MicroRNA-101 suppresses colorectal cancer progression by negative regulation of Rap1b

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Abstract. Colorectal cancer (CRC) is the fourth most lethal malignancy and is the second most common cause of cancer-associated mortality worldwide. The development of high-throughput sequencing has enabled the identification of potential biomarkers for the diagnosis and treatment of various types of cancer. Although microRNA-101 (miR-101) has been demonstrated to be a potential biomarker of CRC, its detailed mechanisms remain to be fully discovered. In the present study, overall survival analysis was applied to determine the association between miR-101 and CRC prognosis. Reverse transcription-quantitative PCR (RT-qPCR) was used to examine gene expression levels in tissues and cells. Cell proliferative and apoptotic activities were determined by MTT and flow cytometry assays, respectively. Wound healing and Transwell assays were used to examine CRC cell migration and invasion, respectively. In the present study, RT-qPCR analysis indicated that miR-101 was significantly downregulated in CRC tissues and cells. However, clinical data collected from The Cancer Genome Atlas revealed no significant association between the expression levels of miR-101 and the prognosis of CRC. Additionally, miR-101 inhibited the progression of CRC by directly binding to the 3'-untranslated region of Ras-related protein Rap1b (Rap1b). This was associated with downregulation of Rap1b expression. Furthermore, the overexpression of Rap1b promoted miR-101 mimic-attenuated CRC cell progression. The present study demonstrated that miR-101 may be involved in the repression of the CRC progression by forming a negative feedback loop with Rap1b. The findings revealed the interaction between miR-101 and Rap1b during the progression of CRC, which could aid the development of therapeutic strategies.

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Introduction

According to the GLOBOCAN 2018 statistics, colorectal cancer (CRC) is the fourth (6.1%) most common malignancy after lung (11.6%), breast (11.6%) and prostate cancer (7.1%), whereas the mortality rate of CRC is the second highest (9.2%) in the world, following lung cancer (18.4%) (1). A total of 1.8 million CRC cases were diagnosed, and nearly 880,000 cases of CRC-associated mortality occurred in 2018 worldwide (1). An improvement in the treatment options of CRC is a significant challenge that requires attention. Additionally, the prioritization of primary prevention and early detection is also required.

MicroRNAs (miRNAs/miRs) belong to a class of short non-coding RNAs that are involved in cancer progression by binding to the complementary site of mRNAs, which often leads to translational repression or mRNA degradation (2). Various miRNAs, including let-7, miR-34a and the miR-200 family, have been identified as potential biomarkers or therapeutic molecules (3). Previous studies have reported that miR-101 is downregulated in various types of cancer and suppresses cancer cell progression, such as bladder cancer (4), breast cancer (5), hepatocellular carcinoma (6) and CRC (7). However, the comprehensive mechanisms underlying the effects of miR-101 on CRC progression have not been fully elucidated.

Ras-associated protein-1 (Rap1) serves a vital role in the regulation of cancer progression (8). This gene is a member of the Ras-like small GTPase family and contains two highly homologous isoforms, Rap1a and Ras-related protein Rap1b (Rap1b) (9). Several studies have proposed potential roles of Rap1b in tumorigenesis and demonstrated the upregulation of Rap1b in several types of cancer (10,11). A previous study reported that the loss of Rap1b can attenuate thyroid cancer (12). Tang *et al* (13) indicated that Rap1b promotes the invasion and migration of hepatocellular carcinoma cells by regulating Twist 1. Jia *et al* (14) discovered that Rap1b can promote the progression of esophageal squamous cell carcinoma. Furthermore, Li *et al* (15) reported that metastasis associated lung adenocarcinoma transcript 1 can promote the proliferation of glioma cells by acting as a sponge for miR-101. In hepatocellular carcinoma, Sheng *et al* (16) reported that miR-101 regulates the progression of hepatocellular carcinoma by interacting with Rap1b. Based on these findings and

assumptions, it was hypothesized that miR-101 may interact with Rap1b to suppress the progression of CRC.

The present study demonstrated that miR-101 inhibited CRC progression via regulation of Rap1b. The present study provided insights into the underlying mechanism of miR-101 in CRC and facilitates development of therapeutic strategies for CRC.

