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Ectopic Expression of miR-147 Inhibits Stem Cell Marker and Epithelial–Mesenchymal Transition (EMT)-Related Protein Expression in Colon Cancer Cells

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Colon cancer is one of the most common cancers in the world. Epithelial-to-mesenchymal transition (EMT) is a crucial step in tumor progression and is also involved in the acquisition of stem cell-like properties. Some miRNAs have been shown to function as either tumor suppressors or oncogenes in colon cancer. Here we investigated the role of miR-147 in the regulation of the stem cell-like traits of colon cancer cells. We observed that miR-147 was downregulated in several colon cancer cell lines, and overexpressed miR-147 decreased the expression of cancer stem cell (CSC) markers OCT4, SOX2, and NANOG in the colon cancer cell lines HCT116 and SW480. Overexpressed miR-147 inhibited EMT by increasing the expression of epithelial markers E-cadherin and α -catenin while decreasing the expression of mesenchymal markers fibronectin and vimentin. Moreover, activation of EMT by TGF- β 1 treatment significantly counteracted the inhibitive effect of miR-147 on the expression of CSC markers OCT4, SOX2, and NANOG, supporting the idea that overexpressing miR-147 inhibited stem cell-like traits by suppressing EMT in colon cancer. In addition, we found that overexpressed miR-147 downregulated the expression of β -catenin, c-myc, and survivin, which were related to the Wnt/ β -catenin pathway. Moreover, treatment of miR-147 mimic-transfected cells with the Wnt/ β -catenin pathway activator LiCl attenuated the inhibitive effect of the miR-147 mimic on the EMT and stem cell-like traits of colon cancer cells, indicating that ectopic expression of miR-147 inhibited stem cell-like traits in colon cancer cells by suppressing EMT via the Wnt/ β -catenin pathway. In summary, our present study highlighted the crucial role of miR-147 in the inhibition of the stem cell-like traits of colon cancer cells and indicated that miR-147 could be a promising therapeutic target for colon cancer treatment.

Key words: MicroRNA-147 (miR-147); Colon cancer; Epithelial-to-mesenchymal transition (EMT); Wnt/ β -catenin pathway

INTRODUCTION

Colon cancer is the fourth most common cancer in the world with high morbidity and mortality worldwide¹. Although improved treatment strategies including surgery, chemotherapy, and radiotherapy have increased the overall survival rates in the early stages, the worldwide mortality of colon cancer is still high at approximately 50%². Colon cancer arises as a result of a combination of several genetic alterations, epigenetic changes, and environmental risk factors. In the last few years, the theory of cancer stem cells (CSCs) has been reported to play an important role in tumorigenesis and tumor progression in colon cancer.

The CSC theory is the concept that cancer tissues contain a minor population of cells with stem cell properties, which are believed to contribute to the development and

expansion of cancer tissues³. CSCs have self-renewal and tumor cell differentiation abilities, which are essential for sustaining the long-term clonal maintenance of the neoplasm². CSCs were first reported by Bonnet and Dick, who observed that cluster of differentiation 44-positive/38-negative (CD44⁺/CD38⁻) leukemia cells possessed CSC properties like self-renewal to promote tumor progression in nude mice⁴. After this report, CSCs have been identified in several types of solid tumors, including colon cancer⁵. Nowadays, CSC theory provides a universal basis to understand tumor initiation and progression.

Epithelial-to-mesenchymal transition (EMT), which is a physiological process observed in embryonic development, tissue remodeling, and wound healing, is also a crucial step in tumor progression. EMT reduces intercellular adhesion and causes epithelial cells to acquire

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mesenchymal properties that increase the motility of tumor cells⁶. A previous study reported that EMT could result in the acquisition of stem cell-like properties. Mani et al. found that induction of EMT in immortalized human mammary epithelial cells resulted in the expression of stem cell markers⁷. These findings illustrated a direct link between EMT and the gain of stem cell-like properties, which promoted tumor progression.

