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## miR-133a-3p Targets SUMO-Specific Protease 1 to Inhibit Cell Proliferation and Cell Cycle Progress in Colorectal Cancer

Guo-Qiang Zhou,\* Fu Han,\* Zhi-Liang Shi,\* Liang Yu,\* Xue-Feng Li,\* Cheng Yu,\* Cheng-Long Shen,\* Dai-Wei Wan,† Xin-Guo Zhu,† Rui Li,‡ and Song-Bing He†

\*Department of Gastrointestinal Surgery, Changshu No. 2 Hospital, Suzhou, P.R. China

†Department of General Surgery, The First Affiliated Hospital of Soochow University, Suzhou, P.R. China

‡Department of Gastroenterology, The First Affiliated Hospital of Soochow University, Suzhou, P.R. China

Dysregulation of SUMO-specific protease 1 (SENP1) expression has been reported in several kinds of cancer, including human colorectal and prostate cancers, proposing SENP1 as an oncogene with a critical role in cancer progression. miR-133a-3p has been reported as a tumor suppressor in several malignant neoplasias. However, the precise molecular mechanisms underlying its role in colorectal cancer remain largely unknown. The aim of this work was to investigate the relationship between miR-133a-3p and SENP1 in colorectal cancer cells. We found that miR-133a-3p expression was downregulated in colorectal cancer tissues. In silico analyses indicated that SENP1 is one of the target genes of miR-133a-3p. Overexpression of miR-133a-3p mimics was able to inhibit cell growth with G<sub>1</sub> arrest of colorectal cancer cells. Overexpression of miR-133a-3p antisense promoted cell growth of colorectal cancer cells. The luciferase reporter experiments showed that miR-133a-3p regulated the expression of SENP1 by combining with its 3'-UTR and resulted in downregulation of SENP1 and upregulation of CDK inhibitors such as p16, p19, p21, and p27. These results suggest that the miR-133a-3p-SENP1 axis might play a role in cell proliferation and cell cycle regulation of colorectal cancer cells.

**Key words:** Colorectal cancer; miR-133a-3p; SUMO-specific protease 1 (SENP1); CDK inhibitors

### INTRODUCTION

Colorectal cancer is the fourth leading cause of cancer-related deaths worldwide and the second leading cause in the US<sup>1,2</sup>. Because of early detection, the death rates of colorectal cancer have declined over the past 20 years; however, its incidence still remains high, and new treatments have been slowing down. Surgical removal of tumors followed with chemotherapy remains the first choice of treatment<sup>3</sup>. Novel drugs that have been recently approved are primarily biologics that target tumor angiogenesis or the epidermal growth factor receptor. However, these drugs have had limited success because the oncogenic mutations in KRAS confer resistance. Thus, it is critical to provide new or complementary targets for therapeutic and preventative applications for colorectal cancer.

SUMO (small-ubiquitin-like modifier) posttranslational modification (PTM) affects the function, subcellular localization, and/or stability of target proteins<sup>4</sup>. SUMOylation is a dynamic and reversible process that is catalyzed by SUMO-specific activating (E1), conjugating (E2), and ligating (E3) enzymes and is reversed by

a family of sentrin/SUMO-specific proteases (SENPs)<sup>5</sup>. SENP1 deconjugates a large number of SUMOylated proteins, the mutation of which causes embryonic lethality due to a defective hypoxia-inducible factor-1a (HIF-1a) pathway<sup>6</sup>. SENP1 functions as an oncogene and was overexpressed in several malignant neoplasias, including colon cancers<sup>7-9</sup>. SENP1 played an important role in colon cancer cell proliferation, tumor formation, and cell cycle progression by controlling the expression of CDK inhibitors<sup>7</sup>. However, how to regulate SENP1 in colon cancer cells remains largely unknown.

