

MicroRNA-100 functions as a tumor suppressor by inhibiting Lgr5 expression in colon cancer cells

MING-KAI ZHOU¹, XIAO-JUN LIU¹, ZHI-GUO ZHAO² and YI-MENG CHENG³

¹Intensive Care Unit and ²Department of Gastroenterology, The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450014; ³Department of Neurology, The Third Hospital of Zhengzhou, Zhengzhou, Henan 450000, P.R. China

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Abstract. Previous studies have demonstrated that microRNAs (miRNAs), a class of single-stranded RNA molecules that are 18-27 nucleotides in length, serve a critical function in tumorigenesis, including in the development of colon cancer. In the current study, miR-100 levels were demonstrated to be reduced in colon cancer tissues compared with the levels in matched adjacent normal tissues. Forced overexpression of miR-100 by transfection with miR-100 mimics substantially inhibited the proliferation, migration and invasion of SW480 and HCT116 cells, whereas reduced expression, resulting from transfection of antisense oligonucleotides, promoted these processes. At the molecular level, miR-100 was observed to reduce the levels of leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), by binding to its 3'-untranslated region. As a result of this, Wnt/ β -catenin signaling was affected by fluctuations in the level of miR-100 mimics or antisense. Collectively, the results of the current study elucidate a novel regulatory pathway involving miR-100 and Lgr5 in colon cancer cells, which may present a potential therapeutic target.

Introduction

Colon cancer is the third most common type of cancer and the leading cause of cancer-associated mortality in Western communities (1,2). In China, the incidence is lower than in Western countries, but nevertheless is a substantial burden (3). The molecular mechanisms underlying the development of this type of cancer remain to be fully elucidated, thus there currently exist limited therapeutic options (4).

MicroRNAs (miRNAs) are a family of small non-coding RNA molecules of 18-27 nucleotides in length (5). In general, miRNAs negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of their target double-stranded mRNA, leading to degradation of the mRNA via the Dicer complex (6). The abnormal expression of certain miRNAs has been observed in various types of solid tumor, including colon cancer (7). For example, miRNA-21 has recently emerged as a novel biomarker in colon cancer, with the potential to be used as a diagnostic and therapeutic target (8). Additionally, several miRNAs have been reported to regulate colon cell growth, migration and invasion, including miR-32, -224 and -203 (9-11).

The current study investigated miR-100 using gain- and loss-of-function experiments, and the effect of up- or down-regulation of miR-100 on the proliferation, migration and invasion of colon cells was determined.

Materials and methods

Human tissues. A total of 25 pairs of frozen primary colon cancer samples and corresponding histologically normal mucosa samples were obtained from the Department of Gastroenterology, Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The diagnoses of these tissue samples were verified by pathologists. The current study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University.

Cell culture. The SW480 and HCT116 colon cancer cell lines were purchased from the cell bank of the Type Culture Collection of The Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin (all from Gibco Life Technologies, Carlsbad, CA, USA).

Cell transfection. miR-100 mimics, antisense oligonucleotides and negative controls (NCs) were purchased from Shanghai Genepharma Co., Ltd. (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Correspondence to: Dr Xiao-Jun Liu, Intensive Care Unit, The Second Affiliated Hospital of Zhengzhou University, No. 2 Jingba Road, Zhengzhou, Henan 450014, P.R. China
E-mail: hnxiaojunliu@126.com

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RNA isolation and quantitative polymerase chain reaction (qPCR). RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies), and reverse transcription was performed with the Takara RNA PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's instructions. In order to determine the transcripts of the genes of interest, qPCR was performed using a SYBR Premix Ex Taq master mix (Takara Biotechnology Co., Ltd.) with an ABI 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). PCR cycling conditions included an initial holding period at 94°C for 5 min, followed by a two-step PCR program consisting of 40 cycles of 94°C for 5 sec and 60°C for 30 sec. Expression of U6 small nuclear RNA was determined as an internal control. Primer sequences were as follows: Cyclin D1, F 5'-GCTGCG AAGTGGAAACCATC-3' and R 5'-CCTCCTTCTGCA CACATTTGAA-3'; cyclin E, F 5'-AAGGAGCGGGACACC ATGA-3' and R 5'-ACGGTCACGTTTGCCTTCC-3'.