MicroRNAs (miRNAs) are a group of small noncoding RNAs with 20–22 nucleotides that act as crucial regulators of gene expression⁸. Some miRNAs have been shown to function as either tumor suppressors or oncogenes in colon cancer^{9,10}. miR-147 was reported to act as a tumor suppressor in human hepatocellular carcinoma and breast cancer^{11,12}. Lee et al. indicated that miR-147 was involved in the mesenchymal-to-epithelial transition (MET) and played a tumor suppressor role in colon cancer¹³. However, whether miR-147 regulates stem cell-like traits in colon cancer cells is still unclear.

Our present study reported that overexpressed miR-147 suppressed stem cell-like traits by suppressing EMT via the Wnt/ β -catenin pathway in colon cancer cells. Our findings indicated that miR-147 might provide a promising therapeutic target for colon cancer treatment.

MATERIALS AND METHODS

Cell Culture and Treatment

Colon cancer cell lines (HCT116, SW480, HCT-8, and LS174T) and the human normal colonic epithelial cell line CCD 841 CoN were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Transforming growth factor- β 1 (TGF- β 1) (4 ng/ml; R&D Systems, Minneapolis, MN, USA), an EMT inducer, was used to treat transfected colon cancer cells for 24 h. LiCl (20 mM; Sigma-Aldrich, St. Louis, MO, USA), a Wnt/ β -catenin pathway activator, was used to treat transfected colon cancer cells for 24 h.

Transfection

Cells were seeded into 96-well plates to reach 60% confluence for transfection. miR-147 mimic and mimic negative control (NC) were purchased from GenePharma (Shanghai GenePharma Co. Ltd., Shanghai, P.R. China). Transfections into colon cancer cells were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNAs were prepared from cells using TRIzol reagent (Invitrogen) according to the manufacturer's

instructions. Then total RNA was reverse transcribed into cDNA using specific primers designed for miRNA analysis. Stem-loop qRT-PCR was performed using SYBR Premix Ex TaqTM (TaKaRa, Shiga, Japan) according to the manufacturer's protocol with U6 small nuclear RNA as an internal normalized reference. The relative miRNA level was calculated by the relative quantification ($2^{-\Delta\Delta Ct}$) method. All experiments were performed in triplicate.

Western Blot

Proteins were lysed by radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, P.R. China), separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred into polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. After being incubated with corresponding secondary antibodies (1:5,000; Cell Signaling Technology, Beverly, MA, USA) at room temperature for 1 h, the enhanced chemiluminescence (ECL) system (Bio-Rad Laboratories) was used for detection. The primary antibodies used in our study were anti-octamer-binding transcription factor 4 (OCT4), anti-sex-determining region Y box 2 (SOX2), anti-NANOG, anti-epithelial (E)-cadherin, and anti-vimentin (Cell Signaling Technology); anti- α -catenin, anti- β -catenin, anti-fibronectin, anti-c-myc, and anti-survivin (Abcam, Cambridge, UK); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma-Aldrich).

Statistical Analysis

All data were presented as mean \pm standard deviation (SD) using SPSS 19.0 software. Statistical significance was tested by a Student's *t*-test. The difference was considered statistically significant with a value of $p < 0.05$.

RESULTS

Overexpressed miR-147 Inhibits Stem Cell-Like Traits in Colon Cancer Cells

First, we detected the expression of miR-147 in colon cancer cells, and we observed that miR-147 expression was significantly downregulated in the colon cancer cell lines HCT116, SW480, HCT-8, and LS174T, compared with the human normal colonic epithelial cell line CCD 841 CoN cells ($p < 0.05$, $p < 0.01$) (Fig. 1A). To determine the role of miR-147 in regulating stem cell-like traits, colon cancer cell lines HCT116 and SW480 were transfected with miR-147 mimic to increase the expression of miR-147, respectively ($p < 0.01$) (Fig. 1B). Our data showed that overexpressed miR-147 decreased the relative expression of colon CSC markers OCT4, SOX2,