MicroRNAs (miRNAs) are a significant class of short endogenous noncoding RNAs that are evolutionarily conserved among different species, controlling gene expression at the posttranscriptional level through direct base pairing with their target mRNAs to degradation or by inhibiting their target mRNA translation<sup>10-12</sup>. miRNAs play critical roles in many biological pathways, and more than half of the sequences encoding miRNAs are located in cancer-associated genomic regions or fragile sites, suggesting they may regulate cancer at a systematically

Address correspondence to Song-Bing He, M.D., Ph.D., Department of General Surgery, The First Affiliated Hospital of Soochow University, No. 188 Shizi Street, Suzhou, Jiangsu Province 215006, P.R. China. Tel: +86-512-67780107; Fax: +86-512-67780107; E-mail: [captain\\_hsb@sina.com](mailto:captain_hsb@sina.com)

fundamental level. Recent studies indicate that aberrant expression of miRNAs plays a crucial role in the pathogenesis of cancer. The human genome codes more than 1,900 miRNAs; about 250 miRNAs are reported to have changes in abundance or altered functions in colorectal cancer<sup>13</sup>. For example, miR-155 and miR19a were upregulated to promote colorectal cancer cell growth. miR-141 and miR-455-3p were downregulated in colorectal cancer and exerted an antitumor effect<sup>14-17</sup>. miR-133a-3p has been reported as a tumor suppressor in several malignant neoplasias<sup>18-20</sup>. However, the role of miR-133a-3p in the pathogenesis of colorectal cancer remains largely unknown.

In the current study, we found that miR-133a-3p expression was downregulated in colorectal cancer tissues. miR-133a-3p functioned as a tumor suppressor in colorectal cancer cells by targeting SENP1 and resulted in downregulation of SENP1 and upregulation of CDK inhibitors such as p16, p19, p21, and p27.

## MATERIALS AND METHODS

### *Human Tissues*

Twenty pairs of frozen material from primary colorectal cancers and the adjacent normal colorectal tissues were obtained from our department. The diagnoses of these tissue samples were verified by pathologists from our hospital. This study was approved by the ethics committee of our hospital.

### *Cell Culture*

Human colon cancer cell lines HCT116 and SW480 were purchased from the ATCC (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS and 1,000 U/ml penicillin/streptomycin and incubated in 5% CO<sub>2</sub> at 37°C.

### *Cell Transfection*

miR-133a-3p mimics, antisense, and negative controls (NCs) were purchased from Genepharma Company (Shanghai, P.R. China). Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### *Bromodeoxyuridine (BrdU) Incorporation Assay*

A cell proliferation enzyme-linked immunosorbent assay kit (Beyotime, Shanghai, P.R. China) was used for BrdU incorporation assay to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols.

### *Real-Time PCR*

Total RNAs were isolated from cells and patient tissues by TRIzol reagent (Invitrogen), and reverse transcriptions were performed by the Takara RNA PCR kit

(Takara, Dalian, P.R. China) following the manufacturer's instructions. To determine the transcripts of the genes of interest, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara) on an ABI 7500 machine.

### *Western Blot*

Tissues and cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, and 10% glycerol). After centrifugation at 20,000 rpm for 10 min at 4°C, proteins in the supernatants were quantified and separated by 10–12% SDS-PAGE, and then transferred to a NC membrane (Beyotime). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Beyotime). The signals were detected by Chemiluminescent Substrate Kit (Beyotime) according to the manufacturer's instructions. Anti-SENP1, p16, p19, p21, p27, and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein levels were normalized to GAPDH.

### *Luciferase Assay*

Synthetic oligonucleotides with three copies of the SENP1 3'-UTR (ACCUUGACCAUGUGGGGGACCAG), which were predicted to bind miR-133a-3p, or three copies of a mutated version of the sequence (ACCUUGACCAUGUGGGGGATCCAG) that contained *Bam*HI restriction site were cloned into the pMIR-REPORT firefly luciferase miRNA expression reporter vector (Applied Biosystems, Framingham, MA, USA).

