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ORIGINAL RESEARCH

RETRACTED ARTICLE: MicroRNA-126 Inhibit Viability of Colorectal Cancer Cell by Repressing mTOR Induced Apoptosis and Autophagy

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Objective: Colorectal cancer (CRC) is a fatal disease, and tumor development is a complex cellular event involving a multistep cascade process involving proliferation, invasion, and migration. In recent years, it has been shown that miRNA-126 (miR-126) plays a key role in the tumorigenesis of CRC, but further studies are required to investigate the regulatory mechanisms through which this miRNA affects cell viability, autophagy, and apoptosis in CRC. We aimed to study the effect of miR-126 on gene regulation in CRC HCT116 cells.

Methods: CRC biopsy samples and normal colorectal tissue samples were used for miRNA profiling. Real-time quantitative PCR and WB were utilized to detect RNA and protein levels. MTT and colony formation assays were performed to examine cell viability. Furthermore, an immunofluorescence assay and Annexin V/PI flow cytometry were performed to detect autophagy and apoptosis, respectively.

Results: The expression of miR-126 was downregulated in CRC biopsies and cell lines compared with that in normal cells and tissues. The upregulation of miR-126 resulted in impaired viability and growth of CRC cells. Furthermore, with the overexpression of miR-126, cell autophagy was increased, as evidenced by LC3-I/II transformation and p62 degradation. Meanwhile, apoptosis induction was also observed because of the increased miR-126 levels. The autophagy inhibitor Bafilomycin A1 (BafA1) repressed both autophagy and apoptosis, indicating that miR-126 induced autophagy was responsible for the induction of apoptosis. A dual-luciferase reporter assay (DLRA) and bioinformatics prediction revealed that miR-126 silenced the mTOR gene by targeting the 3′-UTR. mTOR mRNA levels in CRC biopsy tissues and cell lines were upregulated to a greater extent than that in normal cells and tissues. Furthermore, HCT116 cells transfected with an miR-126 mimic showed a decreased expression of mTOR. In addition, the overexpression of mTOR counteracted miR-126 on autophagy and apoptosis.

Conclusion: Our study demonstrated that miR-126-induced can regulate the activity of CRC cells via autophagy and apoptosis and suggested a new mechanism of miR-126–mTOR interaction in CRC pathogenesis.

Keywords: CRC, miR-126, mTOR, apoptosis, autophagy

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Introduction

Colorectal cancer (CRC) is a fatal disease, and the incidence of synchronous liver metastases (SLM) is approximately 15% in all newly diagnosed CRC cases. SLM exhibit widespread resistance to traditional therapy and has a poor prognosis.[1] Therefore, CRC is causes significant burden of cancer, with CRC proliferation and metastasis is most likely leading treatment failure. The extracellular matrix

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degradation, invasion, and adhesion of cancer cells, epithelial mesenchymal transformation, and abnormal expression of microRNA (miR) are the complex mechanisms involved in CRC.

miRNA is a short, conservative, and noncoding RNA that can bind to the 3′-untranslated region (3′-UTR) of target mRNAs and affect the posttranscriptional regulation of several genes.[2,3] MiR-126 is encoded by intron 7 of the *egfl7* gene.[4] It has been shown that miR-126 is obviously related to vascular integrity, angiogenesis, and several human diseases.[5,6] Studies have suggested that miR-126 could inhibit the development of tumors by targeting several genes such as IRS,[7] VEGF,[8] and PI3K.[9] The downregulation of miR-126 plays a key role in tumorigenesis by regulating signaling pathways, indicating that miR-126 has a potential application in cancer treatment.[9,10] In addition, miR-126 can be used as a new biomarker for acute myocardial infarction,[11] liver metastasis of CRC,[12] and type 2 diabetes.[13]

Recently, various studies have shown that miR-126 is related to the regulatory mechanism of CRC (abnormal activation or inactivation of signaling pathways). However, the effect of miR-126 on the proliferation and survival of CRC cells has not been fully elucidated. Therefore, this study aimed to prove the role of miR-126 in the autophagy and apoptosis of CRC cells.