Bromodeoxyuridine (BrdU) incorporation assays. For the BrdU incorporation assays, a BrdU cell proliferation enzyme-linked immunosorbent assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to analyze the incorporation of BrdU during the S phase of the cell cycle in the SW480 and HCT116 cells, in accordance with the manufacturer's instructions. All experiments were repeated a minimum of three times in quadruplicate.

MTT assay. The cell viability was determined by assaying the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyltetrazolium bromide (MTT) to formazan. SW480 and HCT116 cells were cultured in DMEM at a concentration of 60-70%, prior to the addition of 100 μ l MTT (Beyotime Institute of Biotechnology) for 4 h. The cell viability in the different samples was measured using a Raman spectrophotometer (B&W Tek, Inc., Newark, DE, USA) at a wavelength of 470 nm.

Cell migration and invasion assays. Subsequent to transfection of the SW480 and HCT116 cells with miR-100 mimics, antisense or NC for 24 h, cell migration and invasion were analyzed. The cells were seeded in Transwell migration or extracellular matrix-coated invasion chambers (Tumor Cell Transendothelial Migration Assay kit and ECMatrix Cell Invasion Assay kit, respectively; EMD Millipore, Temecula, CA, USA) and incubated for a further 24 h. Subsequently, the Transwell migration assay or invasion assay was conducted according to manufacturer's instructions. Cell migration and invasion were quantified with an iMark Microplate Absorbance Reader (#168-1130; Bio-Rad Laboratories, Hercules, CA, USA) at 570 nm, according to the manufacturer's instructions.

Luciferase reporter assay. Total cDNA from SW480 cells was obtained from mRNA using a Reverse Transcription System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols. The cDNA was used to amplify the 3'-UTR of Lgr5 by PCR. The Lgr5 3'UTR was cloned into pMir-Report (Ambion Life Technologies, Shanghai, China), yielding pMir-Report-Lgr5. Mutations were introduced

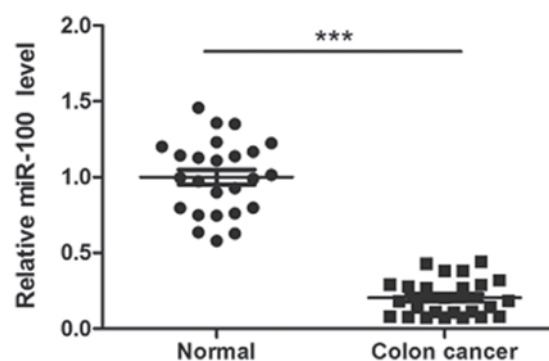


Figure 1. Expression levels of miR-100 in colon cancer tissues. The miR-100 expression levels were determined by RT-qPCR in human colon cancer tissues and adjacent normal tissues. *** $P < 0.001$ vs. normal tissues. miR-100, microRNA-100; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

in potential miR-100 binding sites using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Shanghai, China). Cells were transfected with the pMir-Report vectors containing the 3'-UTR variants with miR-100 mimics or antisense for 36 h. The pRL-TK vector (Promega Corporation) carrying the *Renilla* luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega Corporation). miRWalk software (www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) was used for analysis.

Western blot. Following transfection of mimics, antisense or NC, tissues or SW480 cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology). The protein (40 μ g) was subjected to 7.5% SDS-PAGE (Shanghai Sangong Pharmaceutical Co., Ltd., Shanghai, China) on a PowerPAC HC High-Current Power Supply electrophoresis machine (Bio-Rad Laboratories), and separated proteins were transferred to nitrocellulose membranes (EMD Millipore). The membranes were incubated overnight at 4°C with the following antibodies: Monoclonal mouse anti-human β -actin (1:1,000; sc-130065); polyclonal rabbit anti-human leucine-rich-repeat-containing G protein-coupled receptor 5 (Lgr5; 1:2,000; sc-135238); monoclonal rabbit anti-human β -catenin (1:2,000; sc-376841); and polyclonal goat anti-human histone H1 (1:1,000; sc-247158) (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The immunoreactive bands were detected with a ChemiGlow West Chemiluminescence Substrate kit (ProteinSimple, Santa Clara, CA, USA) with the FluorChem FC2 system (NtureGene Corporation, Beijing, China).

