Wnt/ $\beta$ -catenin activator LiCl was obtained from Sigma-Aldrich (Munich, Germany).

### Plasmids, siRNA, and transfection

The plasmids of the human *RBBP4* gene, siRNA targeting human *RBBP4*, and their controls were synthesized by Genechem (Shanghai, China). The cells were transfected with siRNAs, plasmids, or their controls using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States). In total,  $2 \times 10^5$ – $3 \times 10^5$  cells were transfected with 100 pmol siRNA or 2  $\mu$ g plasmid DNA. Western blotting was used to detect the transfection efficiency 24 h and 48 h after transfection, and real-time polymerase chain reaction was used for verification.

### Real time polymerase chain reaction detection

Total RNA from colon cancer tissues and cells was extracted using TRIzol reagent (Invitrogen). Total RNA (0.5  $\mu$ g) was reversed transcribed to cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). SYBR Green Polymerase Chain Reaction Master mix (Takara) was used to determine the mRNA level of *RBBP4* on an ABI 7900Fast Real-time Detection System (Applied Biosystems, Carlsbad, CA, United States) in 20  $\mu$ L reaction system. All reactions were performed in triplicate. The relative expression of *RBBP4* was normalized to the internal reference *GAPDH*. The 2<sup>-DDCT</sup> method was used to analyze the data. The primers used in the study were as follows: *GAPDH* forward primer: 5'-ATG GGG AAG GTG AAG GTC G-3', *GAPDH* reverse primer: 5'-GGG GTC ATT GAT GGC AAC AAT A-3'; *RBBP4* forward primer: 5'-GCT ATG GGC TTT CTT GGA-3', and *RBBP4* reverse primer: 5'-CAC AGG CAG ATG GTA TGG-3'.

### Cell Counting Kit-8 assay

Cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). Cells were seeded in 96-well plates at  $3 \times 10^3$  cells per well with 200  $\mu$ L culture medium. With supernatant removed, 10  $\mu$ L CCK-8 reagent in 100  $\mu$ L medium was added to each well at 0 h, 24 h, 48 h, 72 h, and 96 h. The plates were incubated in the dark at 37 °C for 2 h, and absorbance at 450 nm was detected with a microplate reader (BioTek, Winooski, VT, United States). The experiments were performed in triplicate.

### 5-Ethynyl-2'-deoxyuridine cell proliferation assay

5-Ethynyl-2'-deoxyuridine (EdU) assay was performed using Click-iT™ EdU Imaging Kit with Alexa Fluor™ 488 Azides (Invitrogen). Briefly,  $1 \times 10^5$  cells were plated in six-well plates and incubated at room temperature overnight. The cells were incubated with 10  $\mu$ M EdU for 1 h at 37 °C and fixed in 3.7% paraformaldehyde. After permeabilization with 0.5% Triton X-100 in phosphate buffered saline for 20 min, the cells were reacted with 1  $\times$  Click-iT® reaction cocktail for 20 min. The nuclei were labeled with Hoechst 33342 for 30 min and photographed under a fluorescence microscope. All studies were conducted in triplicate.

### Western blotting

Total proteins were extracted using the ice cold radioimmunoprecipitation assay buffer with cocktail protease and phosphatase inhibitors (Cell Signaling Technology, Danvers, MA, United States). NE-PER™ Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific, Waltham, MA, United States) was used to extract the nuclear and cytoplasmic proteins. Proteins were quantified using a BCA protein assay kit (Thermo Scientific). Then equivalent proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, United States). The membranes were probed with antibodies against RBBP4 (ab92344), survivin (ab134170), GAPDH (ab181620),  $\beta$ -catenin (ab32572), pro-caspase-3 (ab32150) (Abcam, Cambridge, MA, United States), Cleaved caspase-3 (9661), E-cadherin (14472), N-cadherin (13116), vimentin (5741), and histone H3 (14269) (Cell Signaling Technology) overnight at 4 °C and then incubated with horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The bands were visualized by ECL kit (Millipore).

### Migration and invasion assay

The migration and invasion assays were performed by the transwell method. For the invasion assay, the cells were plated on Matrigel-coated upper chambers (24-well inserts; pore size, 8  $\mu$ m; BD Biosciences, San Jose, CA, United States). For the migration assay, the cells were plated on uncoated upper chambers. In the lower wells, medium was replaced with fresh medium with 5% FBS. The cells were incubated for 24 h in medium containing 1% FBS, trypsinized, and suspended in medium containing 1% FBS at a final concentration of  $1 \times 10^6$  cells/mL. Then, 200  $\mu$ L cell suspension was placed in each of the upper wells, and the chamber was incubated at 37 °C for 24 h. Cells were fixed and stained with hematoxylin and eosin. The nonmigrating cells from the upper surface of the filter were wiped with a cotton swab. The cells that migrated to the lower side of the filter were counted and photographed with an optical inverted microscope. Five random fields in each assay were counted and averaged.

### Flow cytometry analysis of apoptosis

For the assessment of apoptosis, an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences) was used. Colon cancer cells were collected in six-well plates at  $1 \times 10^6$  cells/mL. After transfection for 48 h, the cells were trypsinized and washed once with phosphate buffered saline. After centrifugation at 1000 r/min for 5 min, the cells were stained with 5  $\mu$ L Annexin V-FITC and PI in the dark condition for 30 min, and then were analyzed by flow cytometry (BD Biosciences). At least 10000 events were recorded for each sample. The apoptosis data were analyzed by FlowJo V10 software (Tree Star, San Francisco, CA, United States).

### Luciferase assays

The TOPFlash assay was performed using the T-cell factor Reporter Plasmid Kit (Millipore). The ratio of luciferase activities from a T-cell factor-responsive reporter (pTOPFlash) *vs* a control luciferase reporter gene construct (pFOPFlash) was determined 48 h after transfection with Lipofectamine 2000 in SW480 and HCT116 cells. Luciferase activities were normalized for transfection efficiency by cotransfection with a  $\beta$ -galactosidase-expressing vector. The cells were transfected with siRBBP4 plasmid or RBBP4 plasmid or control plasmids. The cells were harvested after 24 h and processed for luciferase and  $\beta$ -galactosidase activities, and the data were normalized to  $\beta$ -galactosidase levels.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, United States) software and verified by SPSS version 20.0 (SPSS, Chicago, IL, United States). Each experiment was performed at least in triplicate, and the results were expressed as the mean  $\pm$  standard deviation. Student's *t* test and one-way analysis of variance were conducted to analyze the differences between groups, and a *P* < 0.05 was considered statistically significant.