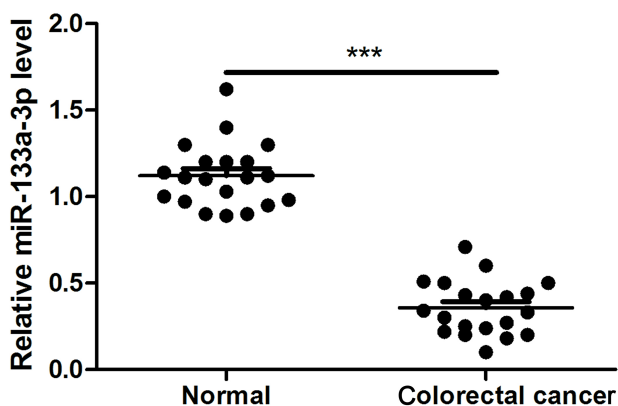
pMIR-REPORT vector and pRL-TK *Renilla* luciferase control vector (Promega, Madison, WI, USA) were co-transfected into HCT116 cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were further transfected with miR-133a-3p or the NC miRNA for an additional 24 h. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase activities were normalized to *Renilla* luciferase activities.

### *Flow Cytometric Assays for Cell Cycle*

Cells were collected, rinsed, and fixed overnight in 75% cold ethanol at -20°C. The cells were treated with Tris-HCl buffer supplemented with RNase A and stained with propidium iodide (BD Biosciences, San Jose, CA, USA). Cell cycle distribution was determined by flow cytometry (Becton Dickinson, San Jose, CA, USA); 10,000 cells were acquired and analyzed for DNA content. All data were collected, stored, and analyzed by ModFit software.

### *Statistical Analysis*

Values are displayed as mean ± standard error of mean (SEM). Statistical analysis was performed with GraphPad



**Figure 1.** miR-133a-3p was downregulated in primary colorectal cancer tissues. miR-133a-3p expression was determined by real-time PCR in human colorectal cancer tissues and adjacent normal tissues. \*\*\* $p < 0.001$ .

version 6.0 software (La Jolla, CA, USA). Statistical significance was considered with a value of  $p < 0.05$ .

## RESULTS

### *miR-133a-3p Was Downregulated in Primary Colorectal Cancer Tissues*

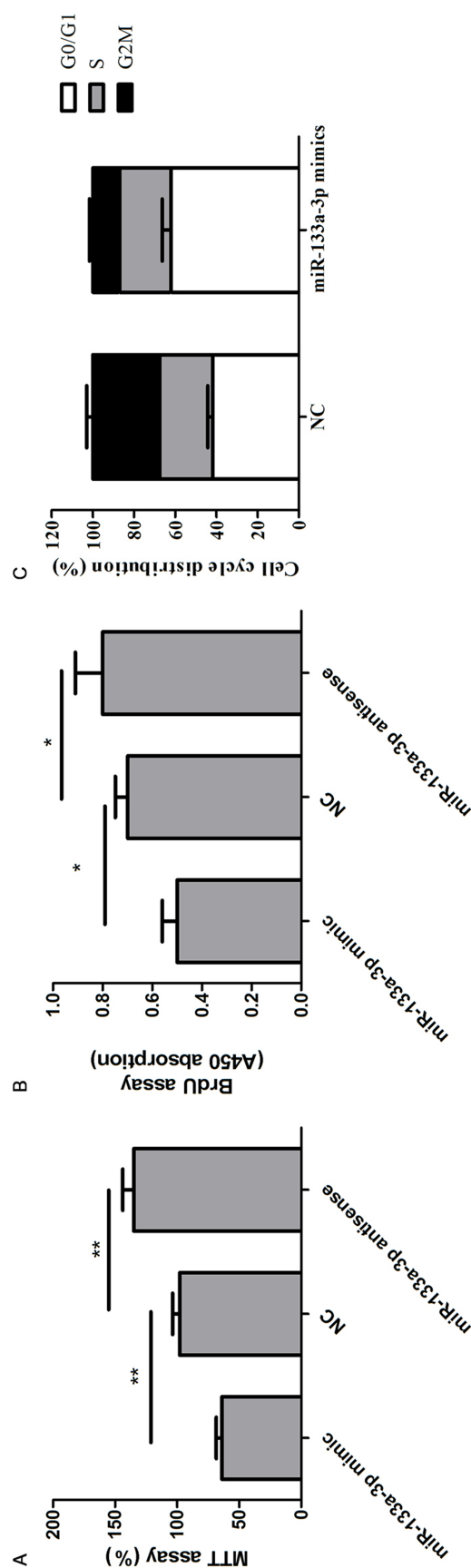
The expression of miR-133a-3p in primary colorectal cancer tissues is still unknown. Thus, we first checked the expression of miR-133a-3p in 20 pairs of frozen material from primary colorectal cancers and the adjacent normal colorectal tissues by means of quantitative real-time PCR. Our data indicated that miR-133a-3p was significantly decreased in colorectal cancer tissues compared with adjacent normal tissues (Fig. 1).