Materials and methods

Clinical specimens. The tissues were collected from 50 patients (31 males and 19 females) with a median age of 48 years (range, 29-78 years) between April 2015 and June 2018 from Shanghai Ninth People's Hospital (Shanghai, China). Written informed consent was obtained from all patients prior to the study start. CRC tumor tissues were obtained via surgical resections of 50 patients, and adjacent normal tissues were obtained from the distal edge of each resection <10 cm away from the tumor. The inclusion criteria for the present study were as follows, the patients were newly diagnosed and agreed to a 5-year follow up period. The exclusion criteria were as follows, patients diagnosed with other diseases and patients that had received chemotherapy or radiotherapy prior to surgery. All tissue samples were obtained and immediately stored at -80˚C prior to further experiments. The present study was approved by the Ethics Committee of Shanghai Ninth People's Hospital (Shanghai, China).

Cell lines. Human CRC cells (SW620), human colon mucosal cells (NCM460) and 293T cells were obtained from American Type Culture Collection. The cell lines were cultured in RPMI-1640 medium and supplemented with 10% fetal bovine serum. The 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc). All cell lines were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from CRC tissues, adjacent normal tissues, SW620 cells and NCM460 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and was reverse transcribed into cDNA by using a PrimeScript RT reagent kit (Takara Bio, Inc.) at 37˚C for 15 min, according to the manufacturer's protocol. The expression levels of miR-101 and Rap1b were determined using a ViiATM 7 Real-Time PCR system (Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using the SYBR®-Green Real-Time PCR Master mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The following thermocycling conditions were used to detect miR-101: An initial predenaturation step at 50˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 min and annealing at 60˚C for 1 min. For other factor detection, the thermocycling conditions were as follows: An initial predenaturation step at 94˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 20 sec. The following primer sequences were used for the qPCR: Rap1b; forward, 5'-TTTATTCCATCACAGCACAGTCC-3' and reverse, 5'-TTTCTGTTAATTTGCCGCACTAGG-3'; miR-101; forward, 5'-CGGCGGTACAGTACTGTGATAA-3' and reverse, 5'-CTGGTGTCGTGGAGTCGGCAATTC-3'; GAPDH; forward, 5'-GATGATCTTGAGGCTGTTGTC-3' and reverse, 5'-CAGGGCTGCTTTTAACTCTG-3' and U6; forward, 5'-CTCGCTTCGGCAGCACATATACTA-3' and reverse, 5'-ACGAATTTGCGTGTCATCCTTGCG-3'. Relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (17) and normalized to the internal reference genes GAPDH and U6.

Western blot analysis. Western blot analysis was performed in order to detect Rap1b expression as previously described by Joseph *et al* (18). Total protein was extracted from transfected SW620 cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid assay and 10 μg protein/lane was separated via SDS-PAGE on a 10% gel. Proteins were subsequently transferred onto polyvinylidene difluoride membrane and blocked in 5% non-fat milk for 2 h at room temperature. The membranes were incubated with the following primary antibodies, anti-Rap1b (1:1,000, cat. no. 2326; Cell Signaling Technology, Inc.) and anti-GAPDH (1:1,000, cat. no. 5174; Cell Signaling Technology, Inc.) overnight at 4˚C. Membranes were washed three times with TBS with 0.1% Tween-20. Following the primary incubation, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:1,000, cat. no. ab205718; Abcam) for 2 h at 37˚C. Protein bands were visualized using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. GAPDH was used as an internal control.

Transfection. miR-101 mimics, anti-miR-101 and their respective negative controls (miRNA mimic/inhibitor) were synthesized by Shanghai GenePharma Co., Ltd. The full length of Rap1b was subcloned into pcDNA3.1 to overexpress Rap1b, with empty pcDNA3.1 vector serving as the control. Transfection of the cells with the miR-101 mimics (10 nM), anti-miR-101 (10 nM), miRNA mimic/inhibitor (10 nM) and vectors (10 nM) were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Co-transfection of the cells with miR-101 mimic and Rap1b was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), the subsequent experiments were performed at 48 h post-transfection. The sequences of oligonucleotides used were as follows: miR-101 mimics, 5'-UACAGUACUGUGAUAACUGAA-3'; miRNA mimics, 5'-UUUGUACUACACAAAGUACUG-3'; anti-miR-101, 5'-UUCAGUUAUCACAGUACUGUA-3' and miRNA inhibitor control, 5'-UCACAACCUCCUAGAAAGAGUAGA-3'.