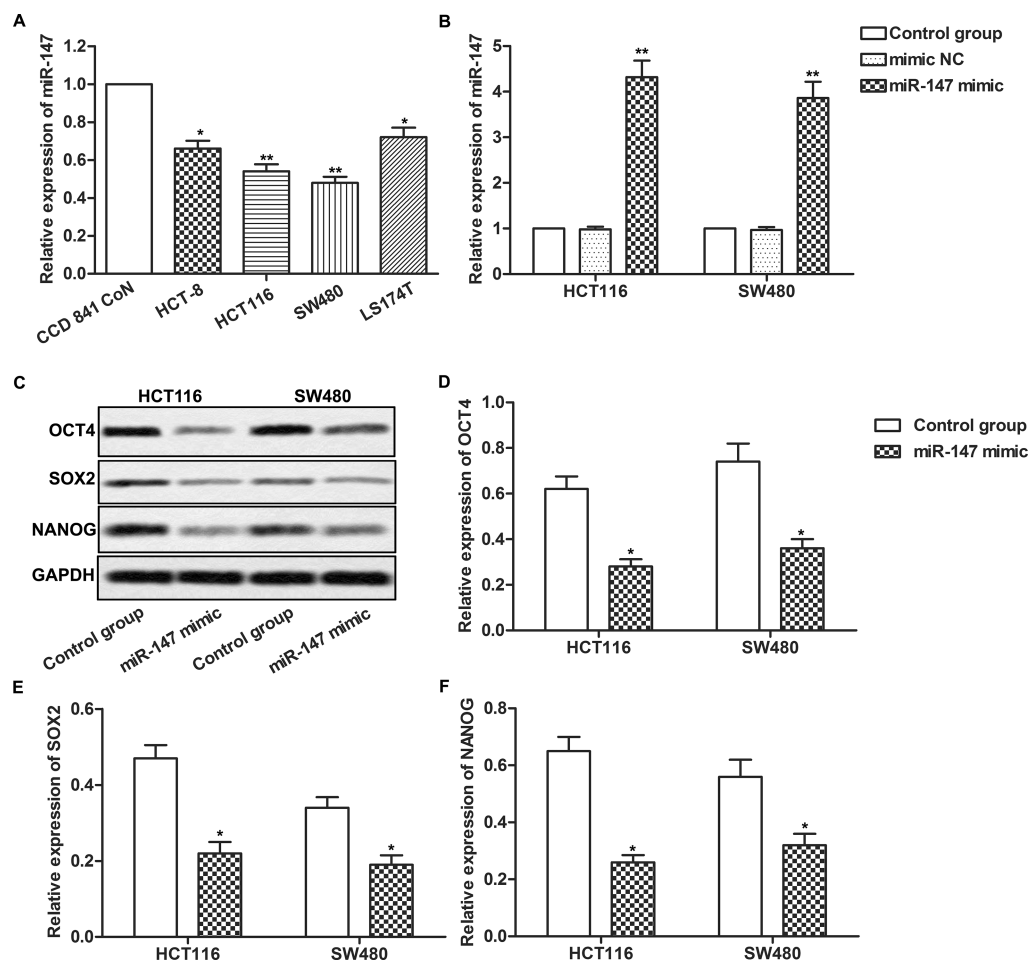


Figure 1. Overexpressed microRNA-147 (miR-147) inhibits stem cell-like traits in colon cancer cells. (A) Relative expression of miR-147 was detected in colon cancer cell lines (HCT116, SW480, HCT-8, and LS174T) and human normal colonic epithelial cell line CCD 841 CoN cells through quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). (B) Colon cancer cells (HCT116 and SW480) were transfected with miR-147 mimic or mimic negative control (NC), respectively, with untreated cells as the control group. Relative expression of miR-147 was compared among these groups through qRT-PCR. (C–F) Relative protein level of OCT4, SOX2, and NANOG was detected by Western blot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The bars showed means \pm standard deviation (SD) of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with control group.

and NANOG compared with the control group ($p < 0.05$) (Fig. 1C–F). These results suggested that overexpressed miR-147 inhibited stem cell-like traits in colon cancer cells.

