

MicroRNA-17 induces epithelial-mesenchymal transition consistent with the cancer stem cell phenotype by regulating CYP7B1 expression in colon cancer

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Abstract. MicroRNA-17 (miRNA-17/miR-17) expression has been confirmed to be significantly higher in colorectal cancer tissues than in normal tissues. However, its exact role in colorectal cancer has not yet been fully elucidated. In this study, we found that miR-17 not only promoted epithelial-mesenchymal transition (EMT), but also promoted the formation of a stem cell-like population in colon cancer DLD1 cells. We also wished to determine the role of cytochrome P450, family 7, subfamily B, polypeptide 1 (CYP7B1) in CRC. miR-17 was overexpressed using a recombinant plasmid and CYP7B1 was silenced by transfection with shRNA. Western blot analysis was used to determine protein expression in the DLD1 cells and in tumor tissues obtained from patients with colon cancer. Our results revealed that miR-17 overexpression led to the degradation of CYP7B1 mRNA expression in DLD1 cells. In addition, we found that the silencing of CYP7B1 promoted EMT and the formation of a stem cell-like population in the cells. Thus, our findings demonstrate that miR-17 induces EMT consistent with the cancer stem cell phenotype by regulating CYP7B1 expression in colon cancer.

Introduction

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality. Nearly 150,000 US residents are diagnosed annually with CRC, and approximately one-third of patients with CRC succumb to the disease (1). The lifetime risk of CRC

in the US is 6%, and the average age at diagnosis is 66 years (2). Primary CRC originates from epithelial cells that line the gastrointestinal tract (3). During progression to metastasis, cancer cells are thought to acquire a mesenchymal phenotype, which allows them to leave the site of the primary tumor, invade surrounding tissues, and migrate to distant organs. After seeding, these cells switch back to an epithelial phenotype and proliferate to form metastases (4). The processes by which cells switch between the epithelial and mesenchymal phenotypes are known as epithelial-to-mesenchymal transition (EMT) and its counterpart, mesenchymal-to-epithelial transition (MET) (5). However, the molecular mechanisms responsible for EMT in CRC are not yet fully understood.

The steroid hydroxylase cytochrome P450, family 7, subfamily B, polypeptide 1 (CYP7B1), a member of the cytochrome P450 enzyme family, has attracted increasing attention over the years due to its multiple reported roles for key events in cellular physiology (6-14). CYP7B1 is widely expressed in tissues of human and other species and metabolizes several steroids involved in hormonal signaling and other processes. Substrates for CYP7B1 include 5 α -androstane-3 β , 17 β -diol (3 β -Adiol), an estrogen receptor (ER) agonist and dehydroepiandrosterone (DHEA), an essential precursor for androgens and estrogens (13-18). CYP7B1 expression is diminished in ER⁺ tumors and is predictive of overall survival in breast cancer (19,20). However, its role in CRC is not yet fully understood.

MicroRNAs (miRNAs or miRs), are small non-coding RNAs which are 21-25 nt in length, and are widely expressed in eukaryotic cells, functioning as post-translational regulators (21). Due to their wide variety of target genes, miRNAs affect a number of biological pathways, including cell proliferation, development and differentiation. The deregulation of miRNAs facilitates cancer development by upregulating oncogenes or silencing tumor suppressor genes (22). miRNAs have been demonstrated to regulate the expression levels of major cancer-related genes and hence may be useful in the treatment of cancer (23,24).

In this study, we found that miR-17 not only promoted EMT, but also promoted the formation of a stem cell-like population in colon cancer DLD1 cells. Our results revealed that miR-17

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degrade CYP7B1 mRNA expression in DLD1 cells. In addition, we found that the silencing of CYP7B1 promoted EMT and the formation of a stem cell-like population in the colon cancer cells. Thus, our findings suggest that miR-17 induces EMT consistent with the cancer stem cell phenotype by regulating CYP7B1 expression in colon cancer.

Materials and methods

Cell culture and tissues samples. DLD1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) (both from HyClone, Ogden, UT, USA) and penicillin-streptomycin. Normal and tumor tissues were obtained from 6 patients were recruited from Shandong Provincial Qianfoshan Hospital, Jinan, China. The tissues were obtained during colon cancer surgery. Normal tissues were adjacent to the tumor tissues. None of the patients received any anti-cancer treatment prior to surgery. The use of human tissue samples was carried out in accordance with internationally recognised guidelines as well as local and national regulations. This study was approved by the Ethics Committee of Shandong Provincial Qianfoshan Hospital and all patients provided written informed consent prior to obtaining the samples.

