

Deregulation of miR-193b affects the growth of colon cancer cells via transforming growth factor- β and regulation of the SMAD3 pathway

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Abstract. MicroRNA-193b (miRNA-193b) is often differentially expressed and is an important regulator of gene expression in colon cancer. The aim of the present study was to determine whether miRNA-193b affects cell growth in colon cancer and to investigate the potential underlying mechanisms. Patients with colorectal cancer (CRC; n=20) and healthy volunteers (n=10) were enrolled from the Department of Gastrointestinal Surgery Center, First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). Western blot analysis was used to evaluate the protein expression of SMAD3 and transforming growth factor- β (TGF- β) in the patient samples. It was determined that miRNA-193b expression was markedly elevated in the CRC tissue samples. Furthermore, silencing of miRNA-193b in SW620 CRC cells by specific inhibitors significantly reduced the cell proliferation and induced apoptosis. In addition, the downregulation of miRNA-193b significantly activated the protein expression of SMAD3 and TGF- β , and promoted caspase-3 activity in SW620 cells. The results of the present study suggested that the deregulation of miRNA-193b may affect cell growth in colon cancer via the TGF- β and SMAD3 signaling pathways.

Introduction

Colorectal cancer (CRC) is a malignant type of cancer with an increasing morbidity (1). Recent data from the International Agency for Research on Cancer indicated that the number of novel cases of lung cancer in 2012 reached 14.1 million (2).

With the improvement of living standards, and changes in lifestyle and the environment, the morbidity of colon cancer is increasing annually (3). Colon cancer has emerged as a serious public health problem, which threatens the health and lives of patients and is a social burden (4). Therefore, further studies on the etiology and pathogenesis of colon cancer are essential (5).

MicroRNA (miRNA; miR) is an endogenous, non-coding, single-stranded small RNA, which is typically composed of 21-25 nucleotides (6). Numerous studies have suggested that miRNA may participate in certain pathophysiological procedures, including cell differentiation, proliferation, apoptosis, multi-drug resistance, fat metabolism, oncogenesis and metastasis (7). Mutation and loss of expression of miRNAs have been closely associated with various types of tumors (8). In breast cancer, colon cancer and glioma, miRNA-21 was able to promote the occurrence and progression of tumors by stimulating cellular invasion and metastasis (8). Similarly, miRNA-10b was shown to promote the invasion and metastasis of breast cancer cells (9). The let-7 miRNA was reported to exert its carcinostatic effect by inhibiting cell adhesion, metastasis and invasion (10). A recently identified miRNA, miRNA-193b, also has an essential role in various types of tumors (11).

TGF- β , which has a relative molecular mass of 25 kDa, is composed of dimers and is located at chromosome 19q13.1 (12). TGF- β is cytokine that exerts various biological effects under physiological and pathological conditions (13). When TGF- β is activated, it is recognized by its receptors and exerts its biological functions through the SMAD or non-SMAD signaling pathways (14). Recent studies have revealed that TGF- β is highly expressed in various types of tumors (breast cancer, liver cancer, lung cancer), and participates in invasion and metastasis through regulating the extracellular matrix and angiogenesis, and by promoting immune evasion (15-17). The current study aimed to determine whether miRNA-193b is able to affect cell growth in colon cancer, and to investigate the possible underlying mechanisms.

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Materials and methods

Clinical tissue samples. Human patients with CRC (n=20) and healthy volunteers (n=10) were enrolled at the Department of

Gastrointestinal Surgery, The First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) from May-July 2013. Cancerous tissues, adjacent mucosal tissues and venous blood were collected from the patients with CRC during surgery, and venous blood was obtained from healthy volunteers. Informed consent was obtained from all participants. The clinical information for all patients and volunteers was recorded. The Institutional Ethics Committee of The First Affiliated Hospital of Sun Yat-Sen University approved the study protocol.