Overexpressed miR-147 Suppresses EMT in Colon Cancer Cells

EMT was reported to result in the acquisition of stem cell-like properties; thus, we explored the effect of miR-147 on EMT in colon cancer cells. Upregulation of miR-147 increased the relative expression of epithelial markers E-cadherin and α -catenin, and decreased the relative expression of mesenchymal markers fibronectin and vimentin in both the HCT116 and SW480 cells

($p < 0.05$) (Fig. 2A–F). Our data supported that upregulation of miR-147 inhibited EMT in colon cancer cells.

EMT Inducer TGF- β 1 Promotes Stem Cell-Like Traits in Colon Cancer Cells

HCT116 cells were used for the following experiments. TGF- β 1 was used to treat cells for activation of EMT. After TGF- β 1 treatment, the expression of E-cadherin was decreased while the expression of vimentin was increased significantly in the miR-147 mimic + TGF- β 1 group compared with the miR-147 mimic group, indicating that TGF- β 1 weakened the inhibitive effect of miR-147 mimic on EMT in colon cancer cells ($p < 0.05$, $p < 0.01$) (Fig. 3A and B). Moreover, activation of EMT by TGF- β 1

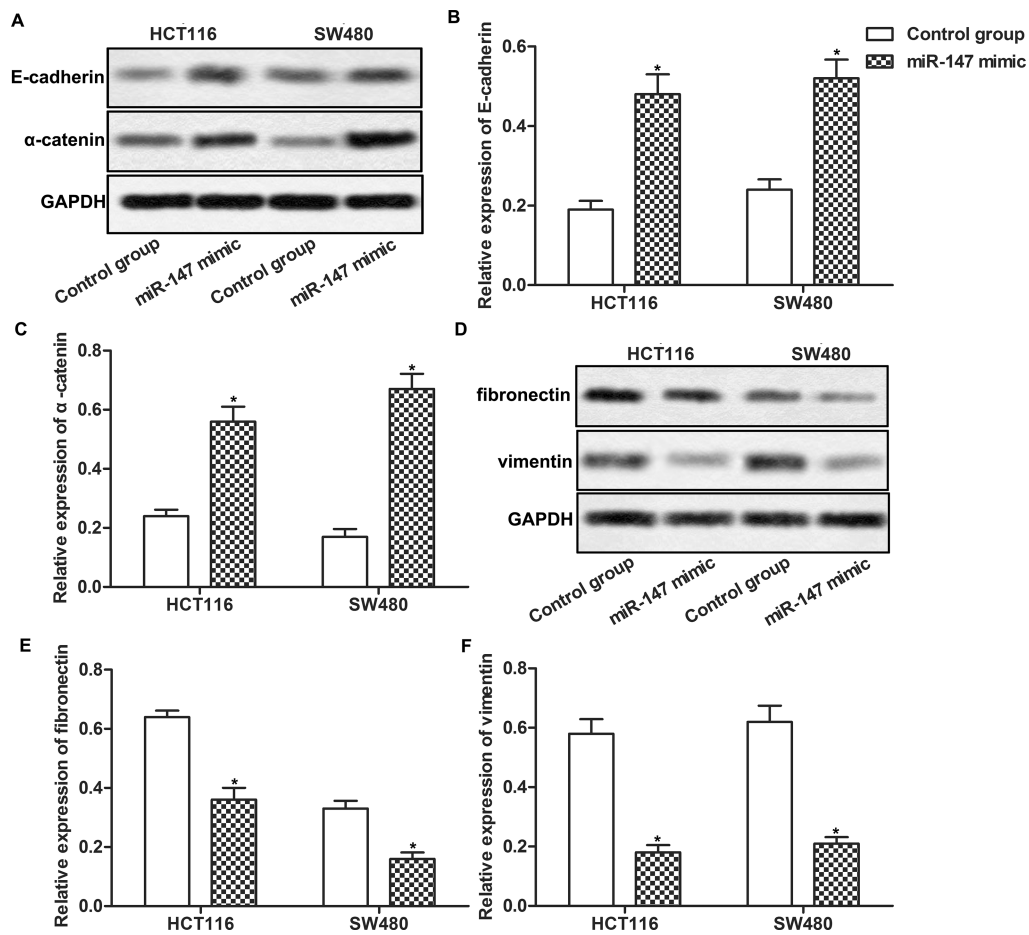


Figure 2. Overexpressed miR-147 suppresses epithelial-to-mesenchymal transition (EMT) in colon cancer cells. HCT116 and SW480 colon cancer cells were transfected with or without miR-147 mimic. (A–C) Relative protein levels of E-cadherin and α -catenin were detected by Western blot. (D–F) Relative protein levels of fibronectin and vimentin were detected by Western blot. GAPDH was used as an endogenous reference. The bars showed means \pm SD of three independent experiments. * $p < 0.05$ compared with control group.