## RESULTS

### RBBP4 is upregulated in human colon cancer cell lines

In our previous study, we detected the expression pattern of RBBP4 in colon cancer



tissues and proved that RBBP4 was upregulated. In the present study, we examined the protein and mRNA levels of RBBP4 in five human colon cancer cell lines and a normal human colon cell line. mRNA and protein levels of RBBP4 were significantly higher in colon cancer cell lines compared with the normal human colon cell line. We selected SW480 and HCT116 cells with high RBBP4 levels for subsequent experiments (Figure 1A and 1B).

### ***RBBP4 knockdown inhibited cell growth in vitro***

To investigate the biological function of RBBP4 in colon cancer cells, we knocked down RBBP4 *via* siRNA-mediated gene silencing. The knockdown efficiency was determined by Western blotting (Figure 1C). We then examined the role of RBBP4 in colon cancer cell viability using the CCK-8 assay. Cell viability was decreased in both HCT116 and SW620 cells after RBBP4 knockdown (Figure 1D and 1E). EdU proliferation assay showed that RBBP4 knockdown significantly reduced proliferation of colon cancer cells (Figure 1F and 1G). These results showed that RBBP4 played an essential role in the growth of colon cancer cells.

### ***RBBP4 knockdown receded migration and invasion of colon cancer cells***

We examined the effect of RBBP4 knockdown on colon cancer cell migration and invasion *in vitro* using the transwell assay. The number of migrated and invasive cells in RBBP4 knockdown HCT116 and SW620 colon cancer cells was less than that in the control group (Figure 2A and 2B). The epithelial-mesenchymal transition (EMT) pathway has been proved to play a key role in tumor migration and invasion<sup>[17]</sup>. To investigate the molecular mechanisms of RBBP4 in regulating colon cancer cell migration and invasion, we performed Western blotting to detect expression of EMT-related proteins including N-cadherin, E-cadherin, and vimentin. RBBP4 knockdown markedly decreased mesenchymal proteins, but upregulated epidermal protein expression (Figure 2C). These results indicated that RBBP4 regulated colon cancer cell migration and invasion *via* the EMT pathway.

### ***RBBP4 knockdown aggravated apoptosis of colon cancer cells***

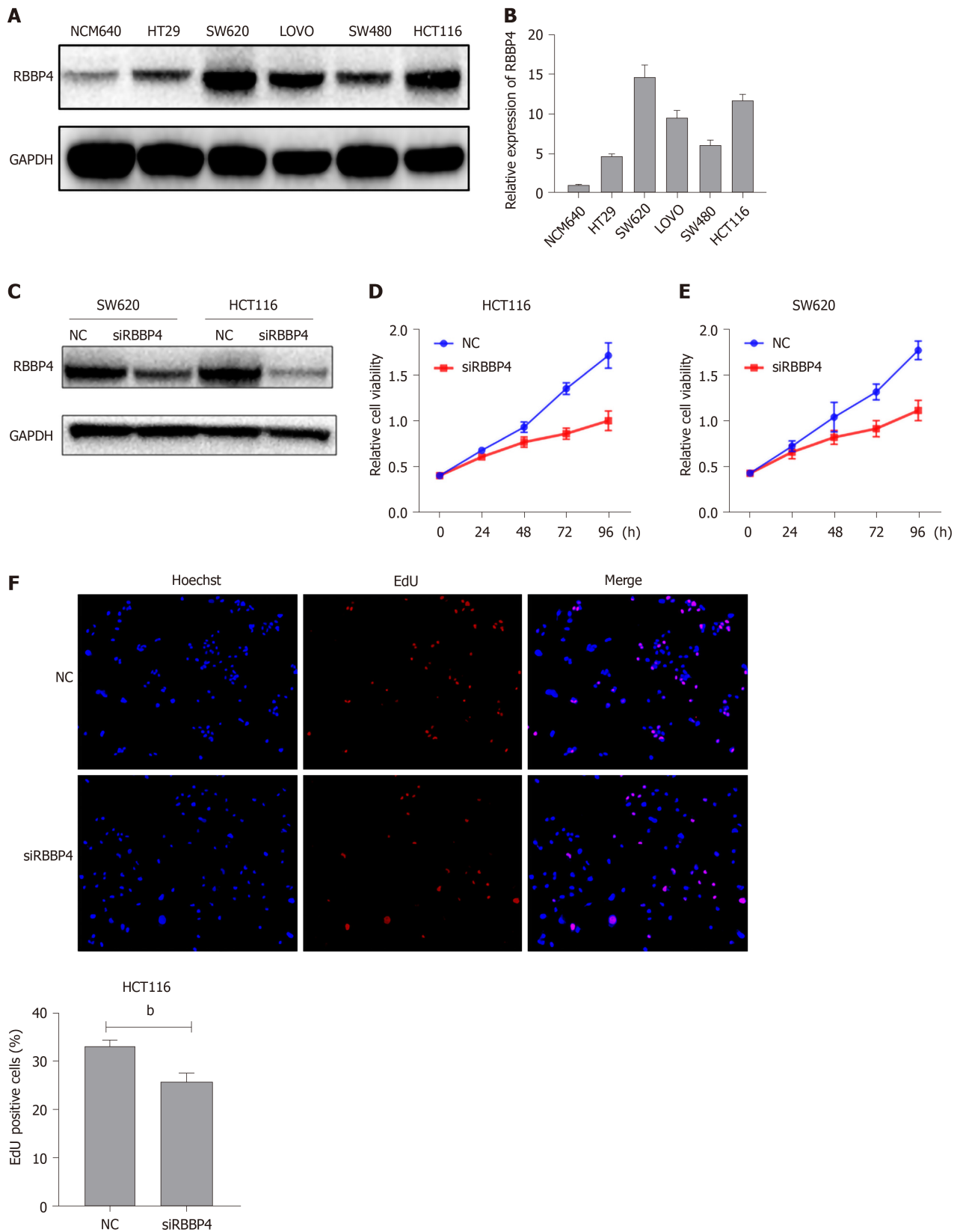
To investigate the mechanism underlying RBBP4-knockdown-induced antiproliferation, we detected the apoptotic rate of colon cancer cells by flow cytometry. Apoptosis rate in HCT116 and SW620 cells with RBBP4 knockdown increased by  $42.0\% \pm 2.2\%$  and  $33.3\% \pm 2.91\%$ , respectively, compared with the control cells ( $12.95\% \pm 1.40\%$  and  $10.83\% \pm 0.93\%$ ,  $P < 0.01$ , Figure 3A and 3B). To investigate the molecular mechanisms of RBBP4 in regulating colon cancer cell apoptosis, we detected apoptosis-related proteins, and showed that survivin, an antiapoptotic protein, was downregulated after RBBP4 knockdown, thus leading to the increase of pro-caspase-3 and cleaved caspase-3.