Cell culture and cell proliferation assay. IEC-6 enterocytes and SW620, SW480 and HT29 colon cancer cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco®) at 37°C in a humidified atmosphere containing 5% CO₂. All cells (1x10⁴) were seeded into 96-well tissue culture plates and incubated at 37°C. Subsequently, 20 µl MTT (50 mg/ml) was added to each well and the wells were cultured at 37°C for 4 h. Old medium was absorbed and 200 µl DMSO was added to each well then agitated at room temperature for 20 min. The absorbance was measured at 490 nm using an ELX-800 microplate assay reader (BioTek Instruments, Inc., Winooski, VT, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cancer tissues, adjacent mucosal tissues, venous blood and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). DNase was used to remove contaminating DNA (Takara Biotechnology Co., Ltd., Dalian, China) and Total RNA (1 µg) was then reverse-transcribed into cDNA using M-MLV reverse transcriptase in the presence of oligo (dT) (1 ng; Takara Biotechnology Co., Ltd.) (12-18). A StepOne™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform RT-qPCR with ABI PRISM 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), as follows: 10 min at 95°C for the initial denaturation, followed by 40 cycles at 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. The primer sequences were as follows: miRNA-193b forward, 5'-TGGCGTTTC TGGTTTCTCTT-3' and reverse, 5'-CGCACTTTTCTCCT CATTT-3'; and U6 forward, 5'-GTGCTCGCTTCGGCAGCA CATATAC-3' and reverse, 5'-AAAAATATGGAACGCTCA CGAATTTG-3'. miRNA-193b was obtained using the 2^{-ΔΔCq} method.

Transfection. The sequence of the small interfering (si)RNA targeting miRNA-193b (final concentration, 100 nM) was 5'-AGCGGGACUUUGAGGCCAGUU-3' and that of the scrambled control was 5'-UUGUACUACAAAAGUA CUG-3'. The siRNAsMAD3 siRNA sequences (final concentration, 200 nM) were sense, 5'-UAGAACUGGUGUCUC UACUTT-3' and antisense, 5'-AGUAGAGACACCAGUUCU ATT-3'. Cell transfections were performed using INTERFERin® reagent (Polyplus Transfection®, Illkirch, France) and transfected at 48 h at other study.

Flow cytometry. The transfected cells were seeded into 6-well tissue culture plates (1-2x10⁶) and incubated at 37°C. Annexin V-fluorescein isothiocyanate (10 µl; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and 5 µl propidium iodide were added to each well and cultured at 37°C for 4 h. The rate of cell apoptosis was analyzed using a FACSCalibur Flow Cytometry System (BD Biosciences, San Jose, CA, USA).

Determination of caspase-3 activity. Transfected cells (1x10³ cell/well) were seeded into 96-well tissue culture plates at 37°C. Ac-DEVD-pNA (10 µl; Beyotime Institute of Biotechnology, Haimen, China) for caspase-3 was added to each well and the plates were incubated for 6 h at 37°C. Caspase-3 activity was evaluated at 405 nm using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. All transfected cells were seeded into 6-well tissue culture plates (1-2x10⁶) at 37°C and lysed using 200 µl protein (RIPA; Sangon Biotech Co., Ltd., Shanghai, China). The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Sangon Biotech Co., Ltd.). The proteins were separated using 12% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Following blocking of the membranes using 5% nonfat milk in TBST, the membranes were incubated with anti-p-SMAD3 (#9520; dilution 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-TGF-β (#3709; dilution, 1:2,000; Cell Signaling Technology Inc.) and β-actin (#4970; dilution 1:2,000; Cell Signaling Technology, Inc.) antibodies at 4°C overnight. Following washing with TBST, the membranes were incubated with a goat anti-rabbit secondary antibody (#A16110; 1:5,000; Pierce; Thermo Fisher Scientific, Inc.) at 37°C for 1 h, and the blots were detected using an enhanced chemiluminescent reagent (#G-21234; Pierce; Thermo Fisher Scientific, Inc.) and quantified using Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Continuous variables are expressed as the mean ± standard error of the mean. All statistical analyses were carried out using the SPSS 16.0 statistical software package (SPSS, Inc., Chicago, IL, USA) and with the Student's *t*-test. P<0.05 was considered to indicate a statistically significant result.

