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MicroRNA-300 inhibits the growth of hepatocellular carcinoma cells by downregulating CREPT/Wnt/β-catenin signaling

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Abstract. A number of studies have demonstrated that altered expression levels of microRNA-300 (miR-300) are associated with tumor progression; however, little is understood regarding the role of miR-300 in hepatocellular carcinoma (HCC). The present study aimed to investigate the expression, biological function and potential regulatory mechanism of miR-300 in HCC. A miR-300 mimic and miR-300 inhibitor were transfected into liver cancer cells using RNAiMAX reagent. The expression levels of miR and mRNA were detected by reverse transcription-quantitative polymerase chain reaction. Protein expression levels were detected by western blot analysis. Cell growth was determined using Cell Counting Kit-8, a colony formation assay and cell cycle assay. miRNA targeting sites were analyzed using bioinformatics analysis and dual-luciferase reporter assay. The results revealed that miR-300 expression was significantly decreased in HCC tissues and cell lines. *In vitro* experiments demonstrated that overexpression of miR-300 could inhibit cell proliferation, colony formation and cell cycle progression of liver cancer cells. By contrast, inhibition of miR-300 was associated with increased rates of cell proliferation, colony formation and cell cycle progression. Notably, regulation of nuclear pre-mRNA domain-containing protein 1B (CREPT) was identified as a putative target gene of miR-300 by bioinformatics analysis. A luciferase reporter assay revealed that miR-300 directly targets the 3'-untranslated region of CREPT. Further data demonstrated that miR-300 can regulate CREPT expression levels in liver cancer cells. Notably, miR-300 was identified to regulate the Wnt/β-catenin signaling pathway in liver cancer cells. The restoration of CREPT expression partially reversed the antitumor effect of miR-300. In conclusion, the current results revealed a tumor suppressive role of miR-300 in HCC and indicated that the underlying mechanism was associated with a regulation of CREPT. The present study suggests that miR-300 and CREPT may serve as potential therapeutic targets for liver cancer.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is emerging as the second leading cause of cancer-associated mortality worldwide (1). Numerous factors, including inherited genetic factors, chronic viral infection, non-alcoholic fatty liver disease, tobacco consumption and chronic alcohol abuse, contribute to the development and progression of HCC (2,3). However, to the best of our knowledge, the precise molecular mechanisms that induce hepatocarcinogenesis remain unknown. Despite improvements in therapeutic strategies, including surgical resection, radiotherapy, chemotherapy, adjunctive therapy and liver transplantation, the survival rate of HCC remains low due to high rates of recurrence and metastasis (4-6). Therefore, there is an urgent requirement to understand the detailed molecular mechanisms that underlie hepatocarcinogenesis and identify novel targets for the development of HCC treatments.

MicroRNAs (miRNAs/miRs) are a group of endogenous, small, non-coding and single-stranded RNAs, consisting of ~22 nucleotides, which have emerged as novel regulators of gene expression (7). miRNAs modulate gene expression by binding to complementary sequences within the 3'-untranslated region (3'-UTR) of mRNAs, which leads to mRNA degradation or translational repression (8). miRNAs are involved in the development of disease by regulating cell proliferation, apoptosis and differentiation (9). Altered expression levels of miRNAs have been identified in numerous types of cancer and these dysregulated miRNAs can contribute to carcinogenesis by functioning as oncogenes or tumor suppressors (10,11). A number of studies have suggested that various miRNAs are involved in the progression of HCC and are potential therapeutic targets and prognostic biomarkers (12-15). However,

to the best of our knowledge, the precise role of miRNAs in HCC remains largely unknown. Therefore, miRNA-mediated molecular mechanisms that affect HCC development and progression should be further investigated.

Cell-cycle-related and expression-elevated protein in tumor (CREPT), also termed the regulation of nuclear pre-mRNA domain containing 1B gene, has previously been identified as a potential oncogene in various types of cancer (16). The CREPT gene is located on human chromosome 20, which is a highly amplified region in numerous types of cancer (17,18). CREPT encodes a protein of 326 amino acids, which contains a regulation of nuclear pre-mRNA domain and is highly conserved across species (16). CREPT mRNA and protein have been revealed to be highly expressed in numerous types of clinical cancer tissues and cancer cell lines, including lung, liver, breast, prostate, stomach, colon, uterus endometrium and uterine cervical cancer (16,19). A high expression level of CREPT is correlated with tumor stage, metastasis and a poor survival rate (19-21). Previous studies have demonstrated that CREPT promotes tumor growth *in vivo* and *in vitro* by accelerating cell growth and cell cycle progression (22,23). Therefore, CREPT may serve as a potential and promising target for the development of anticancer treatments.

Previous studies have reported that miR-300 is aberrantly expressed in multiple types of human cancer and serves an important role in tumor progression (24-26). However, there is limited understanding regarding the role of miR-300 in HCC. The present study aimed to investigate the expression, biological function and regulatory mechanism of miR-300 in liver cancer. It was demonstrated that miR-300 expression was significantly decreased in HCC tissues and cell lines. Functional experiments revealed that miR-300 can regulate the proliferation, colony formation and cell cycle progression of liver cancer cells *in vitro*. Notably, CREPT was identified as a target gene of miR-300. In addition, it was revealed that miR-300 can regulate CREPT expression and the Wnt/β-catenin signaling pathway in HCC cells. Restoration of CREPT expression partially reversed the antitumor effect of miR-300. In conclusion, the present results demonstrate that miR-300 inhibits the growth of HCC cells by targeting CREPT, which may provide a novel miRNA target for HCC treatment.

Materials and methods

Collection of clinical specimens. Hepatocellular carcinoma (HCC) tissue samples (n=20) and adjacent non-tumor tissue samples (n=20) were obtained from The Affiliated Hospital of Changchun University of Chinese Medicine (Changchun, China). All HCC samples were obtained from patients with HCC who underwent radical surgical resection without preoperative chemotherapy or radiotherapy between May 2015 and Dec, 2017 Collected tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C until needed. Patient characteristics are listed in Table I. Written informed consent was obtained from all patients. The present study was approved by the Institutional Human Experiment and Ethics Committee of Changchun University of Chinese Medicine (Changchun, China) and performed in accordance with the Declaration of Helsinki.