Cell proliferation, migration and invasion assays. An MTT assay was used to determine cell proliferation, whereas wound healing and Transwell assays were conducted to measure cell migratory and invasive activities, respectively. All of these assays were performed as previously described (16).

Flow cytometry. Cell apoptosis was detected in three independent experiments with the Annexin V-FITC/Propidium Iodide Apoptosis Detection kit (40302ES20), according to

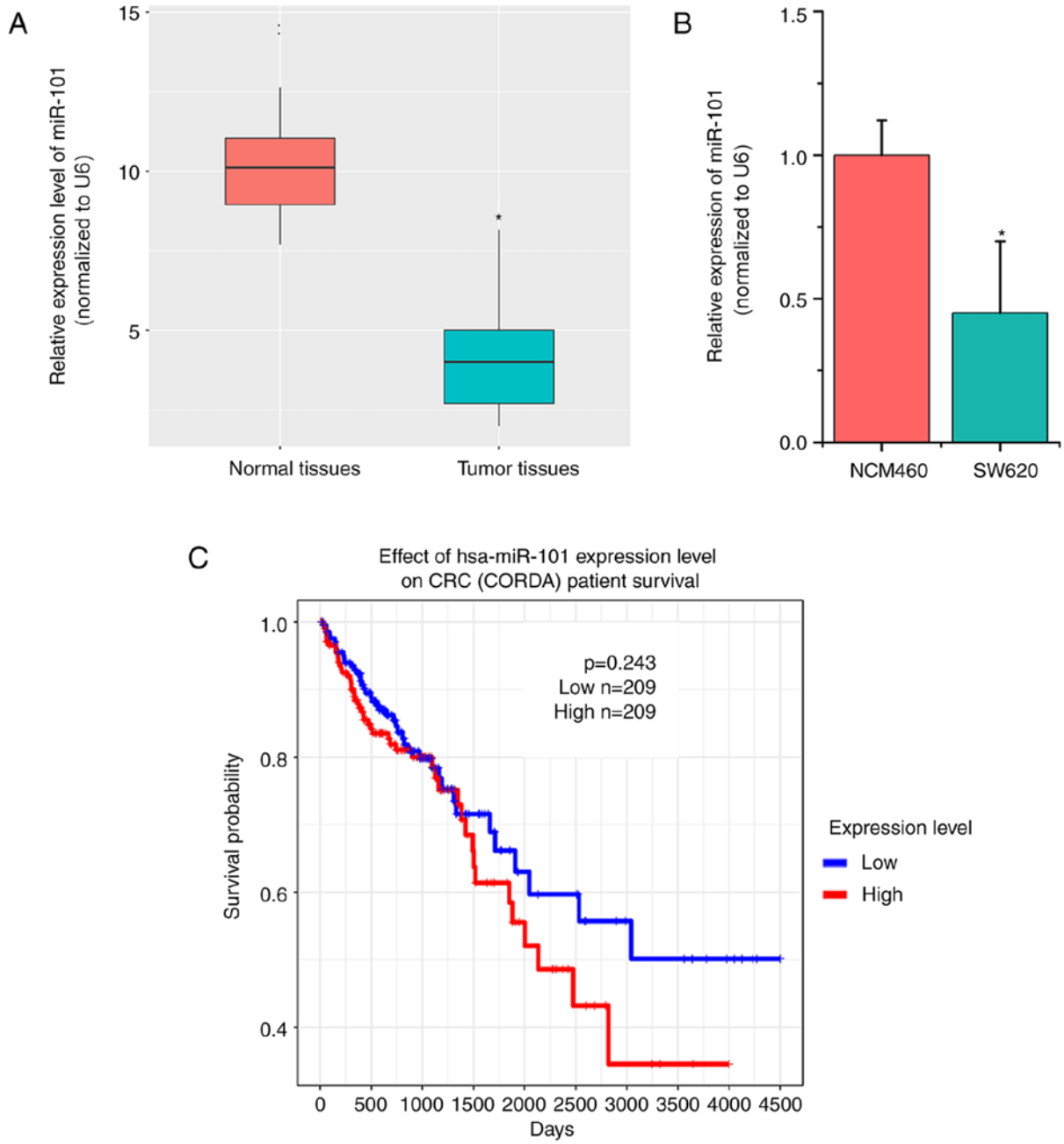

Figure 1. miR-101 expression is downregulated in CRC tissues and cells. (A) miR-101 expression was examined via RT-qPCR in CRC tissues and adjacent normal tissues (n=50). (B) miR-101 expression was examined via RT-qPCR in the CRC SW620 cell line and the normal human colon mucosal NCM460 cell line. (C) Association between miR-101 expression and CRC prognosis. *P<0.05 vs. normal tissues; *P<0.05 vs. NCMA60. CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR; miR-101, microRNA-101; CORDA, colon adenocarcinoma.

the manufacturer's protocol. Cells were subsequently detected using the FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 7.6.1; FlowJo LLC). The apoptotic rate was presented as the percentage of Annexin V-positive cells among the total cells.

Luciferase reporter assay. 293T cells were transfected with the Firefly luciferase plasmid (Promega Corporation) containing wild-type or mutant 3'-untranslated region (UTR) of Raplb, using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). The cells were subsequently transfected with NC, miR-101 mimic or anti-miR-101 (all from Ambion; Thermo Fisher Scientific, Inc.). After 48 h of cell culture, the cells were harvested and lysed. Luciferase activity was measured using the the Dual-Luciferase Reporter Analysis kit (Promega Corporation). Normalized relative light units represent Firefly luciferase activity/*Renilla* luciferase activity.

The Cancer Genome Atlas (TCGA) analysis. The clinical information and miR-101 expression profile of the CRC cases were obtained from TCGA database (https://cancergenome.nih.gov), within the colorecteral cancer dataset (TCGA-COAD) (19), using the R software package (version 3.1.3) (20). The expression levels of each hub gene were defined as either high (expression value ≥ median value) or low (expression value < median value). The survival curves