### *miR-133a-3p Inhibits Colorectal Cell Proliferation and Promotes $G_1$ Arrest*

We then investigate the function of miR-133a-3p in colorectal cells. Gain- and loss-of-function experiments by introducing the mimics or antisense of miR-133a-3p into HCT116 cells were performed. We also used the scramble sequences as NCs. As examined by the BrdU incorporation assay and the MTT assay, we found that the cell viability and proliferation ability were significantly decreased in HCT116 cells when miR-133a-3p was overexpressed (Fig. 2A and B). Moreover, overexpression of miR-133a-3p mimics significantly induced  $G_1$  arrest (Fig. 2C). In contrast, inhibition of endogenous miR-133a-3p by antisense oligos promoted the abilities of cell growth and proliferation (Fig. 2A and B). Similar results were also observed in SW480 cells (data not shown).

### *SENP1 Was a Target of miR-133a-3p in Colorectal Cancer Cells*

To screen the function target of miR-133a-3p in colorectal cancer cells, Bioinformatics software TargetScan



**Figure 2.** miR-133a-3p inhibits colorectal cell proliferation and promotes  $G_1$  arrest. (A) The cell viability assay of HCT116 cells transfected with indicated microRNAs (miRNAs) was determined by MTT assay. \*\* $p < 0.01$ . (B) The cell proliferation assay of HCT116 cells transfected with indicated miRNAs was determined by bromodeoxyuridine (BrdU) incorporation assay. \* $p < 0.05$ . (C) The cell cycle distribution of HCT116 cells transfected with indicated miRNAs was determined by FACS assay.

and miRWalk were used to screen the target genes of miR-133a-3p. Among them, SENP1 was predicted to be a direct target of miR-133a-3p (Fig. 3A). Luciferase activity assay found that miR-133a-3p mimics significantly suppressed the activity of the wild-type 3'-UTR of SENP1, whereas its antisense had the opposite effect on the activity of the wild-type 3'-UTR of SENP1. However, either miR-133a-3p mimic or antisense had a detectable effect on the mutant 3'-UTR of SENP1 (Fig. 3B). Consistently, miR-133a-3p mimics dramatically reduced, whereas miR-133a-3p antisense increased, SENP1 protein content (Fig. 3C and D).