### ***RBBP4 knockdown suppressed activity of the Wnt/ $\beta$ -catenin pathway in colon cancer cells***

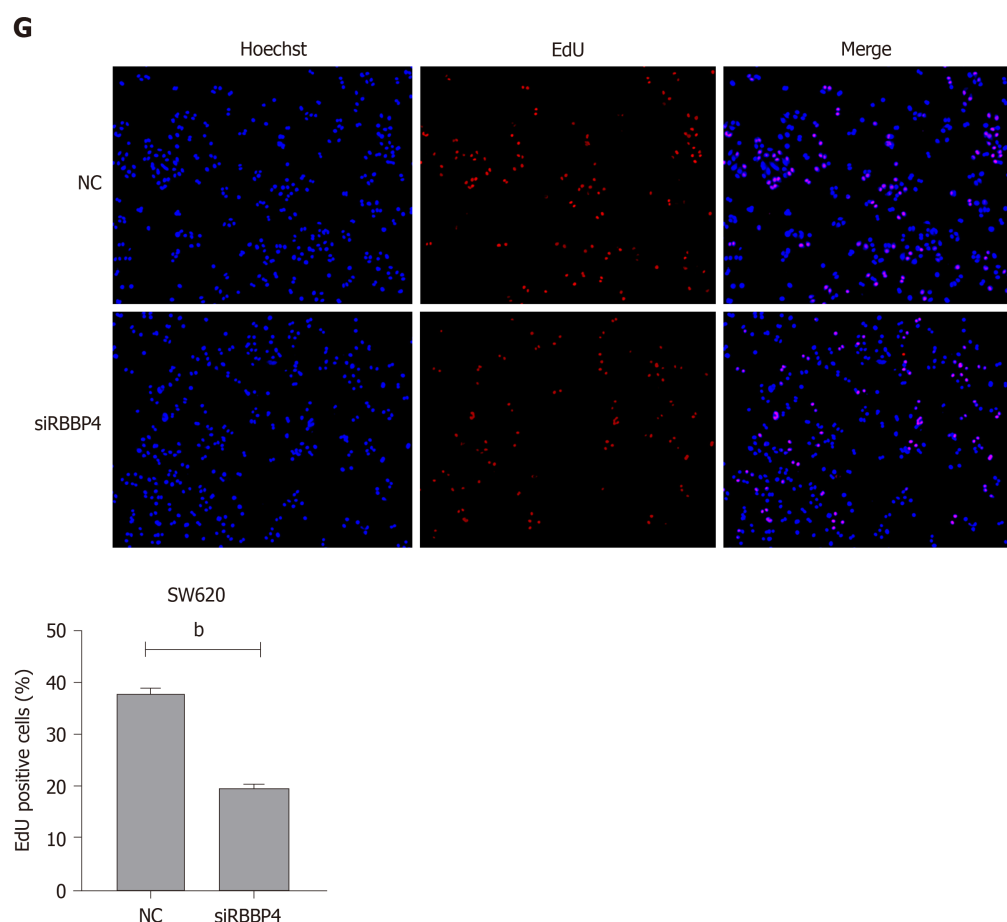
The Wnt/ $\beta$ -catenin pathway is one of the important signaling pathways inducing EMT, and survivin is a known downstream target of the pathway<sup>[17-19]</sup>. We hypothesized that the biological role of RBBP4 was executed through the Wnt/ $\beta$ -catenin pathway. To clarify this hypothesis, the TOP/FOP flash luciferase reporter assays were used. Compared with the control cells, overexpression of RBBP4 led to an increase of TOP flash luciferase reporter activity in HCT116 and SW620 cells (Figure 4A and 4B). However, RBBP4 knockdown inhibited the activity of the TOP flash luciferase reporter (Figure 4A and 4B). As reported previously,  $\beta$ -catenin nuclear translocation is an essential event for Wnt/ $\beta$ -catenin pathway activation. To elucidate further the underlying mechanism, we examined the influence of RBBP4 on  $\beta$ -catenin nuclear translocation in colon cancer cells. The level of  $\beta$ -catenin in the nucleus was decreased, while that in cytoplasm was increased by RBBP4 knockdown (Figure 4C). All these data indicated that activity of the Wnt/ $\beta$ -catenin pathway was regulated by RBBP4 *via* regulation of the nuclear translocation of the  $\beta$ -catenin protein in colon cancer cells.

### ***Function of RBBP4 in colon cancer cells was mediated by the Wnt/ $\beta$ -catenin pathway***

To examine whether the function of RBBP4 in colon cancer was mediated by the Wnt/ $\beta$ -catenin pathway, we used the Wnt/ $\beta$ -catenin pathway activator LiCl in a rescue experiment. The role of RBBP4 knockdown on the inhibition of  $\beta$ -catenin







**Figure 1 Expression of RBBP4 in colon cancer cell lines and its effect on cell proliferation.** A: Protein level of RBBP4 in the colon cancer cell lines quantified by western blotting; B: mRNA level of *RBBP4* in the colon cancer cell lines quantified by polymerase chain reaction; C: RBBP4 siRNA efficiency verified by western blotting in SW620 and HCT116 cells; D: Cell viability was detected by the Cell Counting Kit-8 assay in HCT116 cells; E: Cell viability was detected by the Cell Counting Kit-8 assay in SW620 cells; F and G: Cell proliferation was detected by 5-ethynyl-2'-deoxyuridine assay in HCT116 cells and SW620 cells. <sup>a</sup> $P < 0.01$  vs controls. EdU: 5-Ethynyl-2'-deoxyuridine.

nuclear translocation was partly reversed by LiCl (Figure 5A). CCK-8 assays showed that the viability of HCT116 and SW620 cells treated with RBBP4 siRNA was significantly enhanced when they were cotreated with LiCl compared with untreated cells ( $P < 0.05$ , Figure 5B and 5C). The transwell assay showed that the inhibition of invasion by RBBP4 knockdown was partly reversed by LiCl ( $P < 0.05$ , Figure 5C and 5D). The expression of EMT-related proteins was also partly reversed by LiCl compared with the shRBBP4 group (Figure 5E). All these results suggested that the role of RBBP4 in colon cancer progression is mediated by the Wnt/ $\beta$ -catenin pathway.