resulted in upregulation of CSC markers OCT4, SOX2, and NANOG. The expressions of these stem cell markers were all increased in the miR-147 mimic + TGF- β 1 group compared with the miR-147 mimic group, suggesting that activation of EMT counteracted the inhibitive effect of miR-147 mimic on stem cell-like properties in colon cancer cells ($p < 0.05$) (Fig. 3C and D). Our results also indicated that overexpressed miR-147 inhibited stem cell-like traits in colon cancer cells by suppressing EMT.

Overexpressed miR-147 Inhibits the Wnt/ β -Catenin Pathway

The Wnt/ β -catenin pathway was reported to be involved in the EMT of cancer cells¹⁴. In our study, we detected the expression of β -catenin, c-myc, and survivin, which were related to the Wnt/ β -catenin pathway by Western blot analysis. The data revealed that overexpression of miR-147 decreased the relative expression of β -catenin, c-myc, and survivin compared with the

control group, indicating that overexpressed miR-147 suppressed the Wnt/ β -catenin pathway ($p < 0.05$) (Fig. 4A and B). Treatment with the Wnt/ β -catenin pathway activator LiCl decreased the expression of E-cadherin and increased the expression of vimentin, suggesting that activation of the Wnt/ β -catenin pathway promoted EMT. Moreover, the expression of E-cadherin was decreased while the expression of vimentin was increased by LiCl treatment in the miR-147 mimic + LiCl group compared with the miR-147 mimic group, suggesting that activation of the Wnt/ β -catenin pathway alleviated the inhibitory effect of miR-147 mimic on EMT in colon cancer cells ($p < 0.05$, $p < 0.05$) (Fig. 4C and D). LiCl treatment increased the relative expression of OCT4, SOX2, and NANOG compared with the miR-147 mimic group, revealing that activation of the Wnt/ β -catenin pathway counteracted the inhibitive effect of miR-147 mimic on stem cell-like traits in colon cancer cells ($p < 0.05$) (Fig. 4E and F). In summary, these data revealed that