Statistical analysis. Data are presented as the mean \pm standard error. Statistical analysis was performed with SPSS, version 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-100 is downregulated in colon cancer tissues. The expression level of miR-100 in 25 pairs of human tissues

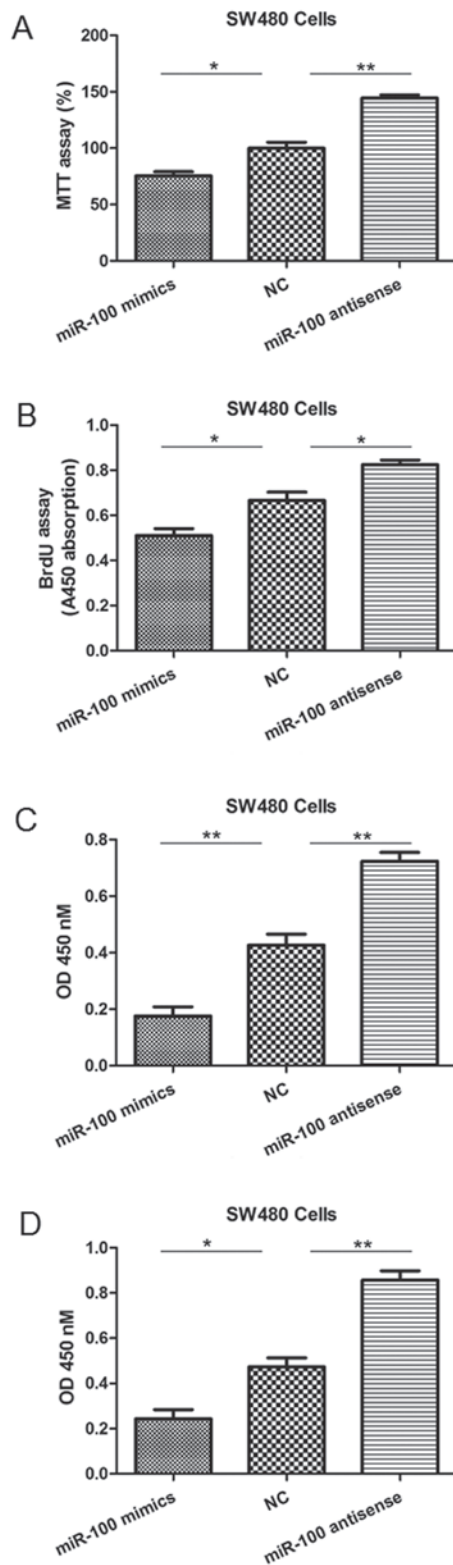


Figure 2. miR-100 mimics or antisense oligonucleotides regulate SW480 cell viability, proliferation, migration and invasion. (A) The MTT assay indicated a reduction or increase in cell viability following transfection of mimics or antisense oligonucleotides, respectively. (B) The BrdU assay indicated a reduction or increase in proliferation following transfection of mimics or antisense oligonucleotides, respectively. (C) The Transwell migration assay indicated a reduction or increase in migration following transfection of mimics or antisense oligonucleotides, respectively. (D) The invasion assay indicated a reduction or increase in invasion capabilities following transfection of mimics or antisense oligonucleotides, respectively. * $P < 0.05$ and ** $P < 0.01$ vs. NC. miR-100, microRNA-100; BrdU, bromodeoxyuridine; NC, negative control; OD, optical density.