Materials and Methods

Specimens

The clinical data of 30 patients undergoing CRC radical resection in the Third Affiliated Hospital of the Sun Yat-sen University were collected between September 2016 and June 2019. All subjects (15 men and 15 women; age range, 22–76 years; mean age, 57.3 ± 8.36 years) were diagnosed with colorectal adenocarcinoma; no other tumors were detected. The patients underwent nonemergency surgery and did not receive any preoperative chemotherapy. Among the 30 patients, there were 4 cases of left-side colon cancer, 4 cases of right-side colon cancer, and 22 cases of rectal cancer. Tumor diameters were < 4 cm (n = 5), 4–6 cm (n = 19), and >6 cm (n = 6). Histopathological examination of specimens obtained after radical operation showed that CRC was malignant In accordance with the NCCN Guidelines, patients were divided based on the cancer stage into stage II and above categories, following which they received postoperative chemotherapy. Samples of the adjacent normal colorectal tissues were obtained 5 cm from the distal end of the tumor. The patients had no history of preoperative radiotherapy or chemotherapy. This study has been approved by the Ethics Committee of the Third Affiliated Hospital of the Sun Yat-sen University. All study participants provided written informed consent before participating in the study.

Cell Culture and Transfection

HCT116 (Colorectal carcinoma, Human, Dukes' type A) and HEK293 (Permanent line established from primary embryonic human kidney, Human) cells were cultured in DMEM with 10% fetal bovine serum (FBS, Invitrogen). NCM460 (Primary ductal carcinoma, Human, Epithelial, Lymphoblast, Grade III), HT29 (Colorectal adenocarcinoma. Human. Grade I), Caco-2 (Colorectal adenocarcinoma. Human, Grade II), SW620 (Colorectal adenocarcinoma. Human. Dukes' type C), and SW480 (Colorectal Adenocarcinoma. Human. Grade IV. Dukes' type B) cells were cultured in RPMI-1640 (Thermo) with 10% FBS. All cell lines were obtained from ATCC.

pcDNA-mTOR plasmids were obtained from Shanghai GenePharmaceutical Co., Ltd., and the miRNA mimics and controls were obtained from Ribobio. Lipofectamine 3000 (Invitrogen) was used as a transfection reagent for transfection based on manufacturer's instructions.

miRNA Array

Total RNA was separated using the phenol–chloroform method, and the quality of RNA was assessed via capillary electrophoresis. Following small RNA sequencing, libraries were generated and the high sensitivity DNA chip in the Agilent Bioanalyzer 2100 system was used for the quantification of these libraries. The quality control of raw sequence files was evaluated using a FastQC quality control instrument. The elimination of adapters using Cutadapt (Ver. 1.2.1) and the cutting of low-quality sequences was performed to exclude low-quality data. The miRDeep2 software was used for the assessing miRNA activity (Ver. 2.0.0.8). The differential expression and change of the samples and controls was detected using differential expression sequencing.

Soft Agar Colony Formation Assay

The cells were transfected with either an NC mimic or an miR-126 mimic and were resuspended in DMEM 2 days after transfection; following this, they were transferred to a 12-well plate with 0.5% bottom agar. After 14 days, the colonies were counted in three random areas of each plate.

MTT Assay

Cell viability was evaluated using an MTT assay; 20 μL of MTT and 100μL of DMSO were added to each well. After adding MTT, formazan dye formed; the sample was further mixed for 10 min to dissolve it. Sample absorbance at 490 nm was determined using an Infinite M200 microplate reader.

Cell Death Assessment

An annexin V-FITC and PI apoptosis detection kit was used for cell death assessment. After transfection, the cells were resuspended in binding buffer (20 μL) with annexin V-FITC (10 μL) and PI (5 μL). The samples were incubated in darkness for 20 min before evaluating cell death via flow cytometry (FC).

Immunofluorescence Assay (IFA) for LC3 Detection

In the LC3 experiment, GFP-LC3B was used to transfect the cells. A fluorescence microscope was used to record the images, and the GFP puncta were observed in 10 different fields.