Plasmids and transfection. The shRNA plasmids were obtained from Tiangen (Beijing, China). Both scramble control sequence and interference sequence (shRNA) of CYP7B1 were designed and synthesized to build the recombinant plasmids; Pre-miR-17 and control miR (purchased from Ambion, Inc. Austin, TX, USA). The cells were cultured in serum-free medium without antibiotics and then separately transfected with shCYP7B1/scramble plasmids or Pre-miR-17/control miR using transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blot analysis. The tissues or cells were homogenized in RIPA lysis buffer containing PMSF and centrifuged at 200 x g for 10 min. Protein lysates were separated by electrophoresis and transferred onto PVDF membranes, and the blots were blocked with 5% non-fat milk for 1 h and incubated overnight at 4°C with primary antibodies against CYP7B1 (ab175889; 1:500), vimentin (ab92547; 1:500), SNAIL (ab82846; 1:500), transforming growth factor beta 1 (TGFB1; ab92486; 1:500), zinc finger E-box binding homeobox (ZEB)1 (ab203829; 1:500), ZEB2 (ab138222; 1:500), Twist (ab50581; 1:500), β -catenin (ab32572; 1:500), Notch1 (ab8925; 1:500), β -actin (ab8227; 1:500) and CD44 (ab157107; 1:500) (all from Abcam, Cambridge, MA, USA). After washing, the blots were incubated with secondary antibodies (anti-rabbit secondary antibodies; ab6721; 1:10,000; Abcam). The protein bands were visualized by chemiluminescence and exposed to the Odyssey™ Infrared Imaging system (Gene Company, Lincoln, NE, USA).

Sphere growth analysis. The DLD1 cells transfected with shCYP7B1/scramble plasmids (1×10^3) in serum-free DMEM/1 mM Na-pyruvate were seeded on 0.5% agar pre-coated 6-well plates. After 1 week, half the medium was exchanged with serum-free medium every third day. Single

spheres were selected and counted under a stereomicroscope (Olympus, Tokyo, Japan).

In vitro migration and invasion assays. The DLD1 cells transfected as indicated above (1×10^5) were placed into the upper compartment of the Transwell insert (Costar, Cambridge, MA, USA). For invasion assay, the two compartments were separated by a porous filter (8 μ m pore) coated with Matrigel (BD Biosciences, San Jose, CA, USA). For migration assay, the filter membranes was not coated with Matrigel. The chambers were incubated for 24 h at 37°C, and the filters were then fixed in methanol and stained with hematoxylin. Quantification of the migration and invasion assays were performed by counting the number of cells at the lower surface of the filters.

Bioinformatics analysis. Potential targets of miRNAs were identified by a combined approach based on the commonly used web tool for bioinformatics algorithms miRanda (<http://www.microrna.org/microrna/home.do>).

Quantitative (real-time) polymerase chain reaction (qPCR) for miR-17. qPCR for miR-17 was performed using the total RNA kit I (Omega Bio-Tek, Norcross, GA, USA) and the miRcute miRNA qPCR detection kit (Tiangen). U6 miRNA was used as a housekeeping control. Small RNA was purified and enriched with the miRcute miRNA Isolation kit (Tiangen). miRNAs were prolonged by *Escherichia coli* poly(A) polymerase, and reverse transcription was performed with the miRcute miRNA First-Strand cDNA Synthesis kit and real-time PCR with the miRcute miRNA qPCR detection kit (Tiangen). Forward primers and reverse primers were provided by Tiangen. The universal reverse primer was provided in the miRcute miRNA qPCR detection kit.

Reverse transcription-quantitative PCR (RT-qPCR) for CYP7B1. Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen). cDNA was synthesized from 1 μ g of total RNA in a 20 μ l reverse transcription (RT) system followed by PCR amplification in a 50 μ l PCR system performed using an RT-PCR kit (Promega, Madison, WI, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an RNA loading control. The PCR primer sequences are as follows: CYP7B1 forward, 5'-CAATCCATGCAGTCACCTTC-3' and reverse, 5'-TGCCTAGAGAAAACAGAAAGACA-3'; and GAPDH forward, 5'-ATTCAACGGCACAGTCAAGG-3' and reverse, 5'-GCAGAGGGGCGGAGATGA-3'. PCR was conducted according to the manufacturer's instructions and the PCR products were analyzed by agarose gel electrophoresis. Gels were photographed and the densities of the bands were determined with a computerized image analysis system (Alpha Innotech, San Leandro, CA, USA). The area of each band was calculated as the integrated density value (IDV). Real-time PCR for CYP7B1 was performed with a Power SYBR-Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Immunofluorescence staining. The cells transfected as indicated above were fixed with paraformaldehyde and permeabilized in Triton X-100 (Beijing Solarbio Biological Technology Co., Ltd., Beijing, China). After blocking,