Culture of cell lines. The human liver cancer cell lines HepG2, Hep3B and Huh-7, and the 293T cell line were provided by the Chinese Academy of Sciences. The normal liver cell line HL-7702 was purchased from the Bena Culture Collection. Cells were routinely cultured, according to the manufacturers' protocols. Briefly, HepG2, Hep3B, Huh-7 and 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin mix (Sigma-Aldrich; Merck KGaA). HL-7702 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS and 1% penicillin/streptomycin mix (Sigma-Aldrich; Merck KGaA). Cells were maintained in a humidified incubator (Thermo Fisher Scientific, Inc.) with 5% CO_2 and a temperature of 37˚C. Cell line authentication was performed using STR profiling.

Cell transfection. The miR-300 mimic and miR-300 inhibitor were purchased from Thermo Fisher Scientific, Inc. The sequences of miR-300 were as follows: Sense, 5'-UAUACA AGGGCAGACUCUCUCU-3'; anti-sense, 5'-AGAGAGAGU CUGCCCUUGUAUA-3'. The sequence of the miR-300 inhibitor was as follows: 5'-GAGAGAGUCUGCCCUUGUAU-3'. The open reading frame fragments of CREPT were inserted into a pcDNA3.1 vector (Thermo Fisher Scientific, Inc.) to generate the CREPT expression vector. A total of $2x10^5$ cells were plated in triplicate overnight in antibiotic-free complete medium in 6-well plates. The cells were grown overnight and then transfected with 200 μl mature miRNA (100 nM) and RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol for 48 h. The transfection efficacy was confirmed by RT-qPCR or western blot analysis. Each experiment was repeated at least 3 times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (human HCC tissue samples and adjacent non-tumor tissue samples; the liver cancer cell lines HepG2, Hep3B, Huh-7 and 293T) was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. For detection of miR-300 expression, complementary DNA was generated using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at the following conditions: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min). PCR amplifications were performed using the TaqMan Small RNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). For detection of mRNA expression, complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) at the following conditions: 37˚C for 15 min, followed by 72˚C for 10 min) and qPCR amplifications were performed using Power SYBR Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR amplifications were performed using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermal parameters: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. U6 small nuclear RNA and GAPDH were used as internal controls for normalizing the relative expression levels of miR-300 and mRNA, respectively.

Table I. Associations between miR-300 expression and the clinicopathological variables of hepatocellular carcinoma.

Parameter	n	Relative miR-300 expression	P-value
Age, years			0.221
>50	11	0.25±0.08	
≤50	9	0.20±0.06	
Gender			0.331
Male	10	0.21±0.06	
Female	10	0.23±0.07	
Histological differentiation			0.404
Low/no	10	0.19±0.06	
Moderate/high	10	0.25±0.07	
Lymph node metastasis			0.471
Yes	10	0.24±0.07	
No	10	0.21±0.06	
TNM stage			0.673
I/II stage	10	0.21±0.07	
III/IV stage	10	0.23±0.07	

Data are presented as the mean ± standard deviation. Significant differences were determined using Student's t-test. Results were considered statistically significant at P<0.05. miR, microRNA. TNM, Tumor-Node-Metastasis; miR, microRNA.

The primer sequences were: miR-300 forward, 5'-TATACA AGGGCAGACTCTCTCT-3'; and U6 reverse, 5'-CGCAAG GATGACACGCAAATTCGT-3'; CREPT forward, 5'-CAC GCGGGACCCATCGTCTC-3'; CREPT reverse, 5'-AGCCTT CATCTGCCTCTCTGGCA-3': cyclin D1 forward, 5'-CTG GCCATGAACTACCTGGA-3'; cyclin D1 reverse, 5'-GTC ACACTTGATCACTCTCC-3'; GAPDH forward, 5'-CATGAG AAGTATGACAACAGCCT-3'; and GAPDH reverse, 5'-AGT CCTTCCACGATACCAAAGT-3'. All RT-qPCR assays were run in triplicate. All results are presented as the mean ± standard deviation of three independent experiments. Relative gene expression analysis was performed using the comparative $2^{-\Delta\Delta Cq}$ method (27).

Cell proliferation assay. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8) assay. HepG2 and Huh-7 cells were seeded into a 96-well plate (Thermo Fisher Scientific, Inc.) at a density of 10,000 cells/well. Cells were transfected with 1 pM miR-300 mimic or inhibitor and cultured for 24, 48 and 72 h. Subsequently, 10 μl CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well. The cells were then cultured for a further 2 h at 37˚C, followed by measurements of the absorbance at 450 nm using a spectrophotometer (Bio-Rad Laboratories, Inc.). Each condition was determined in quintuplicates and all experiments were repeated at least 3 times.

Colony formation assay. After 48 h of transfection, HepG2 and Huh-7 cells were re-seeded into a six-well plate (Thermo Fisher Scientific, Inc.) at a density of 1,000 cells/well. And allowed to grow undisturbed for 7 days. Cells were stained with crystal violet on the plates at room temperature for 15 min. The number of colonies (diameter >1 mm) were counted using an inverted light microscope. Each experiment was repeated at least 3 times.

Cell cycle assay. HepG2 and Huh-7 cells were harvested following the indicated treatment times and fixed with 75% ice-cold ethanol at 4˚C for 24 h. Following washing with ice-cold PBS, cells were treated with 50 μg/ml RNase and 50 μg/ml propidium iodide (BD Biosciences; Becton, Dickinson and Company) in 500 μl binding buffer. Following incubation for 30 min, the cell samples were subjected to flow cytometry with a flow cytometer (BD Biosciences; Becton, Dickinson and Company) and data were analyzed using BD CellFIT software (BD Biosciences; Becton, Dickinson and Company). Each experiment was repeated at least 3 times.

Bioinformatics analysis and dual-luciferase reporter assay. Computer-aided algorithms (http://www.targetscan.org/vert_71/) were adopted to predict the target gene of miR-300. CREPT 3'-UTR fragments containing miR-300-binding sites or mutant miR-300-binding sites were cloned into pmirGLO vectors (Promega Cooperation). The reporter vector was co-transfected with miR-300 mimic into 293T cells using RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following culture for 48 h, relative luciferase activity (firefly/Renilla) was detected using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Each experiment was repeated at least 3 times.