Western Blotting (WB)

A mixture of protease inhibitors (Roche) in RIPA buffer (pH 8.0) was used for cell lysis. Protein levels were detected, separated on 10% SDS-PAGE, and transferred to 0.45 μm PVDF membrane. Further, at room temperature PBST with 5% BSA was used to block the membrane for 60 min. Subsequently, the membranes were incubated with primary antibodies: Bcl-2 ab (ab59348, Abcam, 1:400), Bax ab (ab32503, Abcam, 1:1000), LC3B-I ab (ab51520, Abcam, 1:1000), p62 ab (ab56416, Abcam, 1:100), mTOR ab (ab2732, Abcam, 1:2000), and GAPDH (ab8245, Abcam, 1:5000) at 4°C for 60 min. The membrane and the secondary antibody were incubated at room temperature for 60 min with Amersham ECL peroxidase. A Super Signal West Femto Maximum Sensitivity Substrate Kit was used to determine the immunoreactivity.

RNA Separation and Quantitative Real-Time PCR (Q-PCR)

The total RNA was separated using Trizol, and a Roche Light Cycler 480 Q-PCR system was used to detect the transcription level. The internal reference was GAPDH. A SYBR Green PCR Master Mix was used for Q-PCR (20 μL). The 2-ΔΔCT method was used for quantification; gene expression was normalized to GAPDH, whereas miR-126 expression was normalized to U6 controls. The primer sequences were as follows: miR-126 F: 5′-GTC CGC TCG TAC CGT GAG TAA TA-3′; U6 F: 5′-CTC GCT TCG GCA GCA CA-3′, U6 R: 5′-AAC GCT TCA CGA ATT TGC GT-3′; mTOR F: 5′-C GC TGT CAT CCC TTT ATC G-3′, mTOR R: 5′-ATG CTC AAA CAC CTC CAC C-3′; GAPDH F: 5′-GCA CCG TCA AGG CTG AGA A-3′, GAPDH R: 5′-AGG TCC ACC ACT GAC ACG TT-3′.

Dual-Luciferase Reporter Assay(DLRA)

The mTOR 3′-UTR sequence was amplified using the GV126 luciferase gene. In addition to miR-126, the binding site of the mTOR gene was ablated via site-directed mutation; these samples were used as controls. The transfection efficiency was normalized using a thymidine kinase promoter and plasmid with Renilla luciferase. HEK293T cells were co-transfected with NC as well as miR-126 mimic with luciferase reporter vectors and the luciferase assay was conducted.

Statistical Analysis

The results were presented as means ± SDs. The differences between the groups were assessed using a two-tailed Student's *t*-test or ANOVA with Tukey's post hoc test. A *P* value of < 0.05 indicated statistical significance.

Results

CRC Samples and Cell Lines Showed Reduced miR-126 Expression

miRNA microarray analysis was performed in CRC and normal cells in order to prove the influence of miRs on the occurrence and development of CRC. The results indicated that miR-126 expression was markedly reduced in CRC samples compared with that in healthy controls (Figure 1A and B). The miR-126 expression in CRC cells was obviously downregulated compared with that in the adjacent normal tissues (Figure 1C). Additionally, CRC cell lines (HCT-116, NCM460, SW620, and SW480) showed lower miR-126 expression than the HEK293T cells (Figure 1D). These data confirmed a low expression of miR-126 in CRC cells.

Inhibition of Proliferation and Induction of Autophagy and Apoptosis in HCT-116 Cells by miR-126

Next, we explored the role of miR-126 in the proliferation of HCT-116 cells. An increase in miR-126 expression was found after transfecting HCT-116 cells with an miR-126