Results

miRNA-193b expression in human patients with CRC and healthy volunteers. The expression of miRNA-193b in human patients with CRC and healthy volunteers was evaluated using RT-qPCR. Furthermore, the expression of miRNA-193b in cancer tissues was significantly increased compared with the adjacent mucosa (P<0.01; Fig. 1).

miRNA-193b expression in human CRC cell lines. The expression of miRNA-193b in IEC-6, SW620, SW480 and HT29 cells was determined using RT-qPCR. As presented in Fig. 2, the expression of miRNA-193b in IEC-6 cells was the lowest of all the cells. The expression of miRNA-193b in HT29 cells was low, as compared with that in SW620 and SW480 cells (Fig. 2). The expression of SW620 cells was medium in SW620,

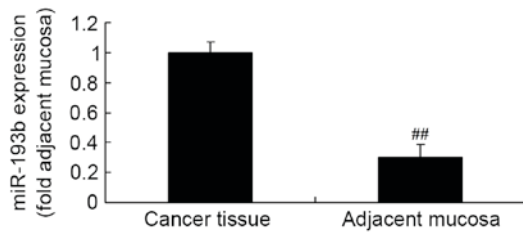


Figure 1. miR-193b expression in human patients with CRC and healthy volunteers. The expression levels of miR-193b in cancer tissues and adjacent mucosa were evaluated using reverse transcription-quantitative polymerase chain reaction. ^{##}P<0.01 vs. the cancer tissue group. miR, microRNA; CRC, colorectal cancer.

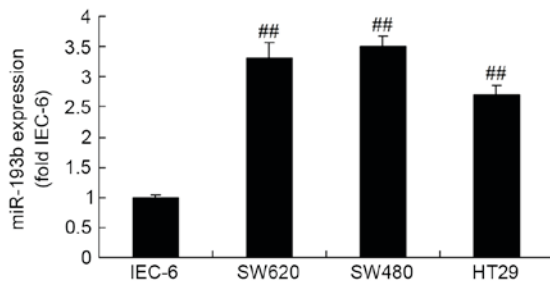


Figure 2. miR-193b expression in human colorectal cancer cell lines was determined using reverse transcription-quantitative polymerase chain reaction. ^{##}P<0.01 vs. the IEC-6 cells. miR, microRNA.

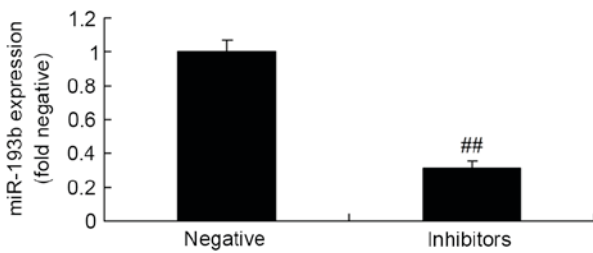


Figure 3. Effect of the deregulation of miR-193b on the expression of miR-193b in SW620 cells. ^{##}P<0.01 vs. the negative group. miR, microRNA.

SW480 and HT29 cells; therefore, SW620 cells were selected for further use in the present study.

Deregulation of miRNA-193b affects the expression of miRNA-193b in SW620 cells. Negative controls or miR-193b siRNAs were transiently transfected into SW620 cells, in order to investigate the expression profile of miRNA-193b in SW620 cells. The results revealed miR-193b inhibitors significantly inhibited the expression of miRNA-193b in SW620 cells, as compared with in the negative control group (P<0.01; Fig. 3).

Deregulation of miRNA-193b affects the proliferation of SW620 cells. The present study investigated how the deregulation of miR-193b may affect the proliferation of SW620 cells using MTT assays. Fig. 4 demonstrates that miRNA-193b inhibitors significantly reduced the proliferation of SW620 cells after 24 or 48 h, as compared with the negative control cells (P<0.01; Fig. 4).

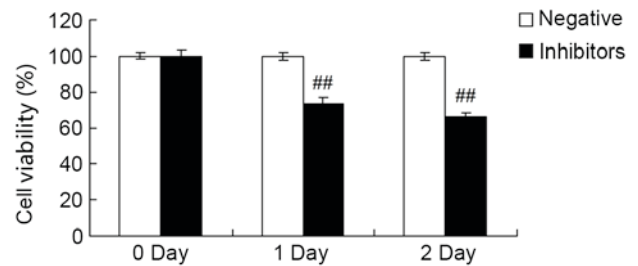


Figure 4. Effect of the deregulation of miR-193b on the proliferation of SW620 cells. The proliferation of SW620 cells transfected with miR-193 inhibitors or negative control was determined using MTT assays. ^{##}P<0.01 vs. the negative group. miR, microRNA.