Wnt/β-catenin-dependent TOP flash reporter assay. Wnt/β-catenin signaling was determined by measurement of TCF-mediated transcriptional activity using a TOP flash reporter assay. Briefly, HepG2 and Huh-7 cells were co-transfected with TOP flash vector (2 μg), pRL-TK vector (1 μg) (Promega Corportation) and 1 nM miR-300 mimic or inhibitor (Thermo Fisher Scientific, Inc.) for 48 h. Relative luciferase activity (firefly/Renilla) was detected using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Each experiment was repeated at least 3 times.

Western blot analysis. Cell lysates were obtained by lysing cells in cell lysis buffer (Thermo Fisher Scientific, Inc.) containing protease inhibitors. Protein concentrations were quantified using the Pierce Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (20 μg) were then loaded onto 10% sodium dodecyl sulfate polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane followed by incubation with 5% skim milk in TBS and 0.1% Tween-20 (TBST) at 37˚C for 1 h. The membrane was then incubated with appropriate antibodies, including anti-CREPT (cat. no. GTX119969; 1:2,000; GeneTex, Inc.), anti-β-catenin (9562; 1:1,000; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. ab9485. 1:2,500; Abcam) at 4˚C overnight. Following washes with TBST, the membrane was incubated with

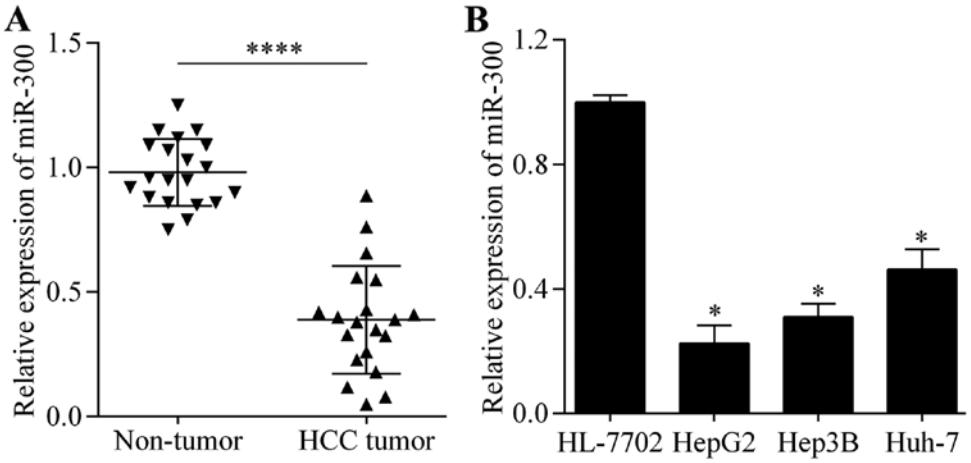

Figure 1. Decreased expression of miR-300 in HCC. (A) RT-qPCR analysis of miR-300 expression levels in tumor tissues and adjacent non-tumor tissues from patients with HCC. ****P<0.0001. (B) The relative expression levels of miR-300 in the liver cancer cell lines HepG2, Hep3B and Huh-7 were detected by RT-qPCR. The normal liver cell line HL-7702 was used as a control. *P<0.05 vs. HL-7702. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-300, microRNA-300; HCC, hepatocellular carcinoma.

horseradish peroxidase-labeled goat polyclonal anti-rabbit IgG secondary antibody (cat. no. ab6721, 1:3,000; Abcam) for 1 h at room temperature. The immunoblots were visualized using enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc.). GAPDH was used as a loading control protein and was visualized using the enhanced chemiluminescence system from Pierce (Thermo Fisher Scientific, Inc.). Gray scale analysis of protein bands was performed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. Significant differences were determined using Student's t-test or one-way analysis of variance followed by Bonferroni's post hoc test. All statistical analysis was performed with SPSS 19.0 software (IBM Corp.). The correlation between miR-300 and CREPT expression was determined using Spearman's correlation test. P<0.05 was considered to indicate a statistically significant difference. Data are representative of three independent experiments performed in triplicate.

Results

miR-300 expression level is lower in HCC tissues and cell lines. To investigate whether miR-300 may serve a role in HCC, the expression level of miR-300 in HCC tissues was examined by RT-qPCR. It was identified that the expression level of miR-300 was significantly decreased in HCC tissue samples compared with adjacent non-tumor tissue samples (P<0.0001; Fig. 1A). However, no correlation was identified between miR-300 expression and tumor stage or histologic grade (Table I). Furthermore, it was revealed that the miR-300 expression level was significantly decreased in a number of liver cancer cell lines compared with the normal liver cancer cell line (P<0.05; Fig. 1B). This altered expression level of miR-300 indicates a possible role of miR-300 in HCC.

miR-300 inhibits the growth of liver cancer cells in vitro. To investigate the biological function of miR-300 in liver cancer, the effect of miR-300 overexpression or inhibition on liver cancer cell growth was determined *in vitro*. Overexpression of miR-300 was achieved by transfection of the miR-300 mimic into HepG2 cells (Fig. 2A). The results demonstrated that overexpression of miR-300 significantly inhibited proliferation and colony formation (P<0.05; Fig. 2B and C). Furthermore, overexpression of miR-300 significantly increased the number of cells in the G_0/G_1 phase and decreased the number of cells in the S phase (P<0.05; Fig. 2D). In addition, inhibition of miR-300 was achieved by transfection of the miR-300 inhibitor into Huh-7 cells (Fig. 3A). Inhibition of miR-300 significantly promoted proliferation and colony formation of Huh-7 cells (P<0.05; Fig. 3B and C). Inhibition of miR-300 significantly decreased the number of cells in the G_0/G_1 phase and increased the number of cells in the S phase compared with the control (P<0.05; Fig. 3D). In summary, these results suggest that miR-300 inhibits liver cancer cell growth by regulating proliferation, colony formation and cell cycle transition.