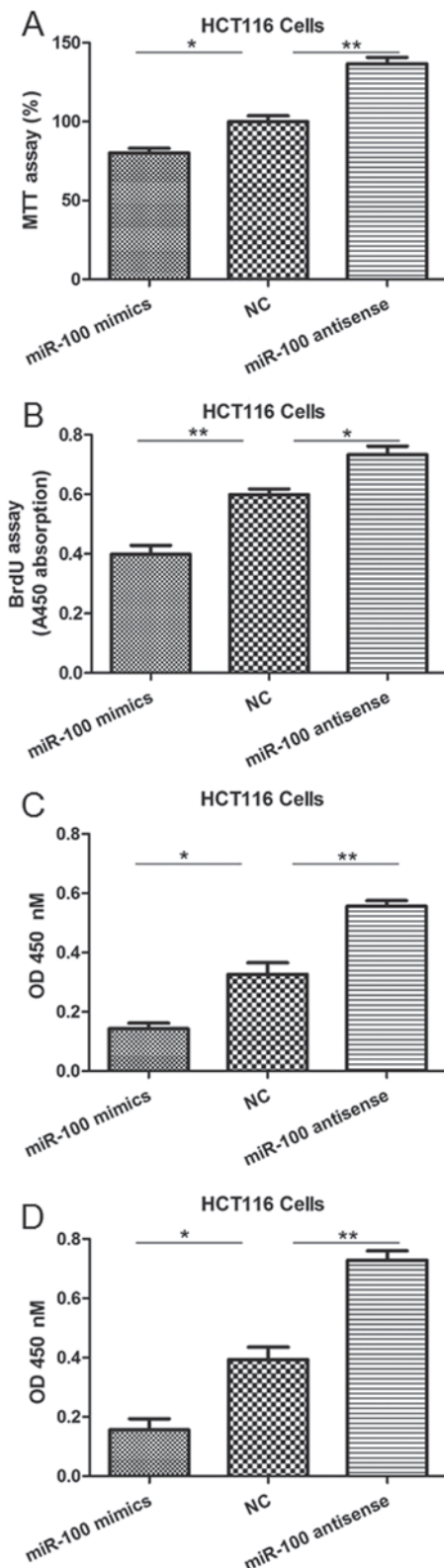


Figure 3. miR-100 mimics or antisense oligonucleotides regulate HCT116 cell viability, proliferation, migration and invasion. (A) The MTT assay indicated a reduction or increase in cell viability following transfection of mimics or antisense oligonucleotides, respectively. (B) The BrdU assay indicated a reduction or increase in proliferation following transfection of mimics or antisense oligonucleotides, respectively. (C) The Transwell migration assay indicated a reduction or increase in migration following transfection of mimics or antisense oligonucleotides, respectively. (D) The invasion assay indicated a reduction or increase in cell viability following transfection of mimics or antisense oligonucleotides, respectively. * $P < 0.05$ and ** $P < 0.01$ vs. NC. miR-100, microRNA-100; BrdU, bromodeoxyuridine; NC, negative control; OD, optical density.