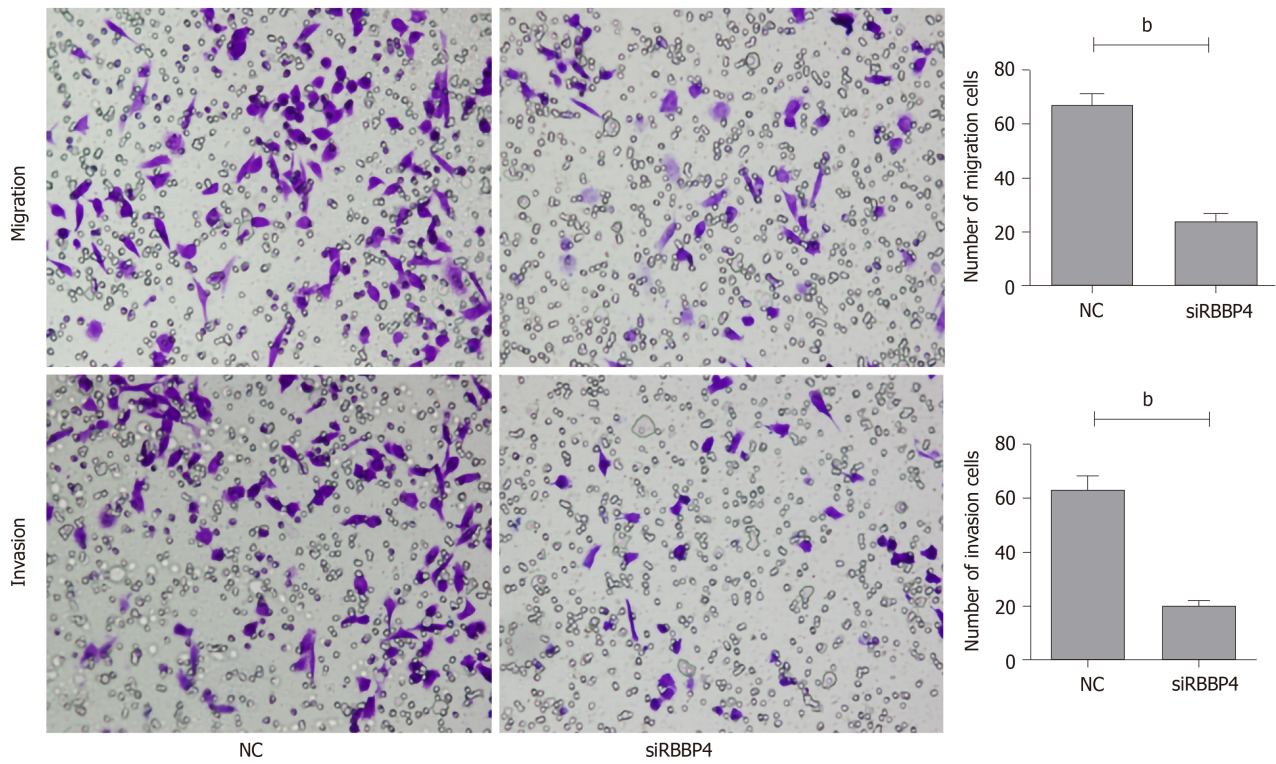
## DISCUSSION

RBBP4, also known as RbAp48, is named for its ability to bind to retinoblastoma proteins *in vivo* and *in vitro*<sup>[20]</sup>. Previous studies showed that the RBBP4 protein is a component of a variety of complexes involved in chromatin assembly, remodeling, and nucleosome modification, such as SIN3<sup>[21]</sup>, polycomb repressor complex 2<sup>[22,23]</sup>, histone acetyltransferase 1<sup>[24]</sup>, and chromatin assembly factor 1<sup>[25]</sup> and plays a different role in each complex. It has been demonstrated that RBBP4 expression is upregulated and correlated with the malignant phenotypes in many types of human tumors, such as lung cancer<sup>[26]</sup>, liver cancer<sup>[27]</sup>, thyroid carcinoma<sup>[28]</sup>, and acute lymphoblastic leukemia<sup>[29]</sup>. However, little is known about RBBP4 in colon cancer.