CREPT is a target gene of miR-300 in liver cancer. It is understood that miRNAs can participate in tumor progression by repressing target genes (13). Therefore, the present study used bioinformatics analysis to predict the potential targets of miR-300. Notably, it was identified that CREPT, an oncogene in numerous types of cancer (16), is a putative target gene of miR-300. The 3'-UTR of CREPT contains putative binding sites for miR-300 (Fig. 4A). A luciferase reporter assay demonstrated that transfection with miR-300 mimic significantly suppressed the luciferase activity of a vector containing wild-type CREPT 3'-UTR; however, no effect on luciferase activity was observed with a vector containing mutant CREPT 3'-UTR (P<0.05; Fig. 4B). Subsequently, the regulatory effect of miR-300 on CREPT expression was examined in liver cancer cells. The results revealed that overexpression of miR-300 significantly decreased the expression level of CREPT (P<0.05; Fig. 4C and D), while inhibition of miR-300 significantly increased the expression level of CREPT (P<0.05; Fig. 4E and F). In summary, these results

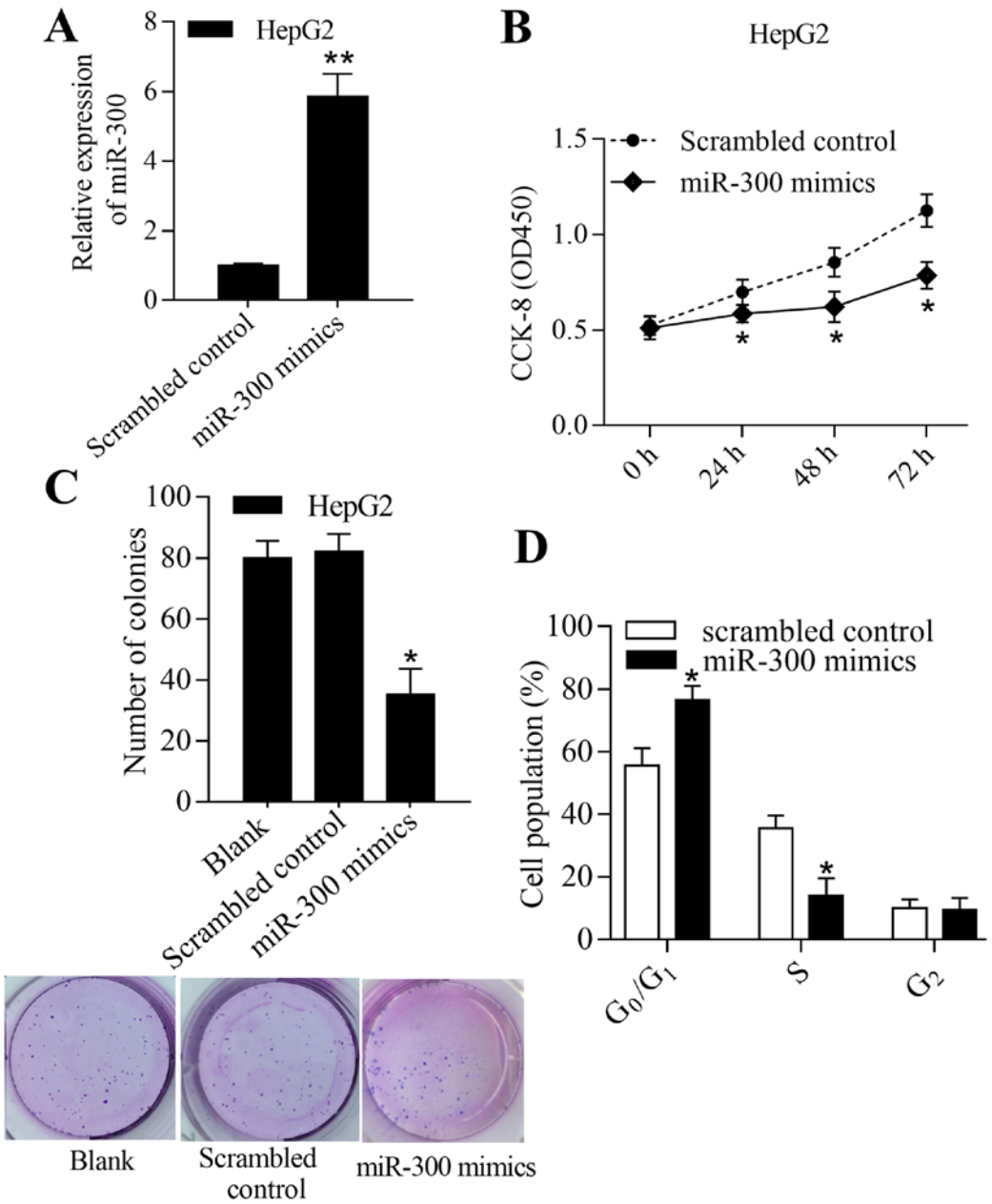

Figure 2. Overexpression of miR-300 inhibits the growth of liver cancer cells. (A) The transfection efficiency of miR-300 mimic on miR-300 expression level was evaluated by reverse transcription-quantitative polymerase chain reaction. HepG2 cells were transfected with miR-300 mimic or a scrambled control for 48 h prior to analysis. (B) The effect of transfection with miR-300 mimic for 24, 48 or 72 h on cell proliferation was assessed by CCK-8 assay. (C) The effect of miR-300 overexpression on colony formation was detected by a colony formation assay. (D) The effect of miR-300 overexpression on cell cycle distribution was examined by flow cytometry. **P<0.01 and *P<0.05 vs. scrambled control. miR-300, microRNA-300; OD, optical density; CCK-8, Cell Counting Kit-8.

indicate that miR-300 binds to the 3'-UTR of CREPT, which regulates the expression level.

miR-300 regulates Wnt/β-catenin signaling in liver cancer cells. CREPT has been reported to be an important regulator of the Wnt/β-catenin signaling pathway (28,29). With the understanding that miR-300 regulates CREPT expression, it was suggested that miR-300 may have a regulatory effect on the Wnt/β-catenin signaling pathway. The present study revealed that overexpression of miR-300 significantly decreased the expression level of β-catenin (P<0.05; Fig. 5A). Furthermore, overexpression of miR-300 significantly inhibited Wnt/β-catenin signaling (P<0.05; Fig. 5B) and significantly decreased the expression level of cyclin D1 (P<0.05; Fig. 5C). By contrast, inhibition of miR-300 induced the opposite effect on the Wnt/β-catenin signaling pathway (Fig. 5D-F). These results suggest that miR-300 exerts a regulatory effect on Wnt/β-catenin signaling in liver cancer cells.

miR-300 inhibits the growth of liver cancer cells and the Wnt/β-catenin signaling pathway. To investigate whether miR-300 exerts its function by targeting CREPT, rescue experiments were performed. Transfection of the CREPT expression vector significantly restored the expression level of CREPT in cells transfected with an miR-300 mimic (P<0.05; Fig. 6A). Furthermore, overexpression of CREPT partially reversed the