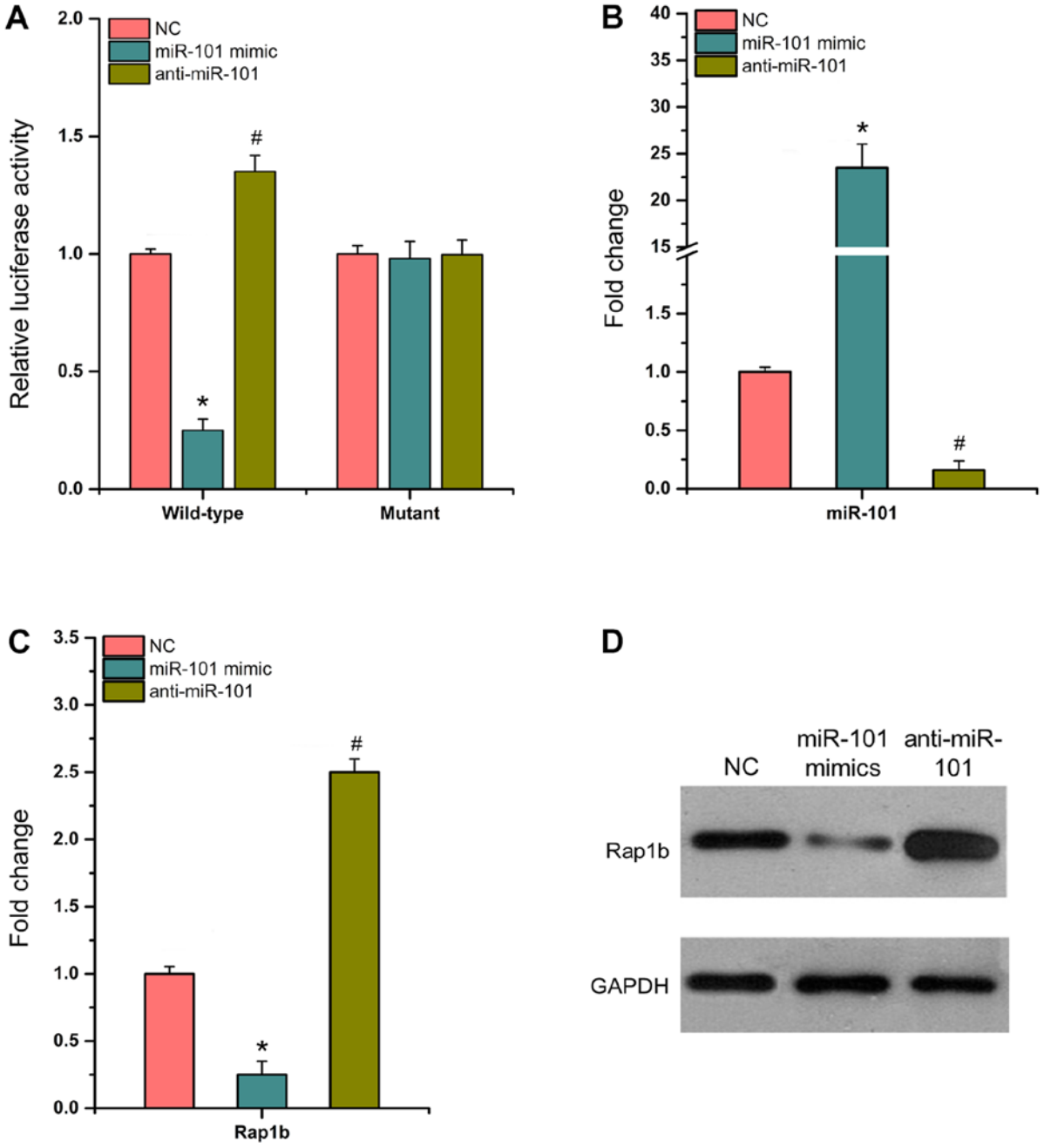

Figure 2. miR-101 directly targets Rap1b and inhibits Rap1b expression. (A) Dual luciferase reporter assays revealed luciferase activities in wild-type or mutant 3'-UTR-containing SW620 cells transfected with NC, miR-101 mimic or anti-miR-101. (B) RT-qPCR revealed the relative expression levels of miR-101 in SW620 cells transfected with NC, miR-101 mimic or anti-miR-101. (C) RT-qPCR revealed RAP1b mRNA expression in SW620 cells transfected with NC, miR-101 mimic or anti-miR-101. (D) Western blotting revealed the Rap1b protein expression in SW620 cells transfected with NC, miR-101 mimic or anti-miR-101. GAPDH was used as the internal control. *P<0.05, #P<0.05 vs. NC. Rap1b, Ras-related protein Rap1b; NC, negative control; miR-101, microRNA-101; RT-qPCR, reverse tanscription-quantitative PCR.

were analyzed via the Kaplan-Meier method, and univariate survival analysis was performed using a log-rank test.

Statistical analysis. The software package SPSS (version 22.0; IBM Corp.) was used for statistical analysis. The data derived from each experiment were presented as the mean ± standard deviation. A paired Student's t-test was used to compare parameters between two groups, and one-way ANOVA and Tukey's test were used to evaluate the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference. The experiments were performed >3 times.

Results

miR-101 expression is downregulated in CRC tissues and cells. In order to explore the detailed role of miR-101 in CRC, 50 paired tumor tissues and their adjacent normal tissues were collected. Subsequently, the expression levels of miR-101 were detected. RT-qPCR results indicated that miR-101 expression was significantly lower in CRC tumor tissues compared with in normal tissues (P<0.05; Fig. 1A;). The present study further examined miR-101 expression in CRC cells (SW620) and human colon mucosal cells (NCM460), which indicated similar results