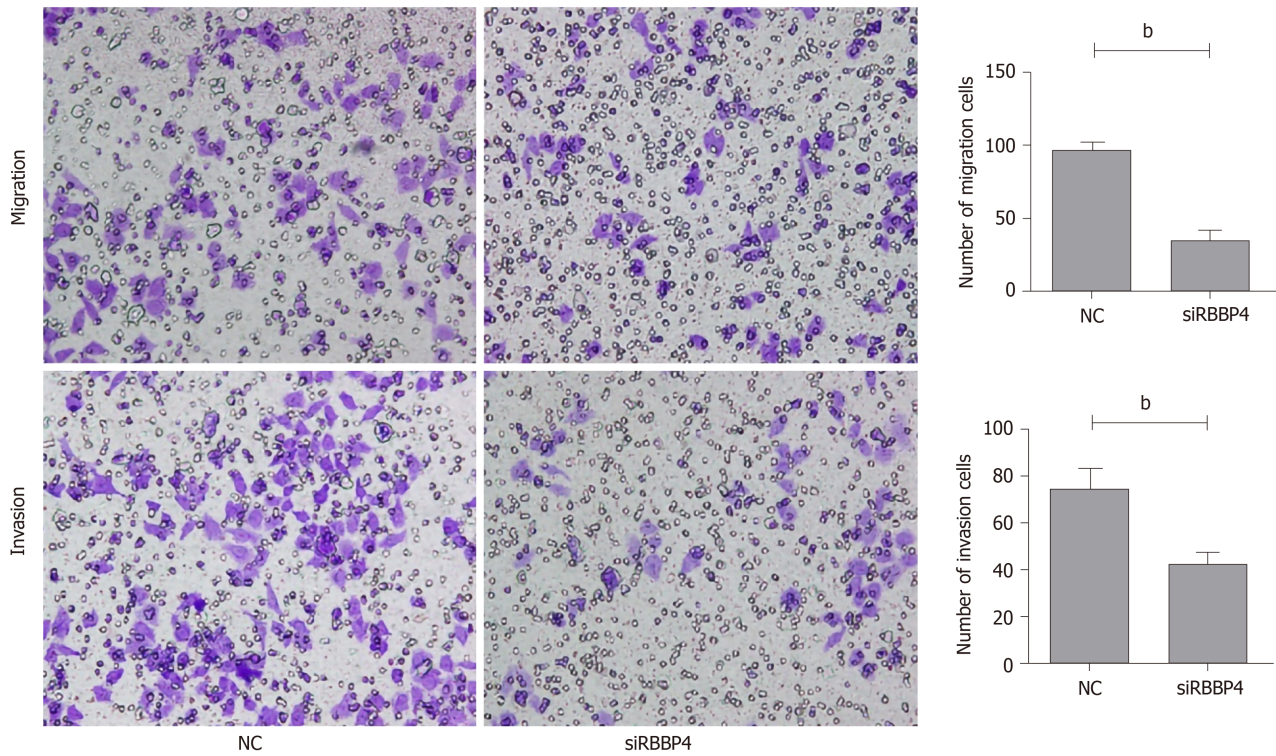
In our previous study, we demonstrated that RBBP4 was upregulated in colon cancer tissues, and elevated RBBP4 level was correlated with poor prognosis and liver metastasis. Nevertheless, the detailed molecular biological function of RBBP4 and the potential mechanisms in colon cancer are unclear. In the present study, our evidence indicated that RBBP4 knockdown decreased the proliferation, apoptosis, and

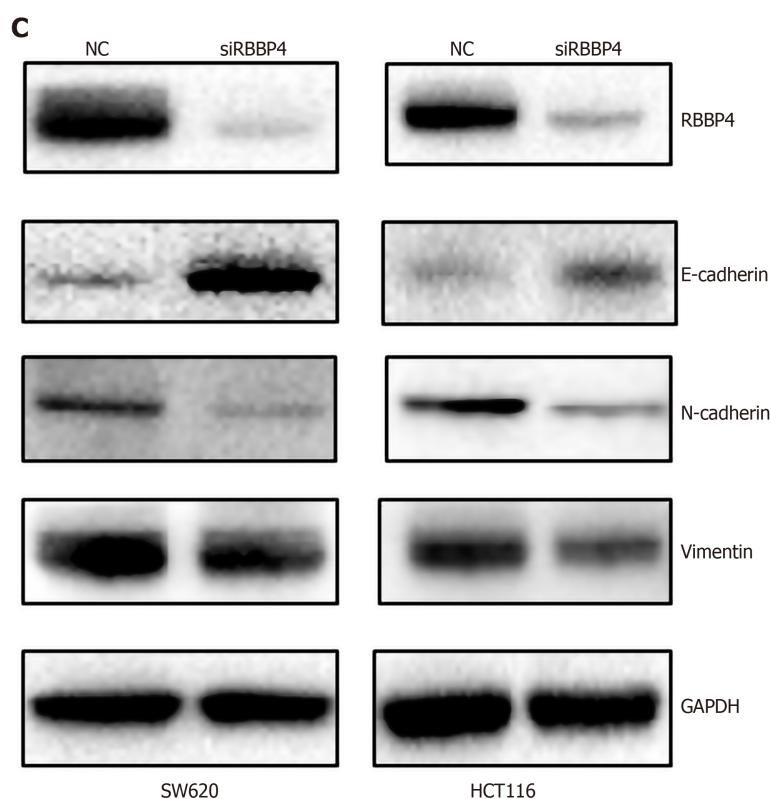
**A**

SW620


**B**

HCT116





**Figure 2 RBBP4 knockdown inhibits migration and invasion of colon cancer cell lines.** A: Migration and invasion of RBBP4 knockdown SW620 cells were measured by the transwell assay. Results were quantitated by counting migrating and invasive cells in five randomly chosen high-power fields for each replicate; B: Migration and invasion of RBBP4 knockdown HCT116 cells were measured by the transwell assay; C: Western blotting examination for epithelial-mesenchymal transition related proteins. <sup>b</sup>*P* < 0.01 vs controls.