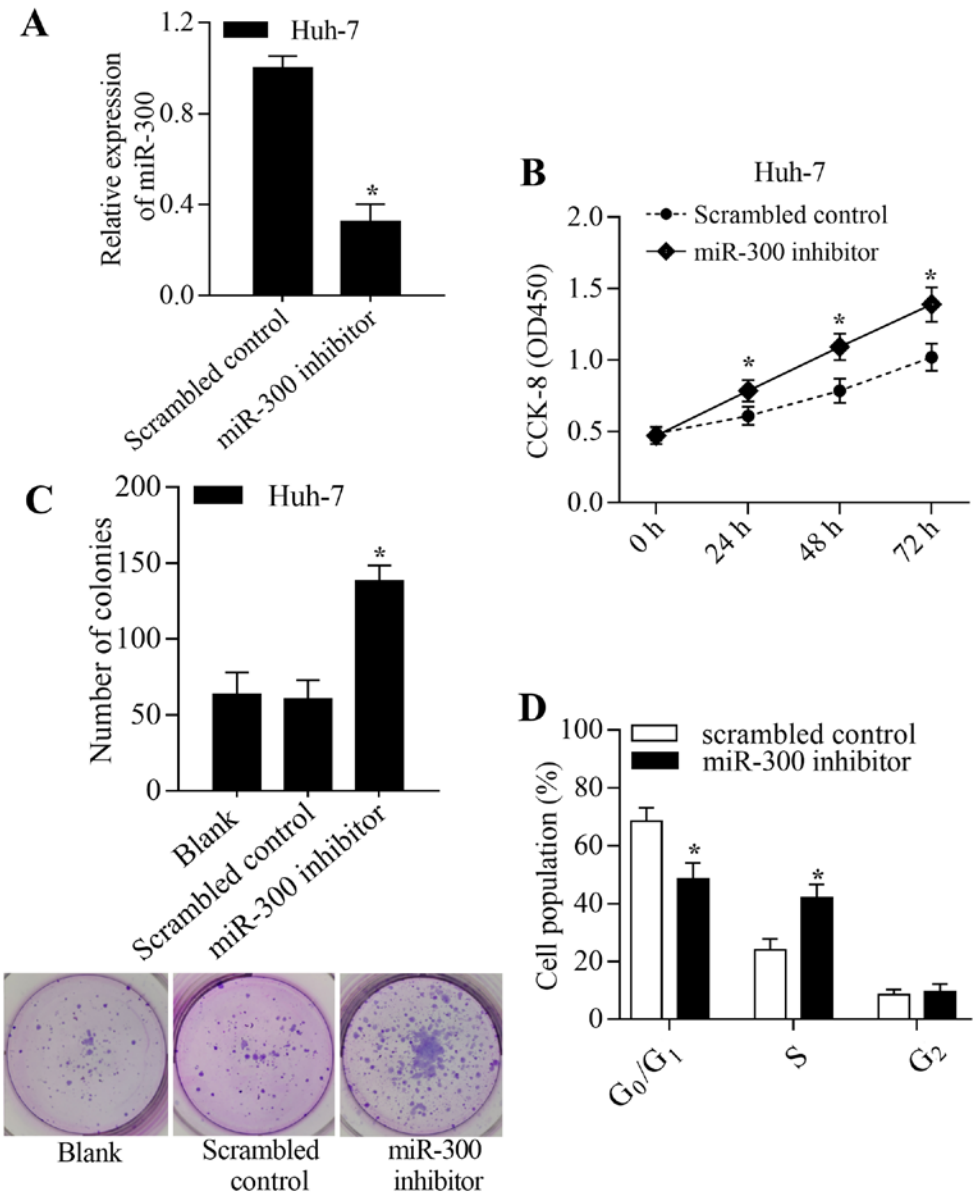

Figure 3. Inhibition of miR-300 accelerates the growth of liver cancer cells. (A) The transfection efficiency of miR-300 inhibitor for 48 h on miR-300 expression level was evaluated by reverse transcription-quantitative polymerase chain reaction in Huh-7 cells. (B) The effect of transfection with miR-300 inhibitor for 24, 48 or 72 h on cell proliferation was assessed by CCK-8 assay. (C) The effect of miR-300 inhibition on colony formation was detected by a colony formation assay. (D) The effect of miR-300 inhibition on cell cycle distribution was examined by flow cytometry. *P<0.05 vs. Blank. Scrambled control. miR-300, microRNA-300; OD, optical density; CCK-8, Cell Counting Kit-8.

inhibitory effect of miR-300 on cell growth (Fig. 6B and C) and the Wnt/β-catenin signaling pathway (Fig. 6D). In conclusion, these results suggest that miR-300 inhibits the growth of liver cancer cells and Wnt/β-catenin signaling in liver cancer cells by targeting CREPT.

Discussion

A number of miRNAs have been reported to be associated with tumorigenesis of HCC (13,30); however, additional miRNAs remain to be identified and characterized. The present study reported miR-300 as a novel miRNA associated with HCC. miR-300 was revealed to inhibit the growth of HCC cells, which indicates it functions as a tumor-suppressive miRNA in HCC (31). Notably, it was identified that the underlying mechanism is associated with a regulatory effect of miR-300 on CREPT. The present study suggests that miR-300 may be used as a therapeutic target for HCC.