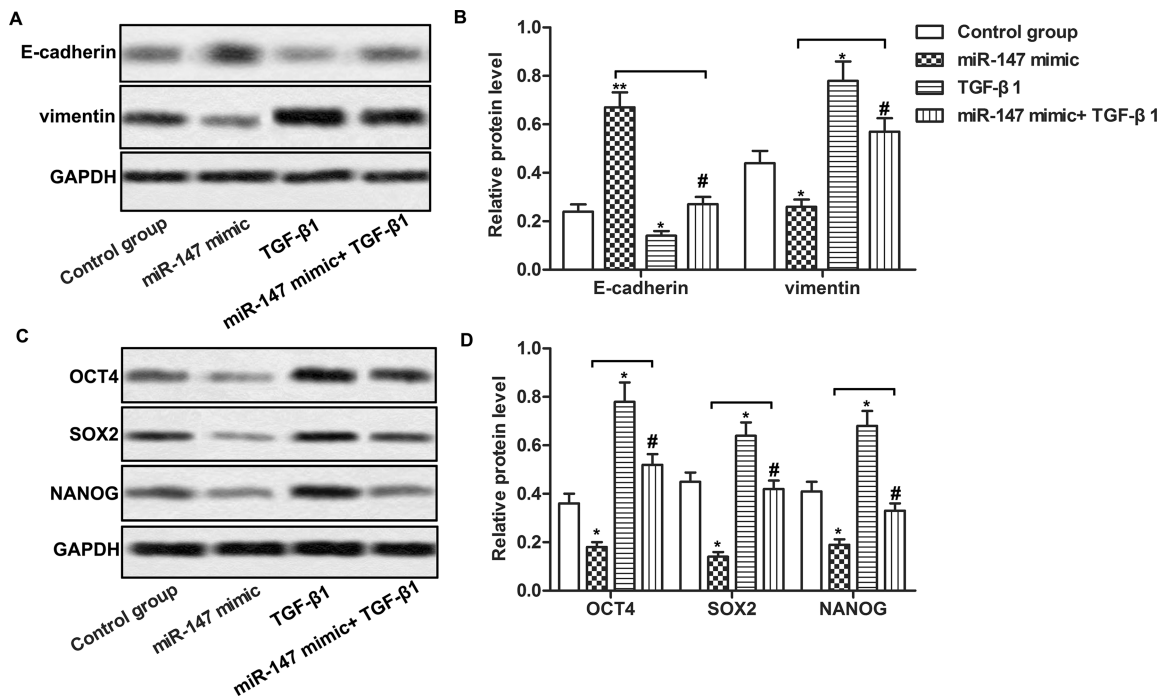


Figure 3. EMT inducer transforming growth factor- β 1 (TGF- β 1) promotes stem cell-like traits in HCT116 colon cancer cells. Colon cancer cells transfected with or without miR-147 mimic were treated with or without TGF- β 1. (A, B) Relative protein levels of E-cadherin and vimentin were detected by Western blot. (C, D) Relative protein levels of OCT4, SOX2, and NANOG were detected by Western blot. GAPDH was used as an endogenous reference. The bars showed means \pm SD of three independent experiments. * p <0.05, ** p <0.01 compared with control group. # p <0.05 compared with miR-147 mimic group.

miR-147 overexpression inhibited the Wnt/ β -catenin pathway and overexpressed miR-147 inhibited stem cell-like traits in colon cancer cells by suppressing EMT via the Wnt/ β -catenin pathway.

DISCUSSION

Colon cancer is a common malignant tumor with high morbidity and mortality worldwide¹⁵. CSCs are considered as the seed cells for tumor initiation, proliferation, and metastasis; therefore, improving our understanding of the CSC mechanism could lead to improvement of therapies for cancer. In our present study, we found that miR-147 was downregulated in colon cancer cells, and overexpression of miR-147 inhibited stem cell-like traits in colon cancer cells by suppressing EMT via the Wnt/ β -catenin pathway, indicating that miR-147 might be an effective target for colon cancer treatment.

With the development of the CSC theory, CSCs have been found in various solid tumors including gastric cancer, pancreatic cancer, and breast cancer^{16–18}. Ricci-Vitiani et al. reported that a small number of undifferentiated tumorigenic CD133⁺ cells might be human colon cancer-initiating cells⁵. Other cell surface proteins have also contributed to the isolation and identification of CSCs.

The transcription factors OCT4, SOX2, and NANOG were also considered as CSC markers^{19,20}.

Several reports revealed the effects of miRNAs in tumor progression, such as regulating the cell proliferation, migration, and invasion of tumor cells^{21,22}. It was reported that a great number of miRNAs were involved in the stem cell-like traits in colon cancer^{11,23,24}. For example, Xi et al. demonstrated that miR-17 induced EMT consistent with the CSC phenotype by regulating cytochrome p450 family 7 subfamily B member 1 (CYP7B1) expression in colon cancer²³. Sakaguchi et al. reported that the expression of miR-137 was downregulated in colon CSCs compared with normal colon stem cells (NCSCs) and that miR-137 regulated the tumorigenicity of colon CSCs through the inhibition of doublecortin-like kinase 1 (DCLK1)²⁴. Here our present study explored the interaction between miR-147 and stem cell-like traits in colon cancer cells. miR-147 was reported to act as a tumor suppressor, which inhibited cell proliferation and increased chemosensitivity to 5-fluorouracil (5-FU) in both gastric cancer and hepatocellular carcinoma^{11,25}. In colon cancer, miR-147 was found to induce cancer cells to reverse EMT and induce cell cycle arrest¹³. However, whether miR-147 regulated stem cell-like traits in colon cancer cells has not been previously reported. Our study is the first to