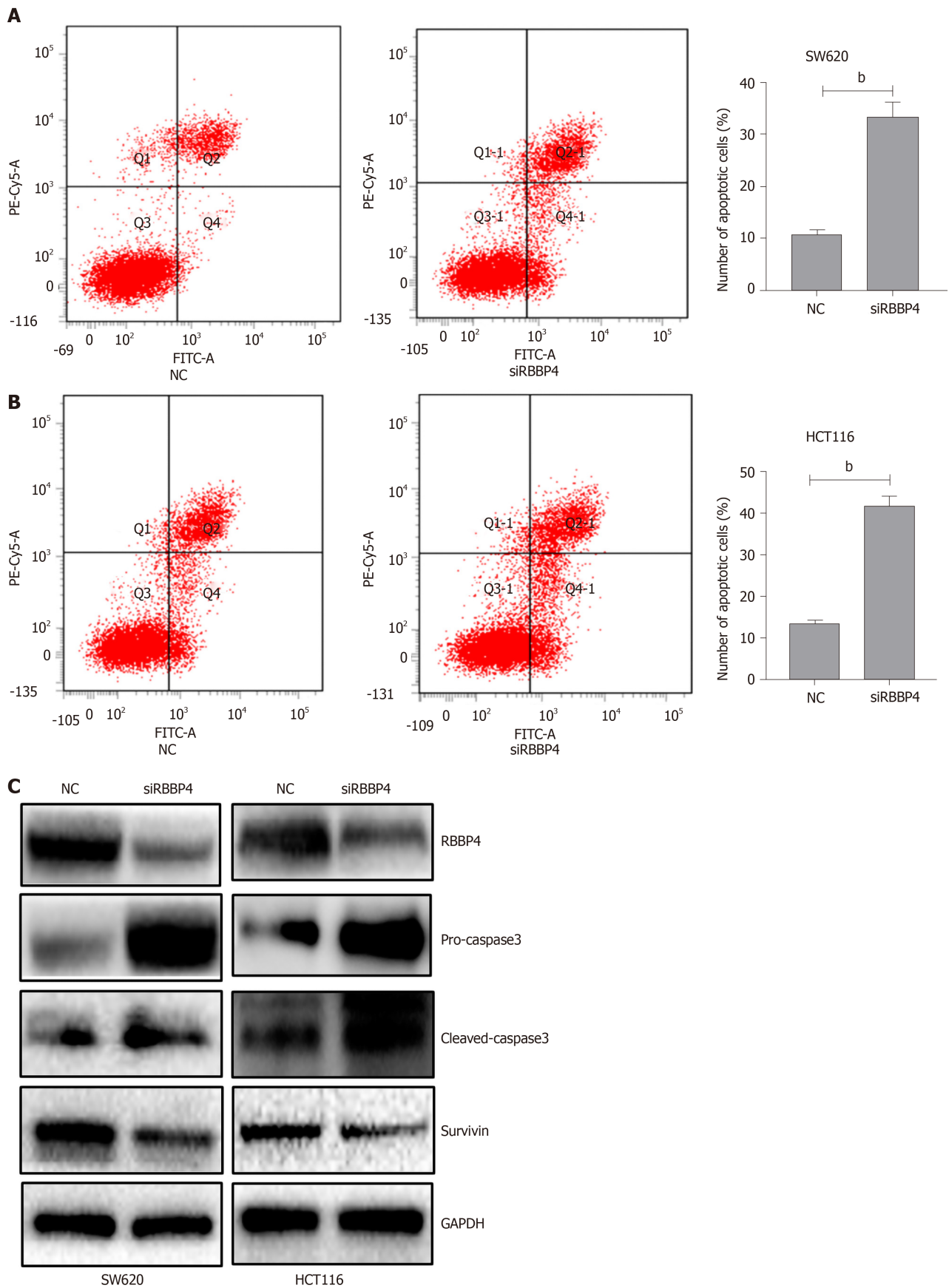
aggressiveness of colon cancer cells, suggesting its oncogenic functions in colon cancer progression. RBBP4 promoted the nuclear accumulation of  $\beta$ -catenin, thus activating the Wnt/ $\beta$ -catenin signaling pathway. The role of RBBP4 in colon cancer progression was partially dependent on Wnt/ $\beta$ -catenin signaling pathway.

Abnormalities in the Wnt signaling pathway are associated with a variety of tumor types, including colon cancer<sup>[30,31]</sup>. The Wnt pathway is classified into canonical and noncanonical pathways; the former of which is  $\beta$ -catenin dependent<sup>[32]</sup>. For the canonical pathway, in the absence of Wnt ligands, free cytoplasmic  $\beta$ -catenin binds to cytoplasmic complexes containing *Adenomatous Polyposis Coli*, axin, casein kinase 1a, and glycogen synthase kinase 3b, which promotes the phosphorylation of  $\beta$ -catenin leading to  $\beta$ -catenin ubiquitination and subsequent proteasomal degradation. The interaction between Wnt and Frizzled leads to the activation of the Dishevelled family proteins. The activated Dishevelled proteins cause the inhibition of glycogen synthase kinase 3b, resulting in the accumulation of free cytoplasmic  $\beta$ -catenin, which is then transported to the nucleus. In the nucleus,  $\beta$ -catenin binds to various transcription factors, such as T-cell factor and lymphoid enhancer factor 1, to activate Wnt target genes<sup>[33-35]</sup>. However, the relationship between RBBP4 and the Wnt pathway is poorly understood. In our study, for the first time we proved that RBBP4 could enhance the nuclear translocation of  $\beta$ -catenin and activate the Wnt/ $\beta$ -catenin pathway in colon cancer.

Studies over the past decade have shown that cells that harbor functionally impaired mutations of Wnt signaling cascades, such as *Adenomatous Polyposis Coli*,  $\beta$ -catenin, and axin, are thought to be prevalent in colon cancer<sup>[36]</sup>. The mutations lead to abnormal transcriptional induction of Wnt/ $\beta$ -catenin downstream genes<sup>[37]</sup>. Many target genes of Wnt/ $\beta$ -catenin have been identified, such as survivin<sup>[38]</sup>. Survivin was recently identified as an inhibitor of apoptosis that directly inhibits caspase-3 and caspase-7 activity<sup>[39]</sup>. The role of survivin in colorectal tumorigenesis has been shown. We found that RBBP4 knockdown inhibited the level of survivin, thus inducing apoptosis of colon cancer cells. This result proved that RBBP4 regulates the Wnt/ $\beta$ -catenin pathway.