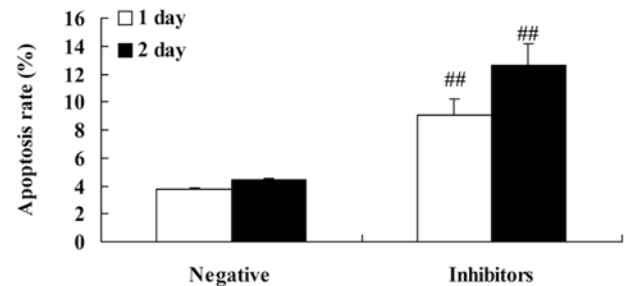


Figure 5. Effect of the deregulation of miR-193b on the apoptosis of SW620 cells. The apoptosis of SW620 cells transfected with miR-193 inhibitors or negative control was determined by flow cytometry. ^{##}P<0.01 vs. the negative group. miR, microRNA.

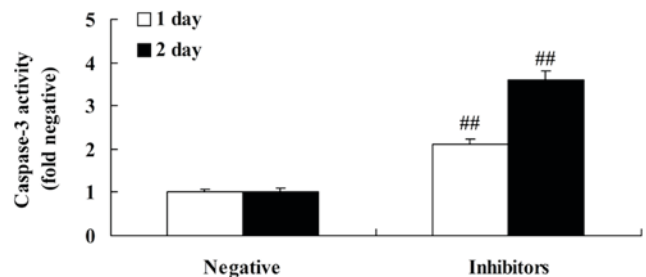


Figure 6. Effect of the deregulation of miR-193b on caspase-3 activity in SW620 cells. ^{##}P<0.01 vs. the negative group. miR, microRNA.

Deregulation of miRNA-193b affects the apoptosis of SW620 cells. In order to explore how the deregulation of miRNA-193b may affect the rate of apoptosis in SW620 cells, the apoptosis rate of SW620 cells was explored using flow cytometry. As shown in Fig. 5, a significant increase in the rate of apoptosis of SW620 cells was observed at 24 and 48 h following transfection with miRNA-193b inhibitors, as compared with the negative control group (P<0.01).

Deregulation of miRNA-193b affects caspase-3 activity in SW620 cells. To determine whether the deregulation of miRNA-193b is able to affect caspase-3 activity in SW620 cells, the caspase-3 activity in SW620 cells was detected. The downregulation of miRNA-193b expression markedly enhanced caspase-3 activity after 24 and 48 h in SW620 cells, as compared with the negative control cells (P<0.01; Fig. 6).

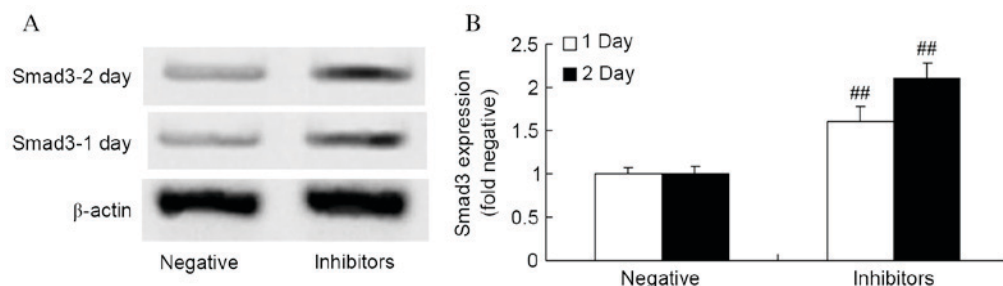


Figure 7. Effect of the deregulation of miR-193b on the SMAD3 signaling pathway in SW620 cells. The effect of the deregulation of miR-193b on SMAD3 protein expression levels in SW620 cells was determined using (A) western blot analysis and (B) densitometric analysis. $^{##}P < 0.01$ vs. the negative group. miR, microRNA.