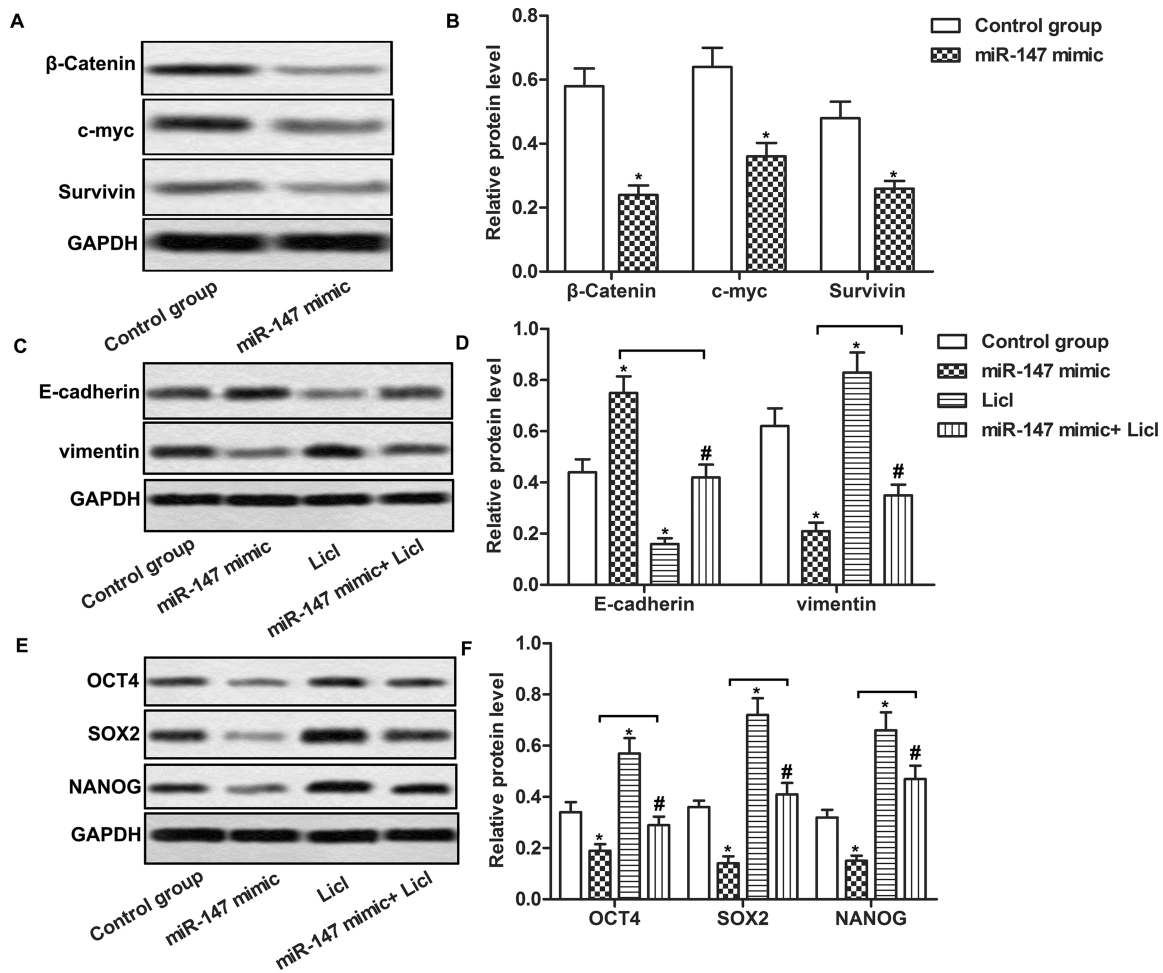


Figure 4. Overexpressed miR-147 inhibits the Wnt/ β -catenin pathway. Colon cancer cells transfected with or without miR-147 mimic were treated with or without LiCl. (A, B) Relative protein levels of β -catenin, c-myc, and survivin were compared between control group and miR-147 mimic group by Western blot. (C, D) Relative protein levels of E-cadherin and vimentin were detected by Western blot. (E, F) Relative protein levels of OCT4, SOX2, and NANOG were detected by Western blot. GAPDH was used as an endogenous reference. The bars showed means \pm SD of three independent experiments. * p < 0.05 compared with the control group. # p < 0.05 compared with miR-147 mimic group.

report that miR-147 was downregulated in colon cancer cells and overexpressed miR-147 in colon cancer cells (HCT116 and SW480), resulting in downregulation of CSC markers OCT4, SOX2, and NANOG significantly, suggesting that ectopic expression of miR-147 inhibited stem cell-like traits in colon cancer cells.

EMT is an important process in tumor progression. A number of studies demonstrated that cancer cells, which underwent EMT, reduced intercellular adhesion and increased motility in lung cancer, ovarian cancer, and gastric cancer²⁶⁻²⁸. Studies suggested that EMT was also involved in the acquisition of stem cell-like properties⁷. EMT was a key program in generating CSCs and maintaining their characteristics²⁹. miR-147 was identified to cause MET primarily by increasing the expression of E-cadherin (CDH1) and decreasing that of zinc

finger E-box-binding homeobox 1 (ZEB1) in colon cancer cells¹³. In agreement with a previous study, we found that overexpression of miR-147 in colon cancer cells (HCT116 and SW480) by transfection with miR-147 mimic caused upregulation of epithelial markers E-cadherin and α -catenin and downregulation of mesenchymal markers fibronectin and vimentin¹³. Our results indicated that miR-147 inhibited stem cell-like traits by suppressing EMT in colon cancer. To further verify our conjecture, an EMT inducer TGF- β 1 was used