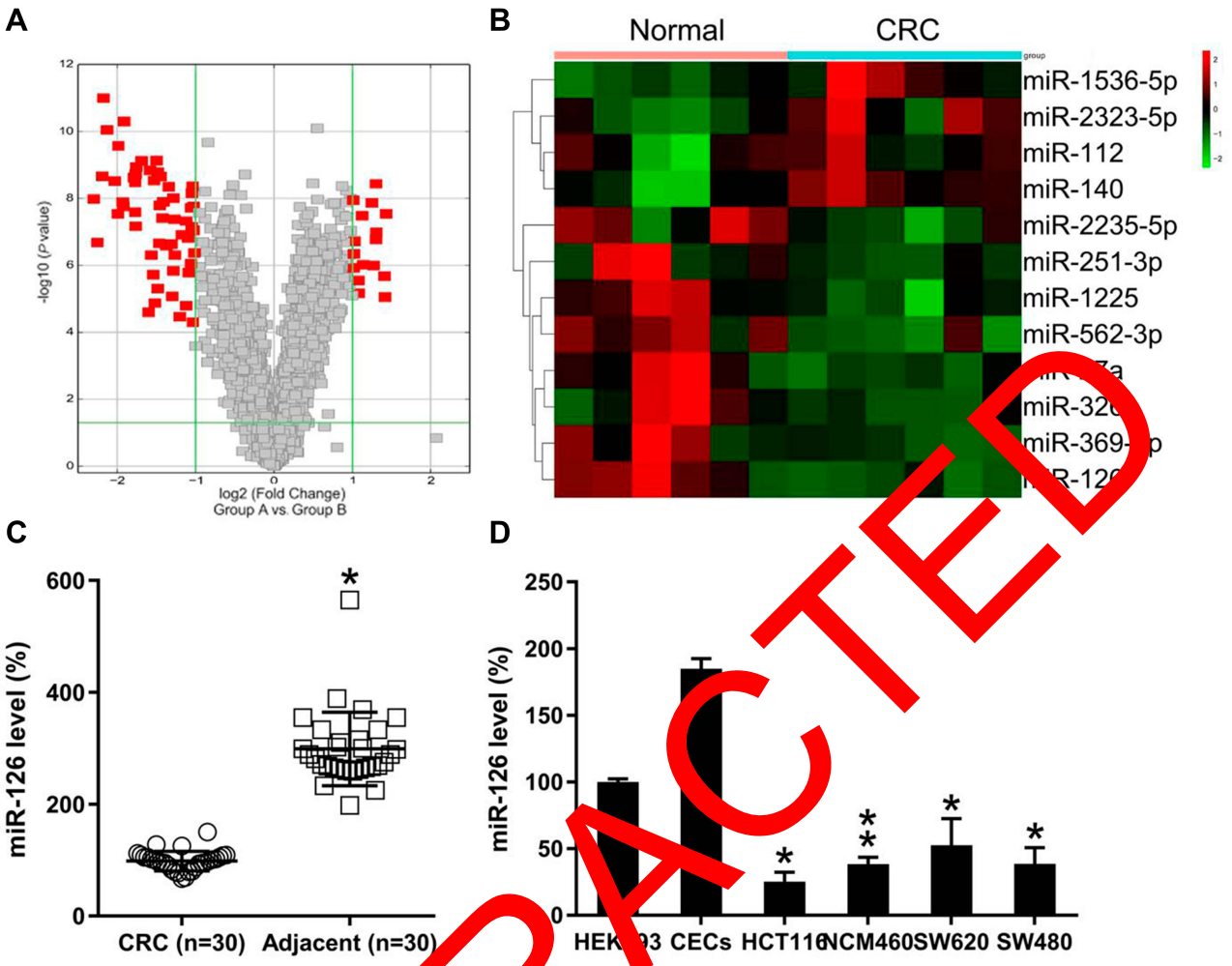

Figure 1 miR-126 expression in CRC cell lines. (**A**) The expression of miR and p are shown in the volcanic plot, and the CRC patients were compared with the healthy control group by microarray detection. (**B**) The expression of miR was different between CRC patients and the healthy control group. Green: low expression; red: high expression. (**C**) Q-PCR was used to detect the relative expression of miR-126 in the adjacent normal control tissue (n = 30) and CRC cells (n = 30). (**D**) The expression of miR-126 was analyzed in HEK293T cells, colon epithelium cells (CECs) and CRC cells using Q-PCR and normalized to U6. Results are shown as mean ± SD. *P < 0.05, **P < 0.01 vs colon epithelium cells group.

mimic (Figure 2A). Compared with the NC mimic group, MTT data collected at 24, 48, and 72 h after transfection revealed that the proliferation rate of HCT-116 cells were greatly decreased after the transfection of miR-498 mimic (Figure 2B). However, the colony formation assay indicated that miR-126 could decrease the number of colonies formed by HCT-116 cells (Figure 2C).