Numerous studies have demonstrated that dysregulation of miR-300 is involved in the development and progression of cancer (24-26). A low expression level of miR-300 is present in glioma tissues and overexpression of miR-300 inhibits the proliferation and invasion of glioma cells *in vitro* (32). Furthermore, miR-300 has been reported to suppress the epithelial to mesenchymal transition and metastasis of head, and neck squamous cell carcinoma and breast cancer cells by targeting Twist (26). The expression level of miR-300 has been revealed to be lower in laryngeal squamous cell carcinoma and overexpression of miR-300 represses proliferation and metastasis by targeting c-ros oncogene 1 receptor tyrosine kinase (33,34). Inhibition of miR-300 contributes to cell proliferation and metastasis of gallbladder carcinoma (35). Additionally, recent

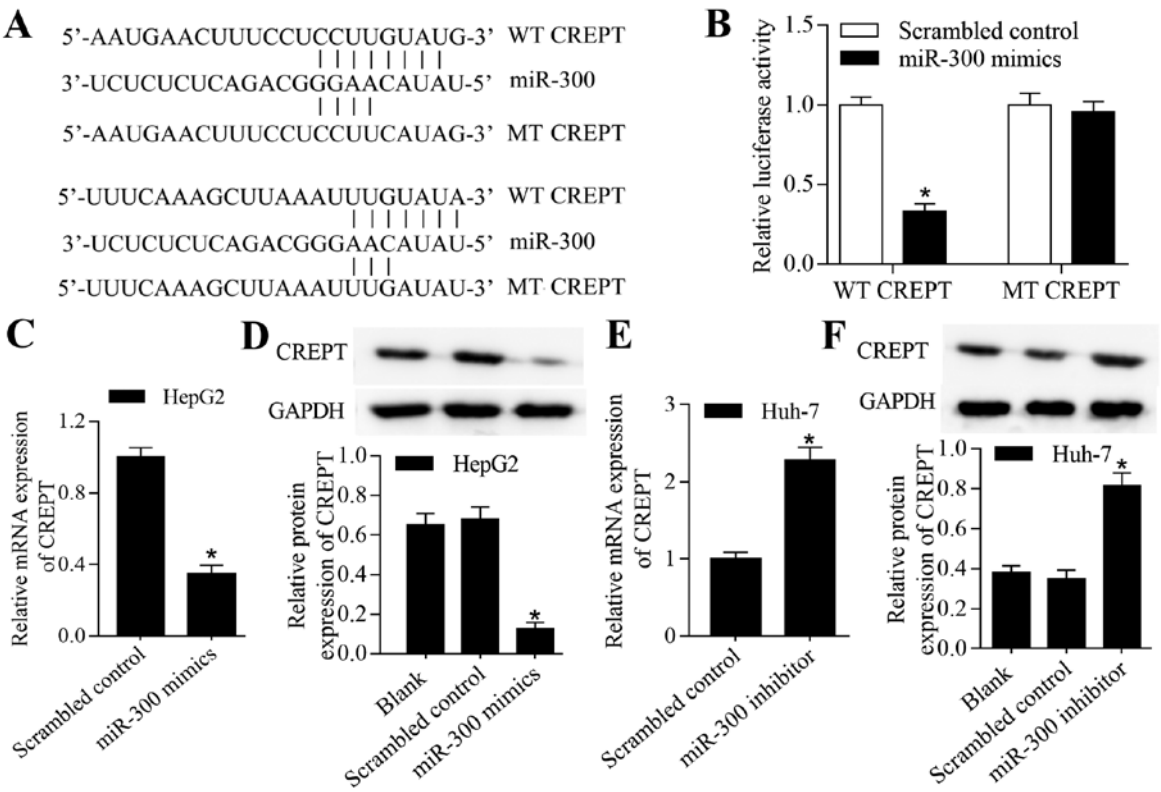

Figure 4. CREPT is a target gene of miR-300. (A) A diagram of the potential target sites of miR-138 in the 3'-UTR of CREPT. (B) A luciferase reporter assay was performed using 293T cells co-transfected with CREPT 3'-UTR reporter vector and miR-300 mimic. *P<0.05 vs. scrambled control. The effect of transfection of miR-300 mimic on CREPT expression was evaluated by (C) RT-qPCR and (D) western blot analysis in HepG2 cells. The effect of transfection with miR-300 inhibitor on CREPT expression was evaluated by (E) RT-qPCR and (F) western blot analysis in Huh-7 cells. *P<0.05 vs. Blank. Scrambled control. CREPT, regulation of nuclear pre-mRNA domain-containing protein 1B; miR-300, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; UTR, untranslated region; WT, wild-type; MT, mutant.

studies have demonstrated that miR-300 inhibits the progression of pancreatic cancer and osteosarcoma by targeting cullin 4B (24,36). These findings suggest a tumor suppressive role of miR-300. Comparable with the aforementioned studies, the present results support a tumor suppressive role of miR-300 in tumor progression. The current study demonstrated that the miR-300 expression level was lower in liver cancer and overexpression of miR-300 could inhibit proliferation and colony formation, and induce G_0/G_1 cell cycle arrest of liver cancer cells, which indicates an antitumor effect of miR-300 in HCC. By contrast, certain studies have suggested an oncogenic role of miR-300 in tumorigenesis. miR-300 has been reported to promote tumorigenesis of colorectal cancer, osteosarcoma and glioma by targeting p53 and bromodomain-containing protein 7 (37,38). Zhang *et al* (39) reported that miR-300 was upregulated in HCC tissues and promoted cancer growth by targeting MDC1. Those controversial results indicated that disregulation of miR-300 might not be the first event during the pathology of HCC. Other master regulators within tumors or tumor microenvironment might exist to regulate the expression miR-300 as disease progress. Wang *et al* (31) reported that miR-300 was downregulated in HCC tissues and cell lines. Those controversial results indicate the complexity of miR-300 regulation during the pathology of HCC. Disregulation of miR-300 might affect disease progress. Moreover, HCC samples from the present study and Wang's study are from patients who did not undergo chemotherapy or radiotherapy. Chemotherapy and radiotherapy are reported to induce expression of some miRNAs in cancers (40,41). It is very possible that the variation of miR-300 expression might come from the treatment difference in different patients. Of note, SMMC-7721 cells used in Wang's study is reported to be Hela contaminated, which make the conclusion unreliable (31). So, future mechanism studies and HCC tissue from a larger patient population are needed to draw a complete picture of miR-300 in HCC. Therefore, the precise role of miR-300 in tumor progression remains to be further investigated.