to treat the miR-147 mimic-transfected HCT116 colon cancer cells. We found that TGF- β 1 treatment activated EMT by decreasing the expression of E-cadherin and increasing the expression of vimentin compared with the miR-147 mimic group. Moreover, TGF- β 1 treatment counteracted the inhibitive effect of miR-147 on the expression of CSC markers

OCT4, SOX2, and NANOG significantly, supporting our conjecture that overexpressed miR-147 inhibited stem cell-like traits by suppressing EMT in colon cancer.

The role of the Wnt/ β -catenin pathway in tumor progression such as proliferation, metastasis, and apoptosis had been well documented in a number of cancers^{30,31}. Evidence suggested that the Wnt/ β -catenin pathway played a regulatory role in EMT. It was reported that activation of Wnt/ β -catenin signaling inhibitors by ten-eleven translocation methylcytosine dioxygenase 1 (TET1) could inhibit EMT of ovarian cancer cells³². Liang et al. also demonstrated that the Wnt/ β -catenin pathway modulated tobacco smoke (TS)-triggered EMT as demonstrated by TS-mediated EMT being attenuated by Wnt/ β -catenin inhibition³³. In our present study, we observed that overexpressed miR-147 decreased the expression of β -catenin, c-myc, and survivin, which are related to the Wnt/ β -catenin pathway, indicating that overexpressed miR-147 inhibited the Wnt/ β -catenin pathway in colon cancer. Moreover, treatment with the Wnt/ β -catenin pathway activator LiCl in miR-147 mimic-transfected cells attenuated the inhibitive effect of miR-147 mimic on EMT and stem cell-like traits of colon cancer cells. Taken together, our present study revealed that the ectopic expression of miR-147 inhibited stem cell-like traits in colon cancer cells through suppressing EMT via the Wnt/ β -catenin pathway.

In summary, the data collected in our present study highlighted the crucial role of miR-147 in the inhibition of stem cell-like traits of colon cancer cells. Therapeutic targeting of miR-147 may be a novel method for colon cancer treatment.

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