Further, we examined whether miR-126 upregulation mediated autophagy of HCT-116 cells. IFA was first utilized to trace the location of LC3B protein following the increase in miR-126 expression. LC3B (green) accumulated as a result of miR-126 mimic transfection (Figure 3A). Furthermore, miR-126 upregulation increased the autophagy rate of HCT-116 cells, as indicated by decreased LC3 transformation and processing (increased LC3-II levels), together with p62 degradation (Figure 3B). These data demonstrated that miR-126 mediates autophagy of HCT-116 cells.

It is assumed that miR-126 can regulate the apoptosis of HCT-116 cells. FC using annexin V-FITC/PI suggested that there was an increase in apoptosis after transfection with the miR-126 mimic compared with transfection with the NC mimic group (Figure 4A). We also explored the role of miR-126 in Bcl-2 and Bax expression. The expression of the antiapoptotic Bcl-2 was decreased as a result of transfection with the miR-126 mimic, whereas that of the proapoptotic Bax was increased (Figure 4B); these findings suggest that miR-126 also triggered apoptosis in HCT-116 cells.

Next, we tested whether the apoptosis induced by miR-126 was dependent on autophagy. miR-126 was

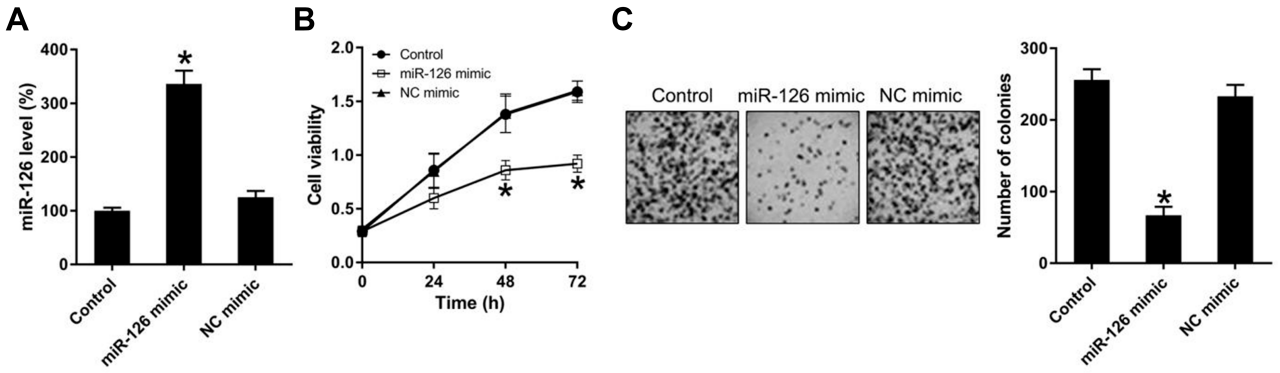

Figure 2 Effect of miR-126 overexpression on the growth of HCT-116 cells. (**A**) Expression of miR-126 in HCT-116 cells transfected with the miR-126 or NC mimic was detected by Q-PCR. (**B**) The proliferation rate of HCT-116 cells was measured at 24, 48, and 72 h after transfection, using an MTT assay. (**C**) A soft agar colony formation assay was performed using CRC cells transfected with the miR-126 or NC mimic. The right panel indicates the number of colonies in each group. Results are shown as mean ± SD. *P < 0.05 vs indicated group.