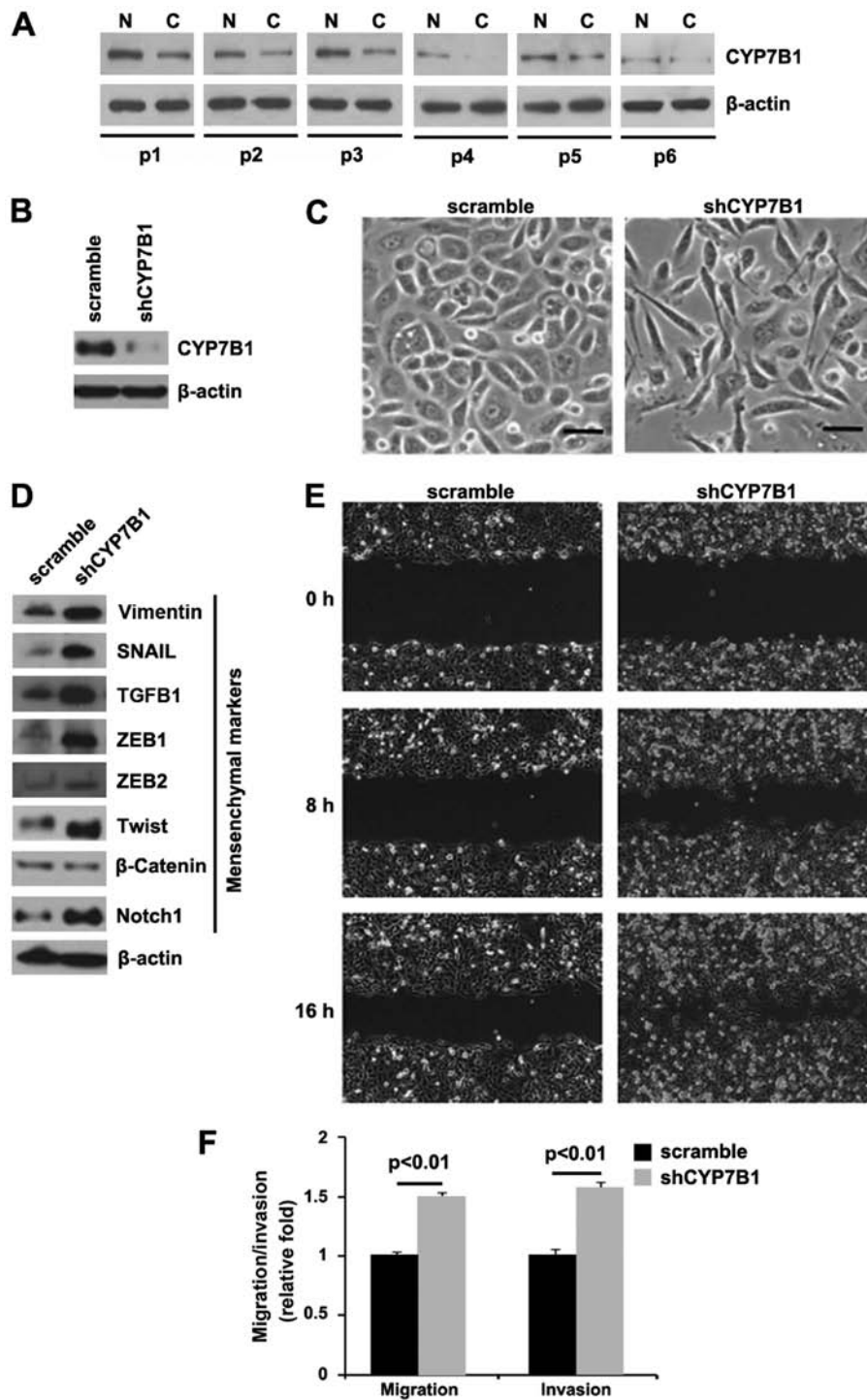


Figure 1. Silencing of CYP7B1 promotes epithelial-mesenchymal transition (EMT) in colon cancer DLD1 cells. (A) Western blot analysis for CYP7B1 in colon cancer tissues (C) and adjacent normal tissues (N). Patients were numbered no. 1-6. All the 6 patients were diagnosed with colon cancer. β -actin was used as a loading control, n=6. (B) Western blot analysis for CYP7B1 in DLD1 cells transfected with shCYP7B1 plasmid or the scramble plasmid. β -actin was used as a loading control, n=3 experiments. (C) DLD1 cells were transfected as indicated. Cells were then photographed following transfection, n=3 experiments. (D) Western blot analysis for vimentin, SNAIL, TGFB1, ZEB1, ZEB2, Twist, β -catenin and Notch1 in DLD1 cells transfected with shCYP7B1 plasmid or scramble plasmid. β -actin was used as a loading control, n=3 experiments. (E) Wound-healing assays for DLD1 cells transfected with shCYP7B1 plasmid or scramble plasmid. The cell layer was photographed following transfection, n=3 experiments. (F) Invasion and migration assays for DLD1 cells transfected as indicated, n=3 experiments.