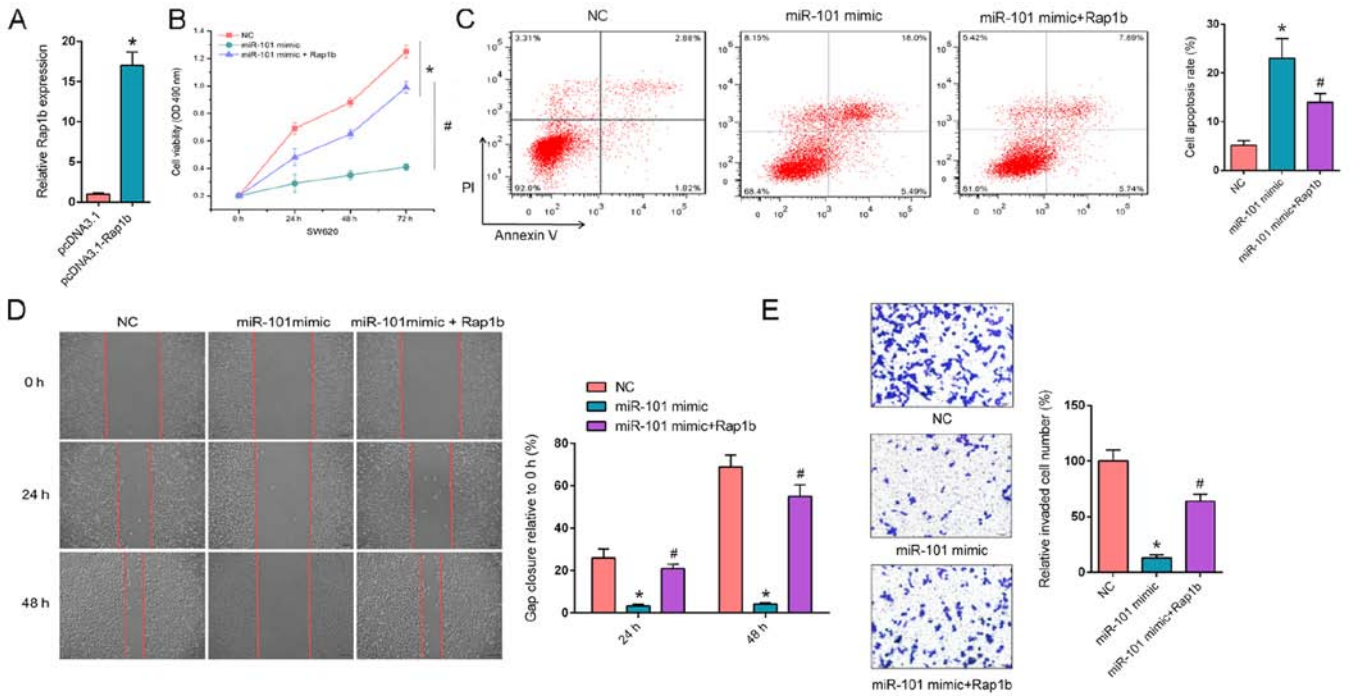

Figure 3. Overexpression of Rap1b rescues miR-101 mimic-attenuated CRC progression. (A) RT-qPCR revealed the relative Rap1b expression in SW620 cells transfected with pcDNA3.1 and pcDNA3.1- Rap1b. (B) MTT assays revealed the cell proliferation rate of SW620 cells transfected with NC, miR-101 mimic or miR-101 mimic + Rap1b at different time points (0, 24, 48 and 72 h). (C) Flow cytometry revealed cell apoptosis of SW620 cells transfected with NC, miR-101 mimic or miR-101 mimic + Rap1b. (D) Wound healing assays revealed migration abilities of SW620 cells transfected with NC, miR-101 mimic or miR-101 mimic + Rap1b at 0, 24 and 48 h. (E) Transwell invasion assays demonstrated invasion abilities of SW620 cells transfected with NC, miR-101 mimic or miR-101 mimic + Rap1b. *P<0.05 vs. NC; #P<0.05 vs. miR-101 mimic. Rap1b, Ras-related protein Rap1b; NC, negative control; miR-101, microRNA-101; PI, propidium iodide; RT-qPCR, reverse transcription-quantitative PCR.

(P<0.05; Fig. 1B). In addition, the association between miR-101 expression and the prognosis of patients with CRC was investigated using TCGA data. The overall survival curves indicated no apparent association between miR-101 expression levels and the prognosis of patients with CRC (P=0.243; Fig. 1C).

miR-101 directly targets Rap1b and inhibits its expression. The present study used dual luciferase reporter assays to detect luciferase activity in 293T cells containing wild-type or mutant 3'-UTR, which were transfected with anti-miR-101, miR-101 mimic and NC. The results indicated that in wild-type SW620 cells, miR-101 mimic significantly decreased luciferase activity by ~75% compared with that noted in NC cells, whereas anti-miR-101 significantly enhanced luciferase activity by ~40% (P<0.05; Fig. 2A). The transfection efficiency of miR-101 mimic and anti-miR-101 in SW620 cells was confirmed (P<0.05; Fig. 2B). RT-qPCR and western blotting assays demonstrated that the expression levels of Rap1b were significantly decreased by miR-101 mimic (Fig. 2C and D; P<0.05). Overall, the data indicated that miR-101 could directly bind to Rap1b and downregulate its expression in CRC cells.