CREPT was initially identified as a potential oncogene in colorectal cancer; it has been identified to be overexpressed at the mRNA and protein levels in colorectal cancer tissues and cell lines (16). High CREPT expression is correlated with tumor differentiation, metastasis and a short survival time for patients with colorectal cancer (20). Functional experiments demonstrated that CREPT can promote the proliferation and cell cycle progression of colorectal cancer cells by regulating the transcription of cell cycle-associated genes (16,20,42). CREPT overexpression has been associated with tumor stage, histology type and depth of myometrial invasion in endometrial cancer, and knockdown of CREPT inhibits cell proliferation and induces G_0/G_1 cell cycle arrest by downregulating cyclin D1, cell cycle dependent kinase (CDK)4 and CDK6 *in vitro* (23). Knockdown of CREPT inhibits the proliferation and migration of non-small cell lung cancer cells, whereas overexpression of CREPT demonstrates an

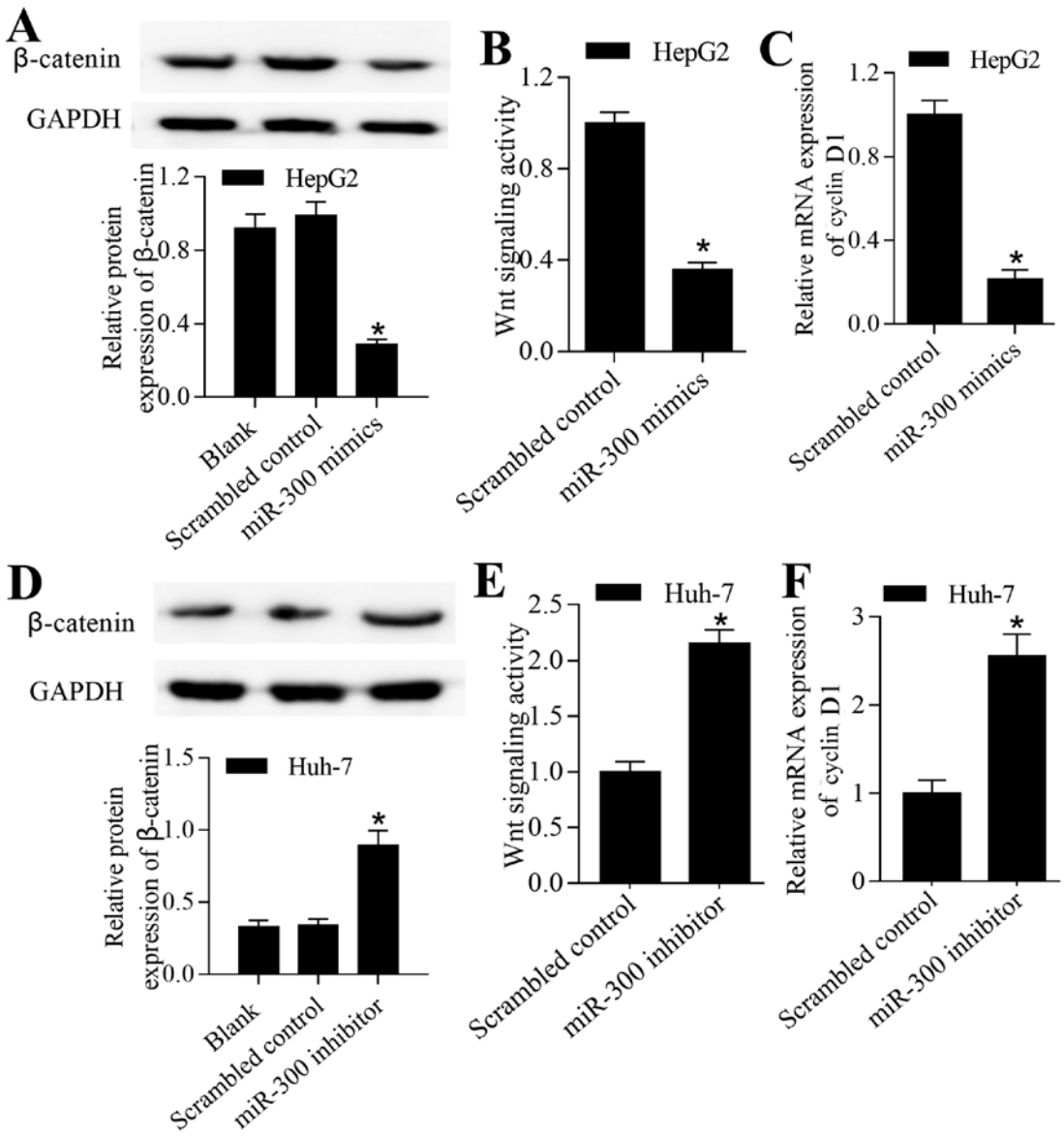

Figure 5. miR-300 regulates the Wnt/β-catenin signaling pathway in liver cancer cells. HepG2 cells were transfected with an miR-300 mimic or a scrambled control for 48 h. (A) The effect of miR-300 overexpression on β-catenin protein expression level in HepG2 cells was then evaluated by western blot analysis. (B) In addition, the effect of miR-300 overexpression on Wnt signaling activation was analyzed by TOP flash reporter assay. (C) The effect of miR-300 overexpression on cyclin D1 expression level was evaluated by RT-qPCR. Huh-7 cells were transfected with miR-300 inhibitor or scrambled control for 48 h. (D) The effect of miR-300 inhibition on β-catenin protein expression was then evaluated by western blot analysis. (E) In addition, the effect of miR-300 inhibition in Huh-7 cells on Wnt signaling activation was analyzed by TOP flash reporter assay. (F) The effect of miR-300 inhibition on cyclin D1 expression in Huh-7 cells was detected by RT-qPCR. *P<0.05 vs. Blank. Scrambled control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-300, microRNA-300.

oncogenic effect (21,43). Similarly, an oncogenic function of CREPT has been observed in oral squamous cell carcinoma and gastric cancer (22,44). Notably, CREPT has been reported to be highly expressed in HCC tissues and cell lines (16). Furthermore, CREPT achieves its oncogenic effects via regulation of HCC cell growth and cell cycle progression (16). These findings suggest that CREPT is a novel oncogene that can be used as a target for cancer treatment. Notably, a recent study demonstrated that CREPT expression is regulated by miR-138, which contributes to breast cancer progression (45). This indicates that high expression of CREPT may be induced by dysregulated miRNAs. However, to the best of our knowledge, the regulatory mechanism of miRNAs against CREPT in HCC remains unknown.

The present study identified that CREPT is targeted and regulated by miR-300 in HCC. It was revealed that miR-300 can inhibit liver cancer cell growth by targeting CREPT, whereas overexpression of CREPT partially reverses the anti-tumor effect of miR-300. Therefore, decreased expression of miR-300 may contribute to a high expression level of CREPT in HCC, which leads to HCC development and progression. Therefore, the miR-300/CREPT axis may serve an important role in the molecular pathogenesis of HCC.