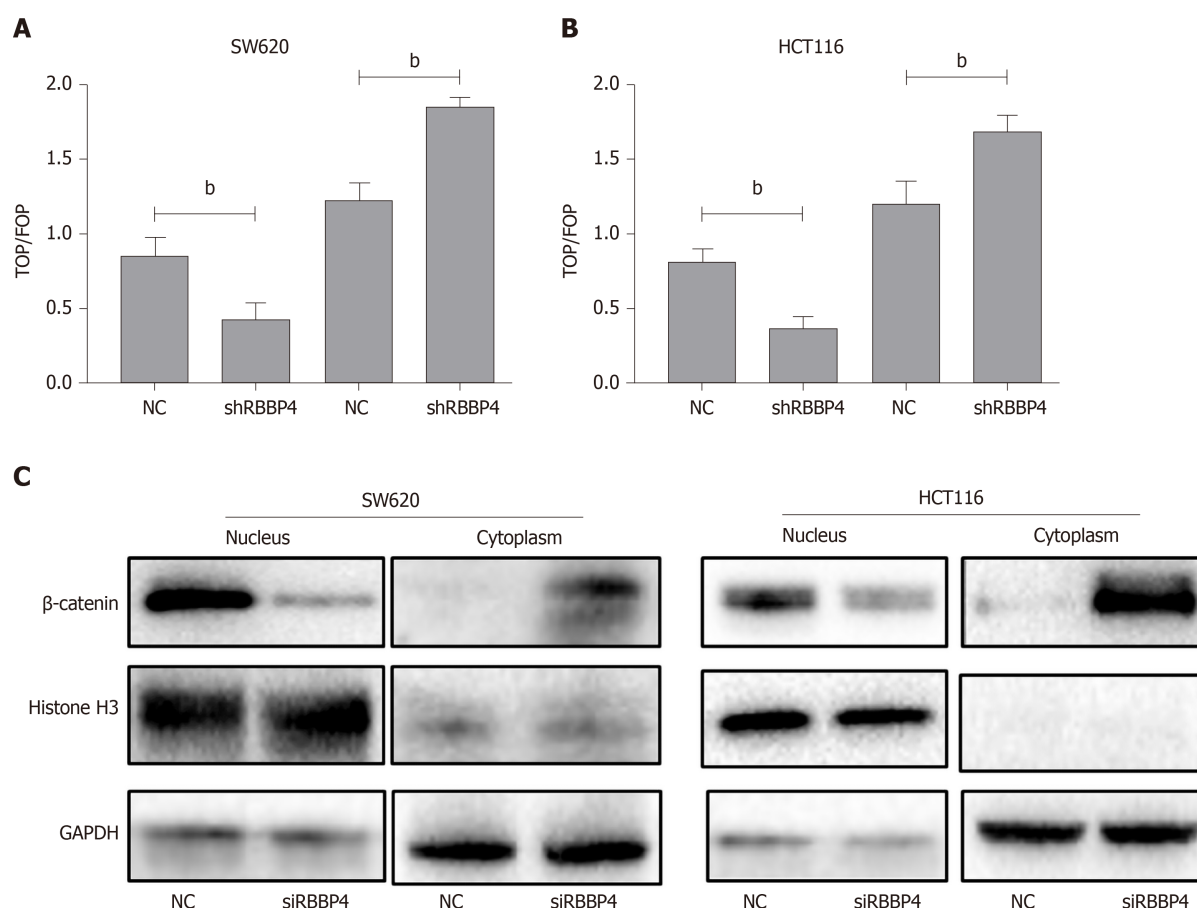
Liver metastasis is an important characteristic of colon cancer, and EMT plays a central role<sup>[40]</sup>. A previous study showed that activation of Wnt/ $\beta$ -catenin signaling





**Figure 3 RBBP4 knockdown inhibits apoptosis of colon cancer cell lines.** A: Apoptosis of SW620 cells examined by flow cytometry with the Annexin V-FITC/propidium iodide kit. The cells in the Q4 quadrant were defined as the apoptotic cells; B: Apoptosis of HCT116 cells examined by flow cytometry with the Annexin V-FITC/propidium iodide kit; C: Western blotting examination for apoptotic proteins. <sup>b</sup>*P* < 0.01 vs controls.

results in expression of target genes that lead to the dedifferentiated phenotype and

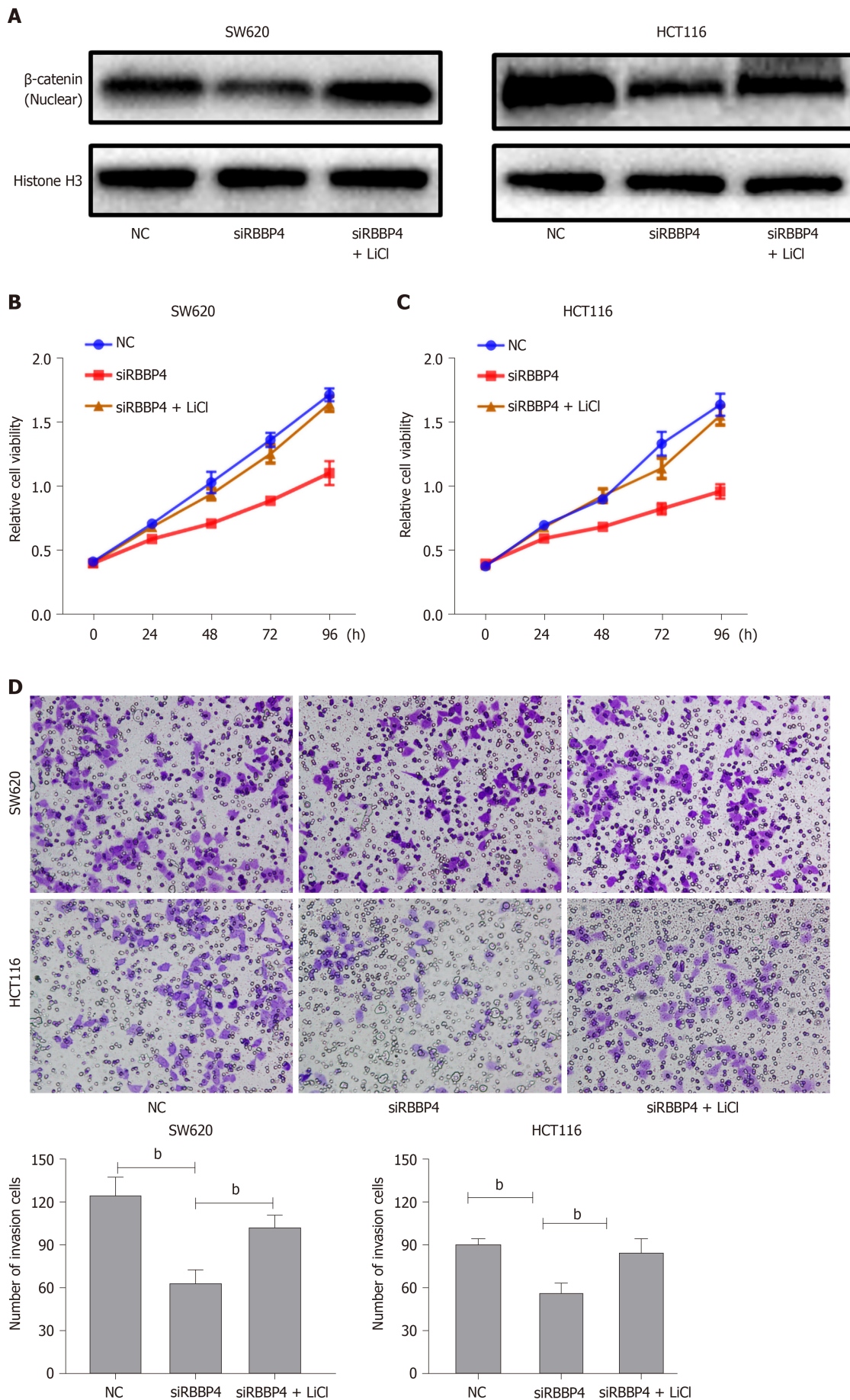


**Figure 4 Effect of RBBP4 on activation of Wnt/β-catenin pathway.** A: The TOPFlash experiment in SW620 cells with RBBP4 knockdown or RBBP4 overexpression; B: The TOPFlash experiment in HCT116 cells with RBBP4 knockdown or RBBP4 overexpression; C: Western blotting of the level of β-catenin in cell nucleus and cytoplasm. <sup>b</sup>*P* < 0.01 vs controls.