miR-101 inhibits the development and progression of CRC cells. MTT and flow cytometry assays indicated that miR-101 overexpression could significantly inhibit cell proliferation and promote apoptosis (Fig. 3A and B). Wound healing assays further demonstrated that miR-101 mimic decreased SW620 cell migration (Fig. 3C). Transwell assays indicated that miR-101 mimic suppressed SW620 cell invasion (Fig. 3D). Overall, the results indicate that miR-101 acts as a tumor suppressor for CRC.

Overexpression of Rap1b rescues miR-101 mimic-attenuated CRC progression. In order to further determine whether the interaction between miR-101 and Rap1b could affect the progression of CRC, Rap1b was transfected into miR-101 mimic-expressing SW620 cells and the transfection efficiency of Rap1b was measured. The expression of Rap1b significantly increased in SW620 cells transfected with pcDNA3.1-Rap1b compared with SW620 cells transfected with pcDNA3.1 (P<0.05; Fig. 3A). Furthermore, MTT assays demonstrated that Rap1b overexpression significantly enhanced miR-101-suppressed cell proliferation (P<0.05; Fig. 3B). Flow cytometry assays indicated that aberrantly expressed Rap1b significantly decreased the apoptosis rate induced by miR-101 mimic (P<0.05; Fig. 3C). In addition, Rap1b reversed the suppressive effect of miR-101 on cell migration and invasion in SW620 cells (P<0.05; Fig. 3D and E, respectively). In summary, the results of the present study suggest that Rap1b is a key downstream effector of miR-101-regulated CRC phenotypes.

Discussion

Abnormal expression of miRNAs is a feature of human malignancies and frequently leads to either oncogenesis or tumor suppression (21,22). Increasing evidence has demonstrated that miR-101 is downregulated in various types of cancer and inhibits the development and progression of cancer (23,24). Liu *et al* (25) reported that miR-101 can interact with cancer susceptibility 2c to promote astrocytoma tumorigenesis. Furthermore, Jiang *et al* (26) demonstrated that miR-101 is involved in CRC metastasis via the miR-101-O-linked β-N-acetylglucosamine/enhancer of zeste

2 polycomb repressive complex 2 subunit regulatory feedback circuit. Xiong *et al* (27) revealed that CRC cell proliferation and migration are regulated by the zinc finger E-box binding homeobox 1 (ZEB1)-transcription factor AS1/miR-101/ZEB1 axis. The present study determined that miR-101 was significantly downregulated in CRC tissues and cells according to RT-qPCR assays. Additionally, SW620 cells transfected with miR-101 mimic exhibited decreased growth rates compared with NC cells. In addition, it was demonstrated that the upregulation of miR-101 promoted cell apoptosis. Wound healing and Transwell assays demonstrated that miR-101 could suppress CRC cell migration and invasion.

Previous studies have demonstrated that Rap1b is involved in the progression of various types of cancer and that it often acts as an oncogene to promote cancer cell proliferation (28-30). Peng *et al* (31) highlighted that miR-100 can regulate CRC cell proliferation and invasion by targeting Rap1b, and Guo *et al* (32) demonstrated that miR-139 acts as a sponge of Rap1b to affect the progression of CRC, which indicated that Rap1b may regulate CRC progression through multiple mechanisms. Therefore, it was inferred that miR-101 could bind to the complementary sequence of Rap1b and may affect the biological functions of CRC cells. Initially, a dual luciferase reporter assay was applied to confirm the interaction between miR-101 and Rap1b. miR-101 mimic significantly downregulated Rap1b expression in wild-type SW620 cells. Furthermore, overexpression of Rap1b could rescue miR-101 mimic-attenuated cell growth, migration and invasion.

Collectively, the present study identified a molecular axis of miR-101/Rap1b underlying CRC progression. However, *in vivo* experiments are required to further validate these findings in follow-up studies. It remains necessary to identify downstream signaling pathways that are regulated by Rap1b.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ and BL designed the study. HX and YD analyzed the data and prepared the figures. ZZ and BL drafted the initial manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Shanghai Ninth People's Hospital (Shanghai, China). Written informed consent was obtained from all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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