anti-CYP7B1 antibody (ab175889; Abcam) was added followed by incubation overnight at 4°C. After washing with phosphate-buffered saline (PBS), fluorescence-conjugated anti-rabbit secondary antibodies (ab6721; 1:10,000; Abcam) were added, and the coverslips were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen-Molecular

Probes, Eugene, OR, USA) for visualization of the nuclei. Microscopic analysis was observed under a Zeiss LSM-510 confocal microscope (Carl Zeiss, Jena, Germany).

Wound healing assay. The cells transfected as indicated above were seeded into a 24-well plate in DMEM containing 10% FBS

and cultured to 90% confluence. The cell monolayer was subjected to a mechanical scratch wound using a sterile pipette tip. After washing with PBS, the cells were further incubated in DMEM without FBS for different periods of time. Digitized images of the wound area were captured using a IX71 fluorescence microscope (Olympus).

Statistical analysis. The results are shown as the means \pm SEM. The Student's t-test was used to perform comparisons between two groups. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Silencing of CYP7B1 promotes EMT in colon cancer cells. In an attempt to examine CYP7B1 expression between colon cancer tissues and adjacent normal tissues, we performed western blot analysis using the cancer tissues and normal tissues. Protein was isolated from 6 pairs of colon cancer tissues and normal tissues (patient nos. 1-6). We found that CYP7B1 protein expression was significantly decreased in the cancer tissues compared with the adjacent normal tissues (Fig. 1A). This suggests that CYP7B1 may be a tumor suppressor gene in colon cancer.

In order to determine the role of CYP7B1 in colon cancer, we transfected the DLD1 cells with shCYP7B1 plasmid and western blot analysis was then performed. We found that CYP7B1 protein expression was significantly decreased in the cells transfected with the shCYP7B1 plasmid (Fig. 1B) and the silencing of CYP7B1 led to significant changes in DLD1 cell morphology (EMT, change in phenotype from a cobblestone-like to a spindle-like morphology) (Fig. 1C).

To further verify that the changes in cell morphology were caused by EMT, the expression levels of mesenchymal markers were compared in the DLD1 cells transfected with the shCYP7B1 plasmid and the cells transfected with the scramble plasmid. The results revealed that the expression of the mesenchymal markers (vimentin, SNAIL, TGFB1, ZEB1, ZEB2, Twist and Notch1) was induced by the silencing of CYP7B1 in the DLD1 cells (Fig. 1D).

EMT can result in increased cell invasion and migration (25-27). Thus, we hypothesized that shCYP7B1 may also affect the invasion and migration ability of the DLD1 cells. To confirm this hypothesis, we performed cell invasion and migration assays, and wound healing assay. We found that the silencing of CYP7B1 enhanced the migration (Fig. 1E and F) and invasion (Fig. 1F) ability of the cells.

Silencing of CYP7B1 promotes the formation of a stem cell-like population in colon cancer cells. EMT not only confers tumor cells with a distinct advantage for metastatic dissemination, but it also provides those cells with cancer stem cell-like characters for proliferation and drug resistance (28-31). To determine whether colon cancer cells with an EMT phenotype have stem-like cell characteristics, sphere forming assay was conducted to assess the capacity of cancer stem cells (CSCs) or CSC-like cell self-renewal in this study. We found that the formation of spheres was increased by the silencing of CYP7B1 in the DLD1 cells (Fig. 2A). CD44 is a robust marker and is of functional importance for colorectal CSCs for cancer initiation (32). We also performed immunofluorescence staining