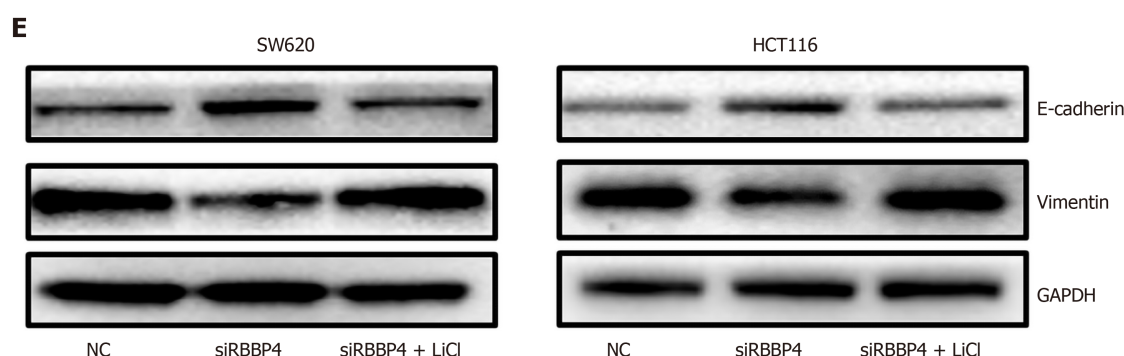
EMT of colon cancer cells<sup>[41]</sup>. The nuclear translocation of β-catenin was reported to induce Slug and inhibit E-cadherin transcription in colon cancer<sup>[42]</sup>. However, the relationship between RBBP4 and EMT has not been clarified. In our study, we found that RBBP4 knockdown markedly decreased mesenchymal proteins but upregulated expression of epidermal proteins, indicating inhibition of the EMT pathway. This process may be mediated by the Wnt/β-catenin pathway.

## CONCLUSION

In conclusion, the results presented in this study demonstrated that RBBP4 plays an important role in the malignant progression of colon cancer. This is probably induced *via* inhibiting Wnt/β-catenin pathway activity and relocating β-catenin from the nucleus to the plasma membrane. Further investigation of the functional mechanism of RBBP4 as a tumor oncogene may provide a potential therapeutic strategy for intervention of colon cancer progression.







**Figure 5 Rescue experiments of RBBP4.** A: SW620 and HCT116 cells with RBBP4 siRNA or negative control transfection were incubated with or without 20 mmol/L LiCl, then the level of  $\beta$ -catenin in the nucleus was detected by western blotting; B: The viability of SW620 cells with RBBP4 siRNA or negative control transfection cultured in medium with or without 20 mmol/L LiCl; C: The viability of HCT116 cells with RBBP4 siRNA or negative control transfection cultured in medium with or without 20 mmol/L LiCl; D: Invasion of SW620 and HCT116 cells with RBBP4 siRNA or negative control transfection cultured in medium with or without 20 mmol/L LiCl; E: The protein expression of E-cadherin and vimentin in SW620 and HCT116 cells with RBBP4 siRNA or negative control transfection cultured in medium with or without 20 mmol/L LiCl.  $^bP < 0.01$  vs controls.

## ARTICLE HIGHLIGHTS

### Research background

Our previous study demonstrated that RBBP4 is upregulated in colon cancer and correlated with poor prognosis of colon cancer and hepatic metastasis. However, the potential biological function of RBBP4 in colon cancer is still unknown.

### Research motivation

To explore the potential mechanisms underlying colon cancer development and discover biomarkers for the treatment of colon cancer.

### Research objectives

To investigate the underlying mechanisms of RBBP4 in colon cancer malignant development.

### Research methods

Real-time polymerase chain reaction and western blot analysis were used to detect the expression of RBBP4 in colon cancer cell lines. The cell proliferation and viability of SW620 and HCT116 cells with RBBP4 knockdown was detected by Cell Counting Kit-8 and 5-ethynyl-2'-deoxyuridine staining. The transwell assay was used to detect the invasion and migration capabilities of colon cancer cells with RBBP4 knockdown. Flow cytometry apoptosis assay was used to detect the apoptosis of colon cancer cells with RBBP4 knockdown. Western blot analysis was used to detect the expression of epithelial-mesenchymal transition and apoptosis related markers in colon cancer with RBBP4 knockdown. The nuclear translocation of  $\beta$ -catenin was examined by western blot analysis in colon cancer cells with RBBP4 knockdown. The TOPFlash luciferase assay was used to detect effect of RBBP4 on Wnt/ $\beta$ -catenin activation. The rescue experiments were performed in colon cancer cells treated with Wnt/ $\beta$ -catenin activator LiCl and RBBP4 knockdown.

### Research results

We found that RBBP4 was highly expressed in colon cancer cell lines. The 5-ethynyl-2'-deoxyuridine assay showed that knockdown of RBBP4 significantly inhibited cell proliferation. RBBP4 inhibition reduced cell invasion and migration *via* regulating proteins related to epithelial-mesenchymal transition. Knockdown of RBBP4 significantly inhibited surviving-mediated apoptosis. Mechanistically, the TOPFlash assay showed that RBBP4 knockdown increased activity of the Wnt/ $\beta$ -catenin pathway. RBBP4 knockdown suppressed nuclear translocation of  $\beta$ -catenin. With a Wnt/ $\beta$ -catenin activator, rescue experiments suggested that the role of RBBP4 in colon cancer progression was dependent on the Wnt/ $\beta$ -catenin pathway.

### Research conclusions

This study demonstrated that RBBP4 promoted colon cancer development *via*

increasing activity of the Wnt/ $\beta$ -catenin pathway. RBBP4 may serve as a novel therapeutic target in colon cancer.

### Research perspectives

In the future, additional research will be carried out to further explore the important role of RBBP4 and whether RBBP4 knockdown can be employed to enhance the sensitivity of chemotherapy of colon cancer and to develop novel anticancer treatments.

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