#### *miR-133a-3p Inhibits the Proliferation of Colorectal Cancer Cells via Downregulation of SENP1*

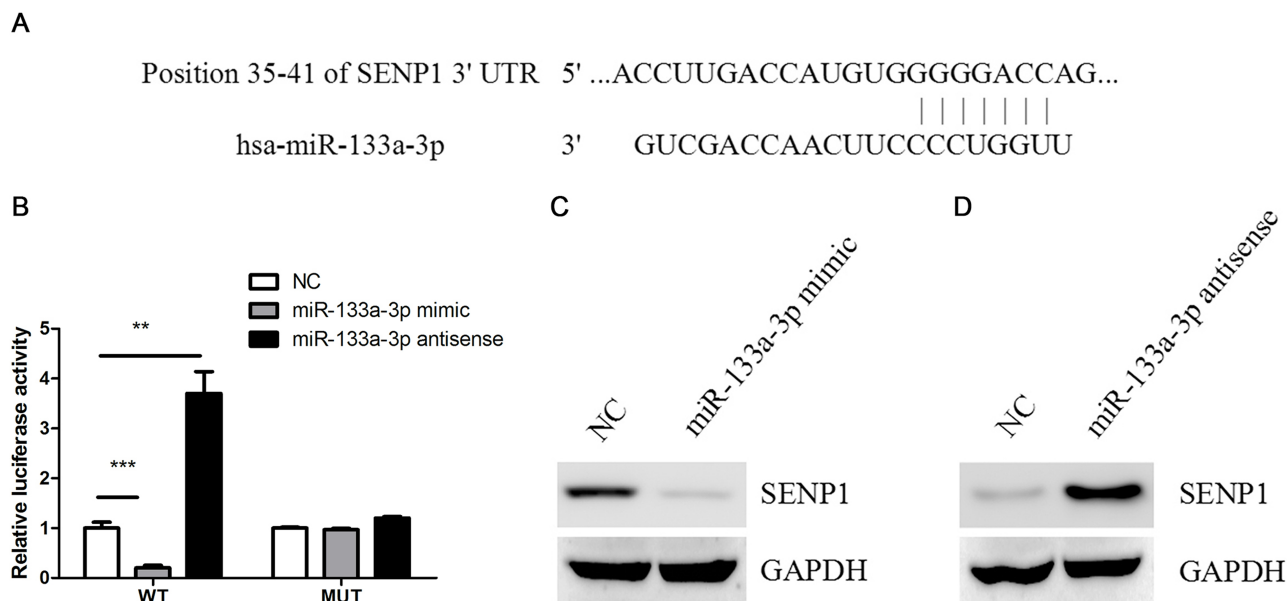
Given the critical role of SENP1 in colorectal cancer cell proliferation and cell cycle progress, we proposed that downregulation of SENP1 might contribute to the biology function of miR-133a-3p. To this end, we silenced the expression of SENP1 in miR-133a-3p antisense-transfected HCT116 and SW480 cells and found that SENP1 inhibition significantly attenuated miR-133a-3p antisense-induced colorectal cancer cell growth and proliferation (Fig. 4A and B). At the molecular level, we found that miR-133a-3p antisense could significantly increase the expression of CDK inhibitors, such as p16, p19, p21, and p27, in HCT116 cells. Consistent with this

observation, silencing the expression of SENP1 in miR-133a-3p antisense-transfected HCT116 cells dramatically decreased the expression of these proteins (Fig. 4C). Taken together, these data suggested that SENP1 and its downstream genes contributed to the impaired proliferation of colorectal cancer cells by overexpression of the miR-133a-3p mimic.