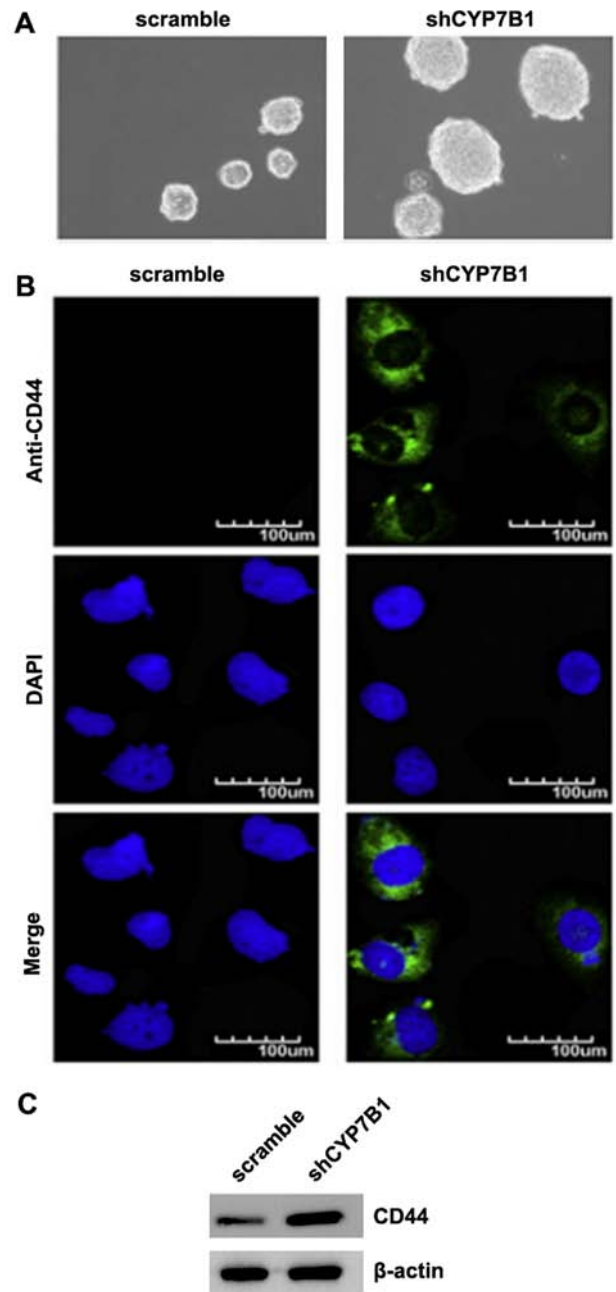


Figure 2. Silencing of CYP7B1 promotes the formation of a stem cell-like population in colon cancer DLD1 cells. (A) Sphere growth in DLD1 cells transfected as indicated. (B) Immunofluorescence staining of DLD1 cells transfected with shCYP7B1 or scramble shRNA, $n=3$ experiments. (C) Western blot analysis for CD44 in DLD1 cells transfected with shCYP7B1 or scramble shRNA. β -actin was used as a loading control, $n=3$ experiments.

to determine whether CD44 was affected by the silencing of CYP7B1 in the cells. The results revealed that CD44 protein was significantly increased by the silencing of CYP7B1 in the DLD1 cells (Fig. 2B). Consistent with the results of immunofluorescence staining, the results of western blot analysis demonstrated that CD44 protein expression was increased by the silencing of CYP7B1 in the cells (Fig. 2C).

miR-17 degrades CYP7B1 in colon cancer cells. Having demonstrated that the silencing of CYP7B1 promotes EMT and the formation of a stem cell-like population in colon