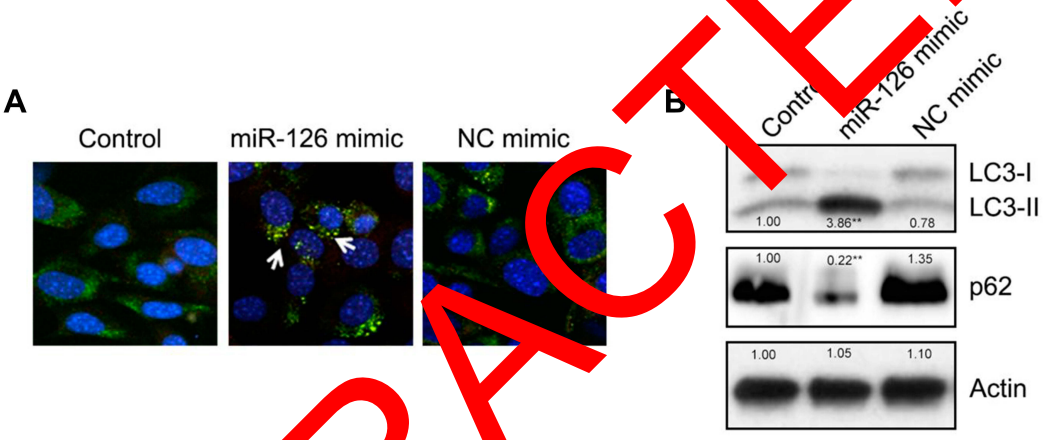

Figure 3 Overexpression of miR-126-induced autophagy in HCT-116 cells. (**A**) HCT-116 cells were inoculated on 24-well plates and were co-transfected with GFP-LC3B and an miR-126 mimic/NC mimic for 36 h before harvesting. The cellular location of GFP-LC3B was then observed using IFA (×400). (**B**) The expressions of LC3 and p62 in HCT-116 cells with miR-126 mimic/NC mimic were detected using WB.

further used to simulate the presence and absence of Baimelimin A1 (BafA1), and the apoptotic cells were detected by annexin V-FITC/PI staining and FC. BafA1 treatment in miR-126-upregulated HCT-116 cells resulted in the downregulation of LC3-II levels and upregulation of p62 levels, indicating that autophagy was successfully inhibited (Figure 5A). On the other hand, BafA1 treatment also led to a significant reduction in annexin-V-positive cells (Figure 5B). In total, these data suggested that miR-126 can induce apoptosis in an autophagic-dependent manner.

MiR-126 Aimed at 3′-UTRs of mTOR

Bioinformatics prediction revealed that miR-126 may target the 3′-UTRs of mTOR (Figure 6A).[14] DLRA was used to study the direct relationship between miR-126 and the 3′-UTRs of mTOR (Figure 6B). The data revealed that, compared with the other control groups, luciferase function was inhibited by 55% by the fusion of the miR-126 mimic with the 3′-UTRs of mTOR after transfection with the miR-126 mimic. Our study indicated that there was an increase in mTOR expression in CRC cells compared with that in the normal adjacent tissue and HEK293T cells (Figure 6C and D). Furthermore, we determined the effect of miR-126 on mTOR expression in HCT-116 cells. The protein and mRNA expressions of mTOR were obviously decreased after transfection with the miR-126 mimic (Figure 6E and F). The results demonstrated that the mTOR expression was decreased after the miR-126 expression was upregulated, and the 3′-UTRs of mTOR were targeted by miR-126.