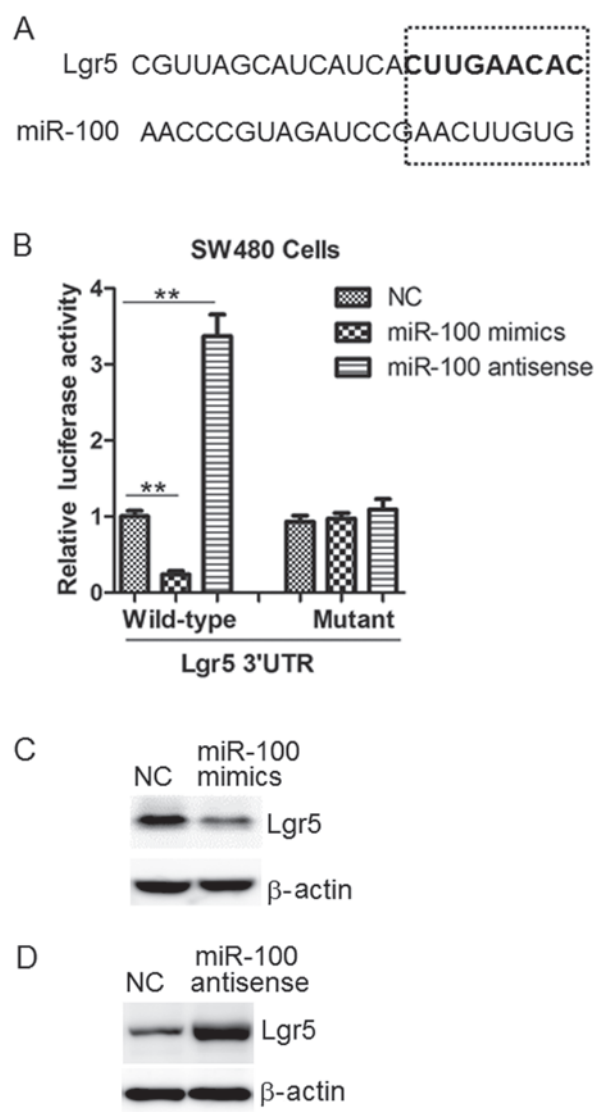


Figure 4. miR-100 downregulated the expression of Lgr5 protein by targeting its 3'-UTR. (A) Prediction of miR-100 binding sites in the 3'-UTRs of the human Lgr5 gene by miRWalk bioinformatics software. A potential binding site is highlighted in bold. (B) Luciferase reporter assays in SW480 cells. Cells were transfected with wild-type or mutant 3'-UTR-reporter constructs with miR-100 mimics or antisense $^{**}P<0.01$ vs. NC. Protein levels of Lgr5 were determined by western blot in SW480 cells transfected with miR-100 (C) mimics or (D) antisense. miR-100, microRNA-100; UTR, untranslated region; NC, negative control.

was measured by RT-qPCR. As demonstrated in Fig. 1, the miR-100 expression level was significantly lower in colon cancer tissues compared with the level in matched adjacent normal tissues ($P<0.001$).

miR-100 reduces the viability, proliferation, migration and invasion of colon cancer cells. To investigate the function of miR-100 in tumorigenesis, gain- and loss-of-function experiments involving the introduction of mimics or antisense oligonucleotides into SW480 and HCT116 cells were performed. Scramble sequences were used for the NC group. As observed with the MTT and BrdU incorporation assays, cell viability and proliferation levels were significantly reduced in SW480 cells in which miR-100 was overexpressed, but

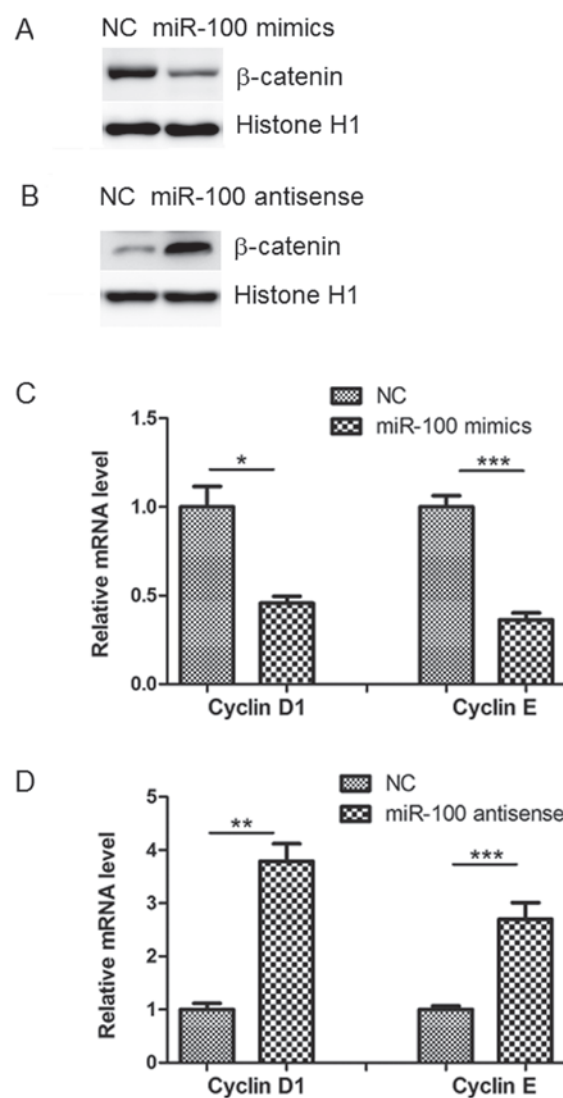


Figure 5. miR-100 regulates Wnt/β-catenin signaling. The nuclear β-catenin expression level was determined by western blot in SW480 cells transfected with miR-100 (A) mimics or (B) antisense. Histone H1 levels were measured as a loading control. mRNA levels of cyclin D1 and E were examined by qPCR in SW480 cells transfected with miR-100 (C) mimics or (D) antisense. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. NC. miR-100, microRNA-100; NC, negative control; qPCR, quantitative polymerase chain reaction.

enhanced by miR-100 antisense (Fig. 2A and B). Additionally, miR-100 mimics significantly inhibited the *in vitro* migration and invasion abilities of SW480 cells, whereas its antisense enhanced these processes (Fig. 2C and D). Similar results were also observed in the HCT116 cells (Fig. 3).