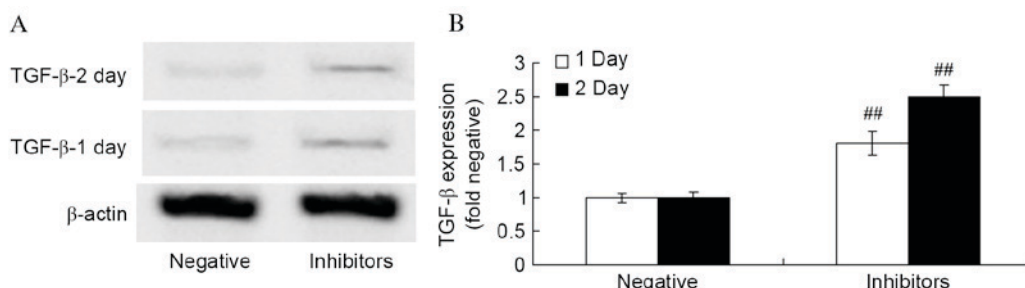


Figure 8. Effect of the deregulation of miR-193b on the TGF- β signaling pathway in SW620 cells. The effect of the deregulation of miR-193b on TGF- β protein expression in SW620 cells was determined using (A) western blot analysis and (B) densitometric analysis. $^{##}P < 0.01$ vs. the negative group. miR, microRNA; TGF- β , transforming growth factor- β .

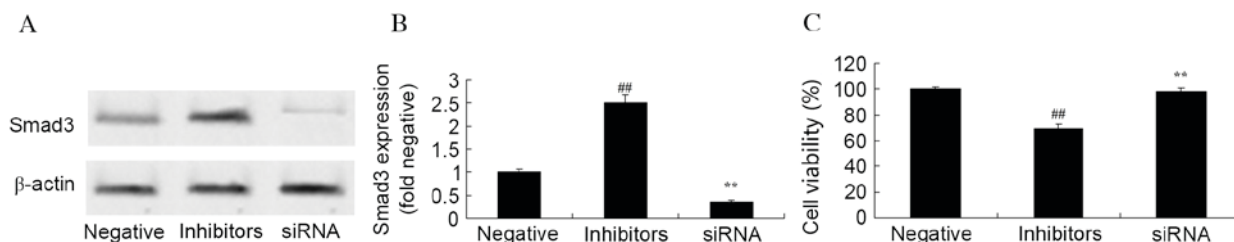


Figure 9. Effect of the deregulation of SMAD3 on the growth of SW620 cells. The effect of the deregulation of SMAD3 on its protein expression levels in SW620 cells was determined using (A) western blot analysis and (B) densitometric analysis. (C) The proliferation of SW620 cells transfected with miR-193b siRNA alone or miR-193b siRNA and siRNA targeting SMAD3. $^{##}P < 0.01$ vs. the negative group; $^{**}P < 0.01$ vs. the inhibitors group. miR, microRNA; siRNA, small interfering RNA.

Deregulation of miRNA-193b affects the SMAD3 signaling pathway in SW620 cells. The effect of miRNA-193b on the SMAD3 signaling pathway in SW620 cells was evaluated by western blot analysis. The downregulation of miRNA-193b expression significantly activated the protein expression of SMAD3 in SW620 cells, as compared with the negative control cells ($P < 0.01$; Fig. 7).

Effect of miRNA-193b deregulation on the TGF- β signaling pathway in SW620 cells. In order to explore the effect of miRNA-193b deregulation on TGF- β expression in SW620 cells, negative controls and miR-193b inhibitors were transiently transfected into SW620 cells and western blotting was performed. The results revealed that miR-193b inhibitors significantly promoted the protein expression of TGF- β in SW620 cells, as compared with the negative control cells ($P < 0.01$; Fig. 8).

Deregulation of SMAD3 affects cell growth in SW620 cells.

To determine the effect of SMAD3 deregulation on the growth of SW620 cells, the cells were transfected with miRNA-193b siRNAs alone or with miRNA-193b siRNAs and siRNA targeting SMAD3, and the protein expression of SMAD3 and the cell proliferation were each evaluated. As presented in Fig. 9A and B, siRNA targeting SMAD3 significantly reduced SMAD3 protein expression levels in SW620 cells, as compared with those in the miRNA-193b inhibitors group ($P < 0.01$). Furthermore, silencing of SMAD3 significantly suppressed the miRNA-193b-mediated reduction of the proliferation of SW620 cells ($P < 0.01$; Fig. 9C).