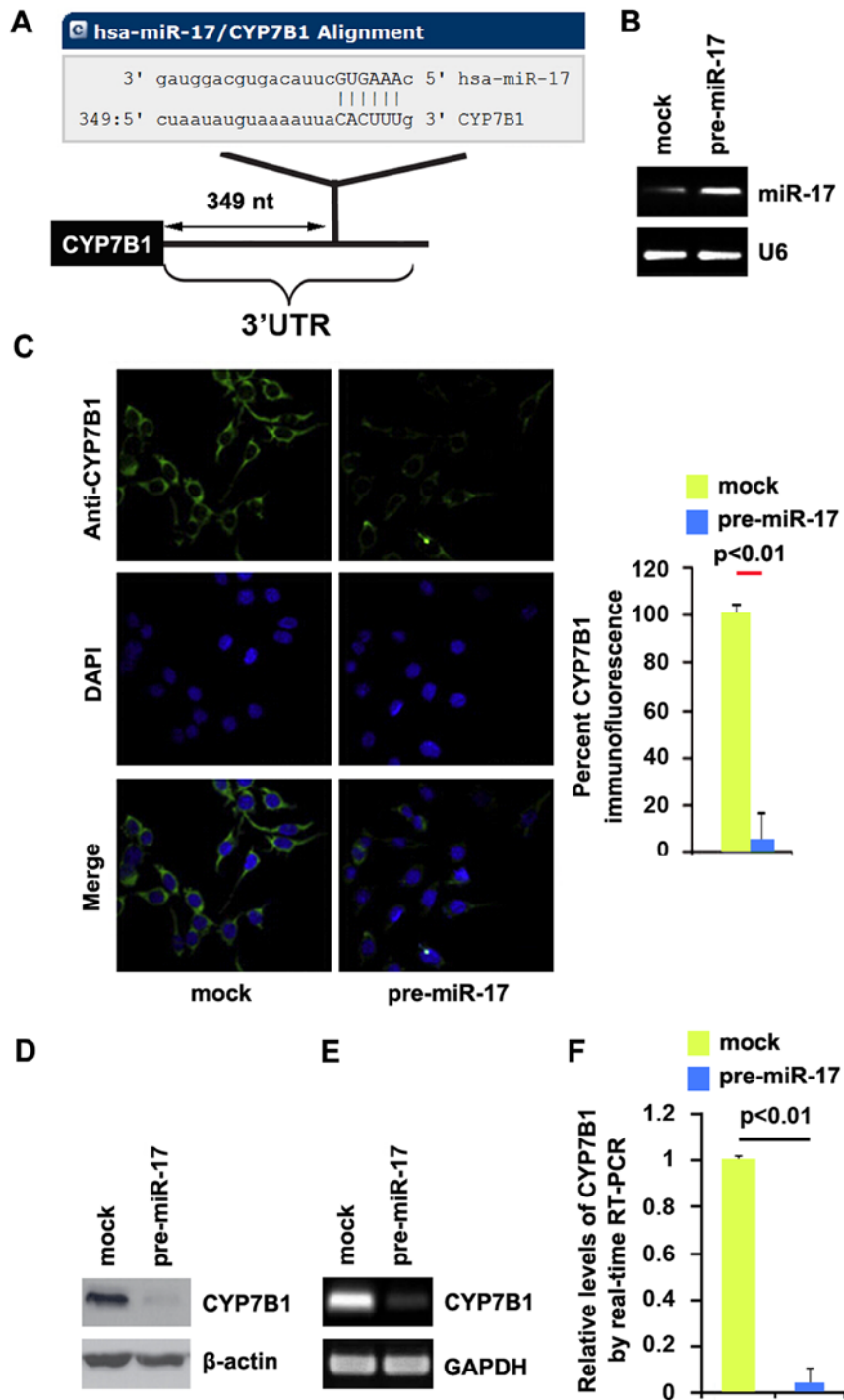


Figure 3. miR-17 degrades CYP7B1 mRNA in colon cancer DLD1 cells. (A) Schematic of predicted miR-17-binding sites in the 3' untranslated region (3'UTR) of CYP7B1 mRNA by miRanda. (B) qPCR for miR-17 in DLD1 cells transfected with pre-miR-17 or control miR (mock). U6 was used as a loading control, n=3 experiments. (C) Immunofluorescence staining for CYP7B1 in DLD1 cells transfected with pre-miR-17 or control miR (mock). Left panel shows microscopic images of immunofluorescence staining of one representative experiment (magnification, x100). Right panel shows graphic presentation of mean fluorescence intensities, n=3 experiments. (D) Western blot analysis for CYP7B1 in DLD1 cells. DLD1 cells were transfected with pre-miR-17 or control miR (mock). β -actin was used as a loading control, n=3 experiments. (E) RT-PCR for CYP7B1 in DLD1 cells. DLD1 cells were transfected with pre-miR-17 or control miR (mock). GAPDH was used as a loading control, n=3 experiments. (F) qPCR for CYP7B1 in DLD1 cells. DLD1 cells were transfected with pre-miR-17 or control miR (mock). GAPDH was used as a loading control, n=3 experiments.

cancer cells, we then wished to determine the mechanisms regulating CYP7B1 expression in the disease. miRNAs are small regulatory non-coding RNAs of 21-25 nucleotides in length. miRNAs are generated from their precursor transcripts by a series of processing steps. Mature miRNAs mediate mRNA degradation or suppress mRNA translation by binding

to the 3' untranslated region (3'UTR) of target mRNAs (33). To further confirm whether CYP7B1 can be regulated by miRNAs, we used the commonly used prediction algorithm, miRanda (<http://www.microrna.org/microrna/home.do>), to analyze the 3'UTR of CYP7B1. A dozen miRNAs were found by the algorithm. However, we focused on miR-17, as miR-17