Lgr5 is a target of miR-100 in colon cancer cells. Bioinformatics software (miRWalk) was used to screen for the target gene of miR-100 in colon cancer cells. The results indicated Lgr5 to be a target of miR-100 (Fig. 4A). The luciferase activity assay established that miR-100 mimics significantly suppressed the activity of the wild-type 3'-UTR, while its antisense upregulated it. However, this effect was not observed in the mutant in SW480 cells (Fig. 4B).

Protein levels of Lgr5 and β-catenin are altered by miR-100. Consistent with the bioinformatic analysis and luciferase assay

results, western blot analysis indicated that miR-100 mimics reduced Lgr5 protein levels compared with NC levels in SW480 cells, whilst miR-100 antisense produced the opposite effect (Fig. 4C and D). A previous study demonstrated that Lgr5 promotes tumor growth and progression through the activation of Wnt/ β -catenin signaling (12). In agreement with this, it was observed in the current study that miR-100 mimics inhibited, while its antisense promoted, nuclear β -catenin accumulation in SW480 cells (Fig. 5A and B). The results of the present study were in accordance with a previous study that demonstrated that cyclin D1 and E were reduced or increased by transfection of miR-100 mimics or antisense, respectively (13). This indicates that downstream targets of β -catenin are also influenced by miR-100 (Fig. 5C and D).

Discussion

Identification of cancer-specific miRNAs may be important for understanding the role they perform in tumorigenesis and exploring novel therapeutic targets. Previous studies have demonstrated that miR-100 is downregulated in several types of malignancy, including osteosarcoma, acute myeloid leukemia, and lung and hepatocellular carcinoma (14-17). miR-100 has been identified as a potential molecular marker of non-small cell lung cancer, and functions as a tumor suppressor by targeting polo-like kinase 1 (17). miR-100 has also been demonstrated to inhibit breast cancer proliferation and survival through the suppression of insulin-like growth factor 2 and β -tubulin (18). This indicates a fundamental function for miRNA as a tumor suppressor. However, the function of miR-100 in colon cancer biology remains unclear.

In the current study, the expression and potential functions of miR-100 in the regulation of the biological properties of colon cancer cells were investigated. miR-100 expression was demonstrated to be downregulated in colon cancer tissues compared with adjacent normal tissues. The subsequent gain- and loss-of-function studies suggested that miR-100 was able to reduce colon cancer cell viability, proliferation, migration and invasion *in vitro*.

To further explore the molecular mechanisms involved in miR-100-mediated effects on biological properties, Lgr5 was selected for further study as it was predicted to be a target of miR-100 by bioinformatics analysis. Lgr5, also known as G protein-coupled receptor 49, is a regulator of Wnt signaling (19). Consistent with the dysregulation of Wnt/ β -catenin signaling, Lgr5 is overexpressed in hepatocellular carcinoma, colon and ovarian cancer, basal cell carcinoma and esophageal adenocarcinoma (20-22). This suggests an important function for Lgr5 in tumorigenesis. Adenomatous polyposis coli mutations observed exclusively in Lgr5-positive cells have been identified to be able to promote adenomatous growth in the colon of mice (23). In addition to patients, the overexpression of Lgr5 has been demonstrated to correlate with poor survival of colon cancer in mice (24). However, the precise molecular mechanisms underlying the upregulation of Lgr5 in colon cancer remain to be fully elucidated, and thorough investigation is required.

Additionally, nuclear localization of β -catenin and expression of its down-stream target genes, including cyclin D1 and cyclin E were demonstrated to be regulated by miR-100. Thus,

the downregulation of miR-100 may be an important mechanism for the aberrant activation of Wnt signaling in human cancer.

To the best of our knowledge, the results from the current study, for the first time explore the function of miR-100 in the progression of colon cancer. Future studies, including the generation of miR-100 knockout mice, are required to establish the physiological function of miR-100 in tumorigenesis.

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