CREPT has been reported to be a positive regulator of the Wnt/β-catenin signaling pathway. Overexpression of CREPT enhances the expression levels of β-catenin, transcription factor 4 (TCF4) and cyclin D1 in chicken fibroblast cells (28). Furthermore, CREPT has been reported

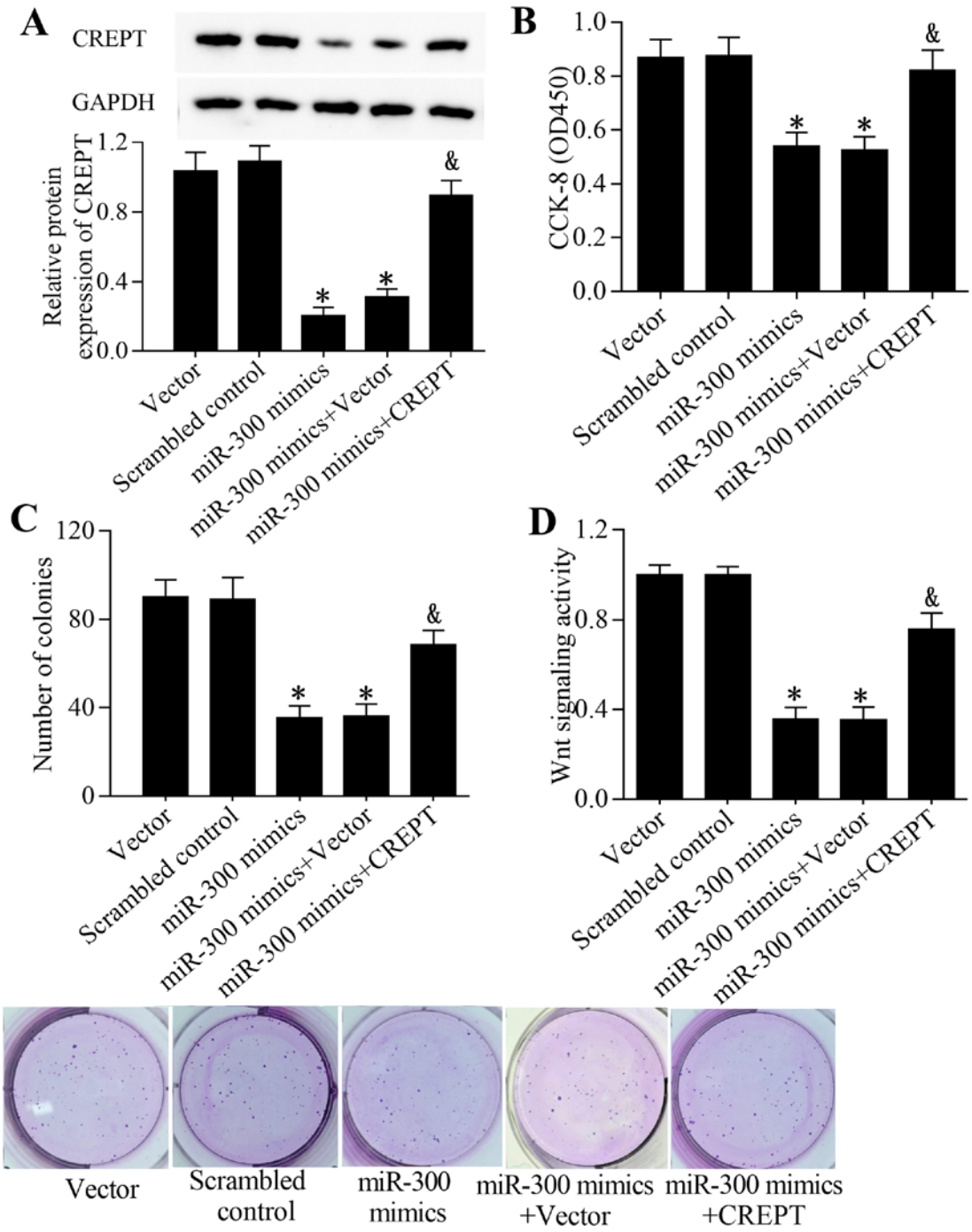

Figure 6. Restoration of CREPT reverses the antitumor effect of miR-300 overexpression. HepG2 cells were co-transfected with miR-300 mimic and a CREPT expression vector for 48 h. (A) Protein expression level of CREPT was measured by western blot analysis. (B) Cell proliferation was analyzed using a CKK-8 assay. (C) Colony formation was evaluated using a colony formation assay. (D) Wnt signaling was detected by a TOP flash reporter assay. *P<0.05 vs. Vector. scrambled control; &P<0.05 vs. miR-300 mimic. miR-300 mimic+Vector. miR, microRNA CREPT, regulation of nuclear pre-mRNA domain-containing protein 1B; OD, optical density; CCK-8, Cell Counting Kit-8.

to promote Wnt/β-catenin signaling by enhancing the association of β-catenin with TCF4 (29). Notably, a recent study demonstrated that CREPT facilitates Wnt/β-catenin signaling by promoting p300-mediated β-catenin acetylation and stabilization (19). Similarly, the present results demonstrated that inhibition of CRPET decreased the activation of Wnt/β-catenin signaling in HCC cells. Therefore, CREPT may serve as a novel target for inhibiting Wnt/β-catenin signaling in tumorigenesis.

In conclusion, the current study provides promising evidence that miR-300 acts as a tumor suppressor in HCC and inhibits the growth of liver cancer cells by targeting and inhibiting CREPT. The present results demonstrate that the miR-300/CREPT axis may be involved in regulating the Wnt/β-catenin signaling pathway, which may serve an important role in the development and progression of HCC. As a limitation of this study, the detail relationship between Wnt signaling and miR-300 is still unknown. This interesting

project is now ongoing in the authors' lab. In conclusion, the current study may increase understanding of the mechanisms involved in tumorigenesis and suggests a novel target for HCC treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JB and XZ designed the study; YG and YD conducted the experiments; XZ contributed new reagents or analytic tools; YG and XY analyzed the data and prepared figures; JB, XZ and YD drafted the manuscript; all authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The present study was approved by the Institutional Human Experiment and Ethics Committee of Changchun University of Chinese Medicine, Changchun, China (approval no. CCZYFYLL2019-020).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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