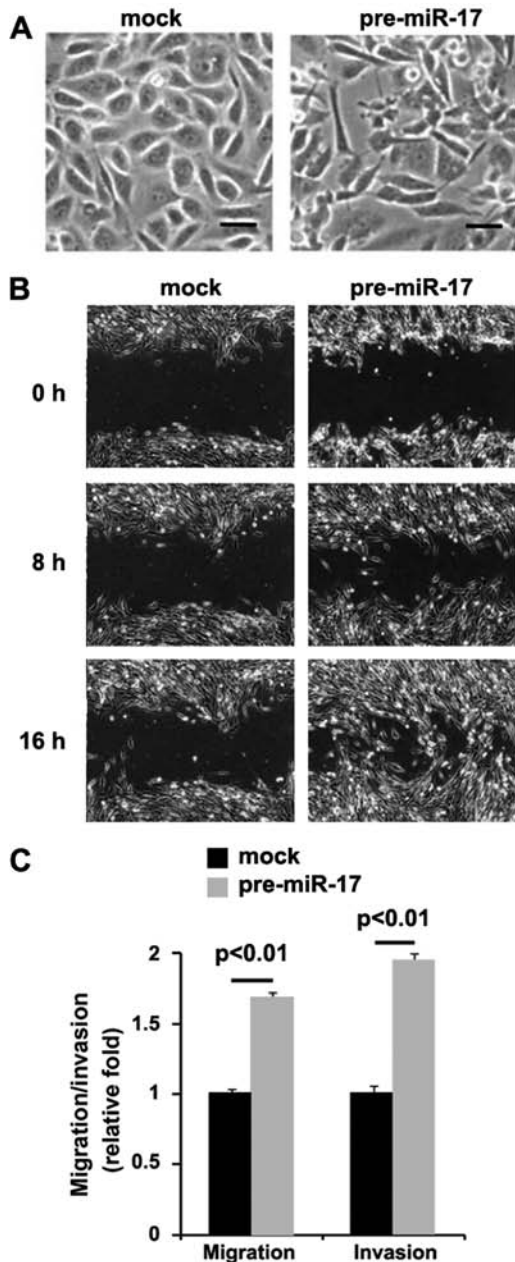


Figure 4. miR-17 promotes the epithelial-mesenchymal transition (EMT) phenotype in colon cancer DLD1 cells. (A) DLD1 cells were transfected with pre-miR-17 or control miR (mock). Cells were then photographed following transfection, n=3 experiments. (B) Wound-healing assays for DLD1 cells transfected with pre-miR-17 or control miR (mock). The cell layer was photographed, n=3 experiments. (C) Invasion and migration assays for DLD1 cells transfected with pre-miR-17 or control miR (mock), n=3 experiments.

expression has been confirmed to be significantly higher in CRC tissues than in normal tissues (34). However, its role in CRC has not yet been fully elucidated. The target sites on the 3'UTR of CYP7B1 are shown in Fig. 3A. We reasoned that miR-17 may downregulate CYP7B1 expression by targeting its 3'UTR in colon cancer. In an attempt to determine the role of miR-17 in regulating CYP7B1 expression in colon cancer cells, the DLD1 cells were transfected with pre-miR-17 or control miR. Following transfection, miR-17 expression was detected by qPCR and the results revealed that miR-17 expression was significantly increased by transfection of the cells

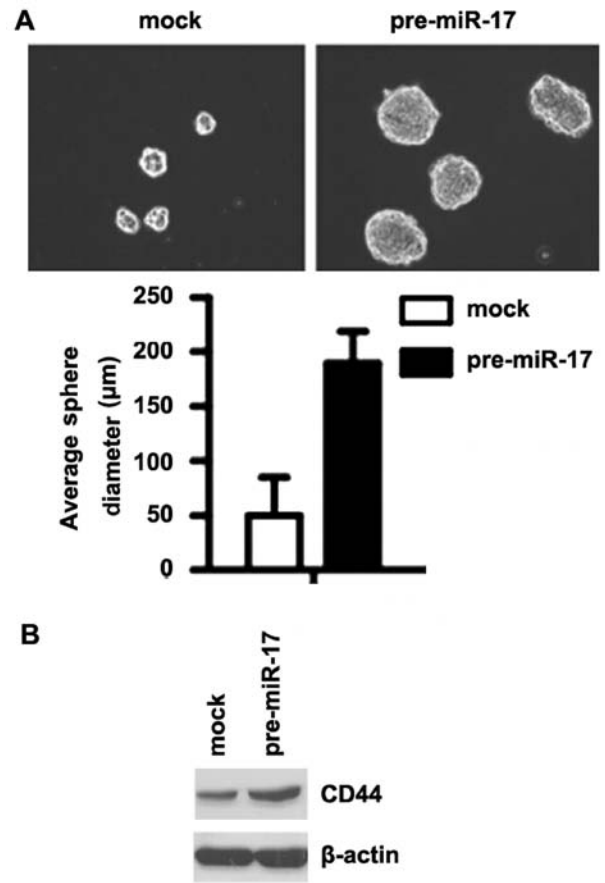


Figure 5. miR-17 promotes the formation of a stem cell-like population of colon cancer DLD1 cells. (A) Sphere growth in DLD1 cells transfected with pre-miR-17 or control miR (mock). Upper panel shows microscopic images of sphere growth. Bottom panel shows graphic presentation of the mean number of spheres, n=3 experiments. (B) Western blot analysis for CD44 in DLD1 cells transfected with pre-miR-17 and control miR (mock). β-actin was used as a loading control, n=3 experiments.