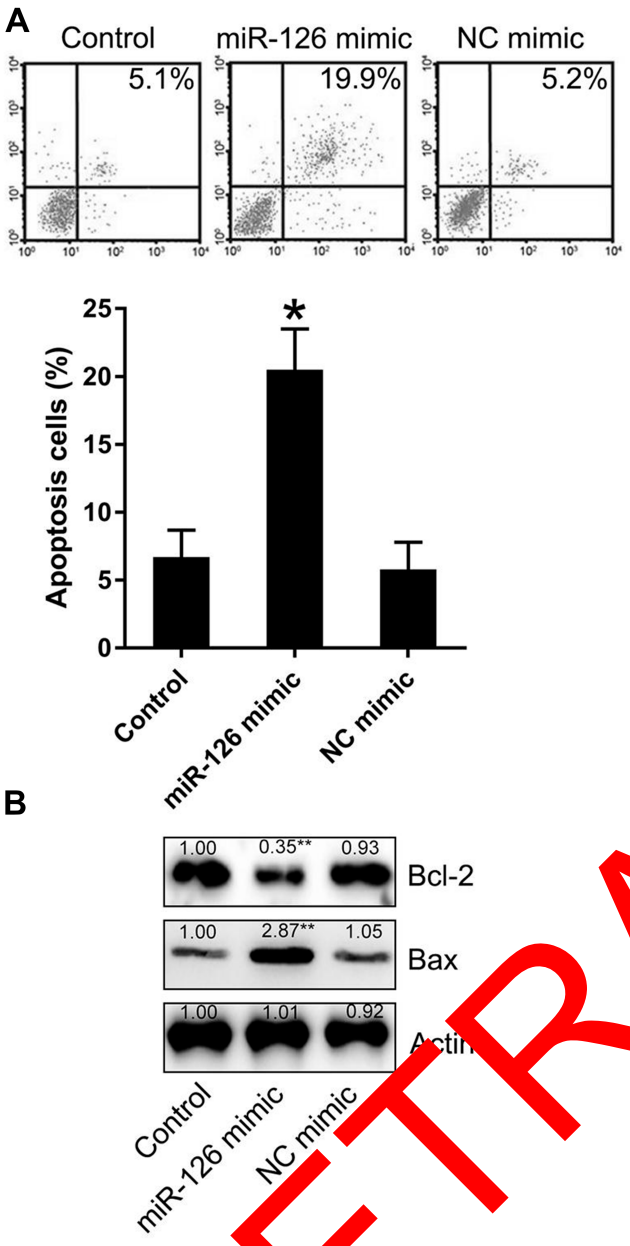

Figure 4 Overexpression of miR-126 induced apoptosis of HCT-116 cells. (**A**) The number of apoptotic cells transfected by miR-126 and NC mimics was detected by flow cytometry (left). The upper right quadrant of each plot represents early apoptotic cells. The apoptosis rate of HCT-116 cells is displayed in the lower panel. (**B**) WB was used to detect the expression of apoptotic markers (Bax and Bcl-2) in HCT-116 cells transfected with miR-126 or the NC mimic. Results are shown as mean ± SD. *$P < 0.05$ vs indicated group.

mTOR Overexpression Diminished the Effect of miR-126 on the Viability of HCT-116 Cells

To explore whether mTOR could reverse the effects of miR-126 on CRC cell viability (autophagy and apoptosis), mTOR was overexpressed in HCT-116 cells and co-transfected with miR-126 mimic. WB and Q-PCR were used to detect the change in mTOR expression (Figure 7A and B). Further, we showed that the overexpression of mTOR rescued the proliferation of HCT-116 cells, was inhibited inhibited by the miR-126 mimic, based on data from the MTT assay (Figure 7C). The overexpression of mTOR led to an obvious decrease in apoptotic cells transfected with the miR-126 mimic (Figure 7D and E). Additionally, our data showed that mTOR overexpression resulted in a significant reduction in the level of LC3-II, as well as in p62 stability (Figure 7F). Therefore, mTOR expression can restore cell proliferation by reducing miR-126-induced autophagy and apoptosis.

Discussion

CRC is one of the most common malignant tumors; thus, an effective treatment is essential for patients with CRC.[15] The present study revealed that the expression of mTOR was downregulated by miR-126, showing an anticancer effect via autophagy and apoptosis modulation in CRC cells. Further validation revealed that mTOR overexpression can promote proliferation of CRC cells. Moreover, miR-126-triggered autophagy was responsible for BafA1-induced apoptosis.