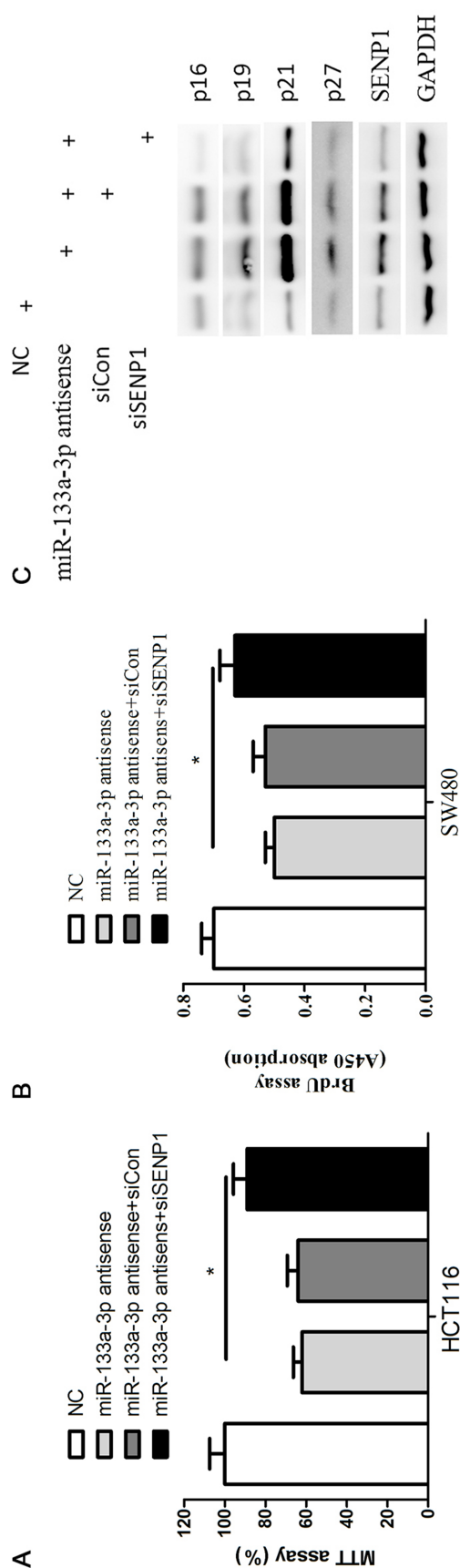
### DISCUSSION

In this study, we found that miR-133a-3p showed significantly lower expression in colorectal cancer tissues compared to adjacent normal tissues. However, the molecular mechanisms underlying the downregulation of miR-133a-3p remain unclear. DNA methyltransferases, such as DNMT3B, was usually abnormally upregulated in colorectal cancer tissue. Therefore, we proposed that epigenetic factors might contribute to the downregulation of miR-133a-3p in colorectal cancer tissue, which requires further investigation in the future.

It has been well established that a single miRNA could regulate multiple targeted genes. A previous study identified miR-133a-3p to exert inhibitory effects on gallbladder carcinoma via targeting RBPJ<sup>18</sup>. Aberrant expression of miR-133a-3p was also associated with tumor differentiation degree and lymph node metastasis<sup>18</sup>. Therefore, the roles of miR-133a-3p in tumorigenesis might be cell or tissue specific. In our study, we found that miR-133a-3p



**Figure 3.** SUMO-specific protease 1 (SENP1) was a target of miR-133a-3p in colorectal cancer cells. (A) Prediction of miR-133a-3p binding sites in the 3'-UTRs of the human SENP1 gene by bioinformatics software TargetScan and miRWalk. (B) HCT116 cells were transfected with wild-type or mutant 3'-UTR reporter constructs together with miR-133a-3p mimics, antisense, or negative control (NC). Luciferase reporter assays were then performed. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . (C) Protein levels of SENP1 were determined by Western blot in HCT116 cells transfected with miR-133a-3p mimics or NC. (D) Protein levels of SENP1 were determined by Western blot in HCT116 cells transfected with miR-133a-3p antisense or NC.



**Figure 4.** miR-133a-3p inhibits the proliferation of colorectal cancer cells via downregulation of SENP1. (A) The cell viability assay of HCT116 cells transfected with indicated miRNAs and siRNAs was determined by MTT assay. \* $p < 0.05$ . (B) The cell proliferation assay of SW480 cells transfected with indicated miRNAs and siRNAs was determined by BrdU incorporation assay. \* $p < 0.05$ . (C) HCT116 cells transfected with indicated miRNAs and siRNAs were subjected to Western blot with indicated antibodies.

overexpression inhibited, while its suppression increased, colorectal cancer cell growth and proliferation. We also identified SENP1 as a direct target of miR-133b in colorectal cancer cells. Therefore, our results suggest that miR-133b could inhibit colorectal cancer progression, at least in part, by repressing SENP1 expression.

It has been reported that expression of SENP1, a SUMO-specific protease, was elevated in colorectal cancer<sup>7</sup>. SENP1 played an important role in colon cancer cell proliferation, tumor formation, and cell cycle progression by controlling the expression of CDK inhibitors<sup>20</sup>. Moreover, SENP1 is also required for the proper accumulation of the HIF-1 $\alpha$  protein during hypoxia response<sup>21</sup>. Given that SENP1 plays a crucial role in promoting progression of human cancers, molecules or chemical compounds targeting SENP1 might be an effective new therapeutic for malignant progression. Indeed, several kinds of small molecule inhibitors targeting SENP1 has been reported and exhibited promising anti-tumor activity<sup>22–24</sup>. We observed that miR-133a-3p could regulate the expression of CDK inhibitors in colorectal cancer cells, further indicating the tumor-suppressive role of miR-133a-3p.

In summary, for the first time, this study explored the function of miR-133a-3p in colorectal cancer progression and identified SENP1 as its critical targeted gene. Future studies, for example, generating miR-133a-3p knockout mice, will be needed to establish the physiological role of miR-133a-3p in colorectal tumorigenesis.

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