with pre-miR-17 (Fig. 3B). Subsequently, we performed immunofluorescence staining in the DLD1 cells transfected with pre-miR-17 or control miR. The results revealed that CYP7B1 protein expression was evidently suppressed in the cells transfected with pre-miR-17 (Fig. 3C). We then performed RT-qPCR and western blot analysis to detect CYP7B1 expression in the DLD1 cells transfected with pre-miR-17 or control miR. The results revealed that the CYP7B1 protein (Fig. 3D) and mRNA (Fig. 3E) expression levels were significantly down-regulated in the cells transfected with pre-miR-17. Consistent with the results of RT-qPCR, qPCR demonstrated that CYP7B1 mRNA expression was decreased in the DLD1 cells transfected with pre-miR-17, compared with the control miR-transfected cells (Fig. 3F). All the data demonstrated that miR-17 degraded CYP7B1 in colon cancer cells.

miR-17 promotes EMT in colon cancer cells. In order to determine the role of miR-17 in colon cancer, we transfected the DLD1 cells with pre-miR-17. We found that the overexpression of miR-17 led to significant changes in DLD1 cell morphology (EMT, change in phenotype from a cobblestone-like to a spindle-like morphology) (Fig. 4A). To further verify that the changes in cell morphology were caused by EMT, we

performed invasion and migration assays, and would healing assay. We found that the overexpression of miR-17 enhanced the migration (Fig. 4B and C) and invasion (Fig. 4C) ability of the cells.

miR-17 promotes the formation of a stem cell-like population in colon cancer cells. To determine whether miR-17 promotes the development of stem-like cell characteristics, we performed sphere-forming assay to assess the capacity of CSC or CSC-like cell self-renewal in this study. We found that formation of spheres was increased by the overexpression of miR-17 in the DLD1 cells (Fig. 5A). We also performed western blot analysis to determine whether CD44 is affected by miR-17 in the cells. The results revealed that CD44 protein expression was significantly increased by the overexpression of miR-17 in the DLD1 cells (Fig. 5B).

Discussion

Mounting evidence suggests that the deregulation of miRNAs is involved in colon cancer pathogenesis, microsatellite stability status, therapeutic outcome and patient prognosis (35-37). miR-17 expression has been confirmed to be significantly higher in CRC tissues than in normal tissues (34,38). However, its role in CRC has not yet been fully elucidated. In line with previous reports, we found that miR-17 not only promoted the EMT phenotype, but also promoted the formation of a stem cell-like population in colon cancer DLD1 cells. The results further confirmed that miR-17 is an oncogene in colon cancer. There is evidence to support that high levels of miR-17-92 cluster inhibit tumor growth and metastasis *in vivo* (39). However, Yu *et al* reported that the miR-17-92 cluster and its paralogs were significantly elevated in patients with colon cancer, and the increased expression of miR-17 was associated with a poor survival (40), further supporting our findings that miR-17 is an oncogene in colon cancer.

Previous studies have demonstrated that CYP7B1 plays an important role in cancer development and progression (20,41,42). The overexpression of CYP7B1 has been detected in prostatic adenocarcinoma (41). However, CYP7B1 expression is diminished in ER⁺ tumors and is predictive of a poor overall survival in breast cancer (20). We found that CYP7B1 protein expression was decreased in colon cancer tissues and that the silencing of CYP7B1 promoted the EMT phenotype and the formation of a stem cell-like population. In addition, CYP7B1 mRNA was degraded by miR-17 in colon cancer cells. We aim to further determine whether the expression of miR-17 inversely correlates with CYP7B1 expression in colorectal tumors in future studies.

In conclusion, miR-17-mediated CYP7B1 regulation in colon cancer cells demonstrated in this study has potential basic and clinical implications. On the one hand, miR-17 is a powerful oncogene by promoting EMT and the formation of a stem cell-like population in human colon cancer cells and the pharmacological suppression of miR-17 may represent a promising therapeutic strategy. On the other hand, CYP7B1 is a tumor suppressor gene and the overexpression of miR-17 can downregulate its expression. Our data lay the foundations for future research into the role of CYP7B1 in CRC and in other types of cancer.

Acknowledgements

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