Discussion

Colon cancer is a common type of malignant cancer, with its morbidity and mortality rates ranking third worldwide (18).

Colon cancer is characterized by various genetic and epigenetic changes (19), as well as molecular alterations. However, further studies are required to identify novel therapeutic targets to develop new treatment strategies (20). In the present study, the expression of miRNA-193b in human patients with CRC was determined to be high, as compared with healthy volunteers. The expression of miR-193b in cancer tissues was also high, as compared with the adjacent mucosa. Furthermore, it was demonstrated that the expression of miRNA-193b in HT29 cells was low compared within SW620 and SW480 cells.

As an important regulatory factor, miRNA-193b is located on chromosome 16 and participates in the pathological processes of cancer (21). In a previous study, the expression of miR-193b-3p was significantly downregulated in patients with myotonic dystrophy type-2 (22). In the current study, compared with the serum miRNA expression levels in healthy patients, the expression of miRNA-193b in patients with septicemia was significantly altered. In addition, miRNA-193b was observed to be significantly decreased in adipose tissues, and participated in the formation of white adipose tissue via a series of transduction factors (23). miRNA-193b is also considered to have an important role in tumor metastasis, drug susceptibility and DNA methylation (24). miRNA-193b exhibited low expression levels in non-small cell lung cancer tissues, and the overexpression of miRNA-193b was revealed to significantly reduce the proliferation, invasion and metastatic capacity of A549 cells (25). The present study demonstrated that miRNA-193b inhibitors were able to effectively reduce cell proliferation, increase apoptosis and enhance caspase-3 activity in SW620 cells. Yang *et al* (7) demonstrated that miRNA-193b was able to suppress breast cancer progression. Mu *et al* (26) reported that the downregulation of miR-193b may be used as a novel and promising prognostic marker in gastric cancer.

The TGF- β signaling pathway is an important factor in the regulation of certain cellular biological behaviors. Typically, TGF- β exhibits tumor suppressive ability and promotes cell differentiation (23). However, during the malignant progression of a tumor, TGF- β may facilitate malignant progression by promoting cell proliferation, invasion and metastasis, enabling immune evasion and altering the cellular microenvironment (27). TGF- β induces the epithelial-mesenchymal transition via the SMAD signaling pathway, and sometimes the Ras signaling pathway (27). Numerous studies have suggested that TGF- β modulates the expression of c-myc via the classical SMAD signaling pathway, in order to regulate the proliferation of cells (28). The results of the present study demonstrated that miR-193b inhibitors significantly promoted the protein expression of TGF- β in SW620 cells. Iwamoto *et al* (29) reported that the downregulation of miR-193b induced the expression of urokinase-type plasminogen activator via the TGF- β signaling pathway.

SMAD3 is a key signaling protein in the TGF- β 1 signaling pathway (30). Following its transcription, the phosphorylation of various proteins determines the functions of the TGF- β 1 signaling pathway (31). When the TGF- β 1/SMAD3 signaling pathway is activated, the expression levels of SMAD3 in the cytoplasm are increased (27). The current study demonstrated that the downregulation of miRNA-193b significantly activated the protein expression of SMAD3 in SW620 cells, and that

SMAD3 silencing suppressed the miRNA inhibitor-mediated reduction in the proliferation of SW620 cells. Zhong *et al* (32) reported that miRNA-193b promotes cell proliferation via the SMAD3 and TGF- β signaling pathways in colon cancer.

In conclusion, the present study demonstrated that miRNA-193b was significantly overexpressed in colon cancer. The silencing of miRNA-193 in SW620 cells was revealed to induce the SMAD3 and TGF- β 1 signaling pathway in colon cancer. The results of the present study suggest that miRNA-193b maybe a tumor oncogene associated with the activation of cell growth in colon cancer via the TGF- β 1/SMAD3 signaling pathways. Therefore, miRNA-193b may be considered for use in the development of miRNA-based therapies in the future.

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