An increasing number of studies have shown that miR-126 is a tumor suppressor and that its expression is downregulated in multiple tumors. Various molecular signal cascades involved in the regulation of cancer cells have been described to be modulated by miR-126 and vice versa in a cell type–specific and context-dependent manner (Figure 8). Furthermore, our previous related studies[16–19] have shown that miR-126 can inhibit the proliferation and invasion of CRC cells in vitro. Liu et al demonstrated VEGF-A to be a target of miR-126, as the infection of lung carcinoma cell lines with lentiviral miR-126 caused a decrease in VEGF-A. This led to a cell cycle arrest in lung cancer cells and to the inhibition of tumor cell growth in vitro and in vivo.[8] Li et al demonstrated that the transformation of mouse embryonic Cx43KO brain cells with the oncogene v-Src caused a decrease in miR-126 expression. Additionally, the 3′ UTR of Crk, a component of the focal adhesion network involved in integrin signaling, has been reported as a target of miR-126.[20] Chen et al suggested that reduced miR-126 expression facilitates angiogenesis of gastric cancer through its regulation of the VEGF-A-mediated Akt/mTOR pathway.[21] Downregulation of miR-126 expression increases the expression of VEGF-A and its downstream mTOR signaling. In contrast, restoration of miR-126 expression reduces the expression of VEGF-A and suppresses the activation of its downstream AKT and mTOR. Banerjee et al and Guo et al

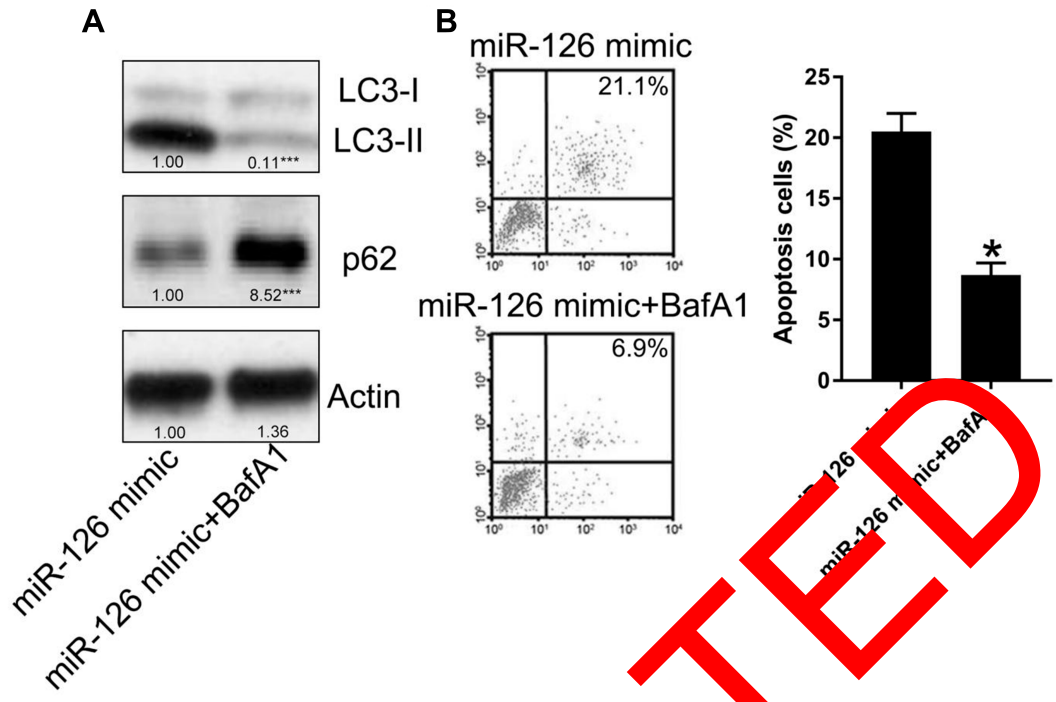

Figure 5 Effect of balA1 on autophagy and apoptosis induced by the miR-126 mimic. (**A**) Autophagy was determined in HCT-116 cells. Cells with overexpression of miR-126 were cotreated with bafA1 for 12 h. WB analysis of cell lysates was performed with anti-p62 and anti-LC3 antibodies. (**B**) Annexin V-FITC/PI and FC was used to detect HCT-116 cells cotreated with BafA1 (100 nM) for 24 h. Representative data from three independent experiments are shown. Results are shown as mean ± SD. *P < 0.05 vs indicated group.

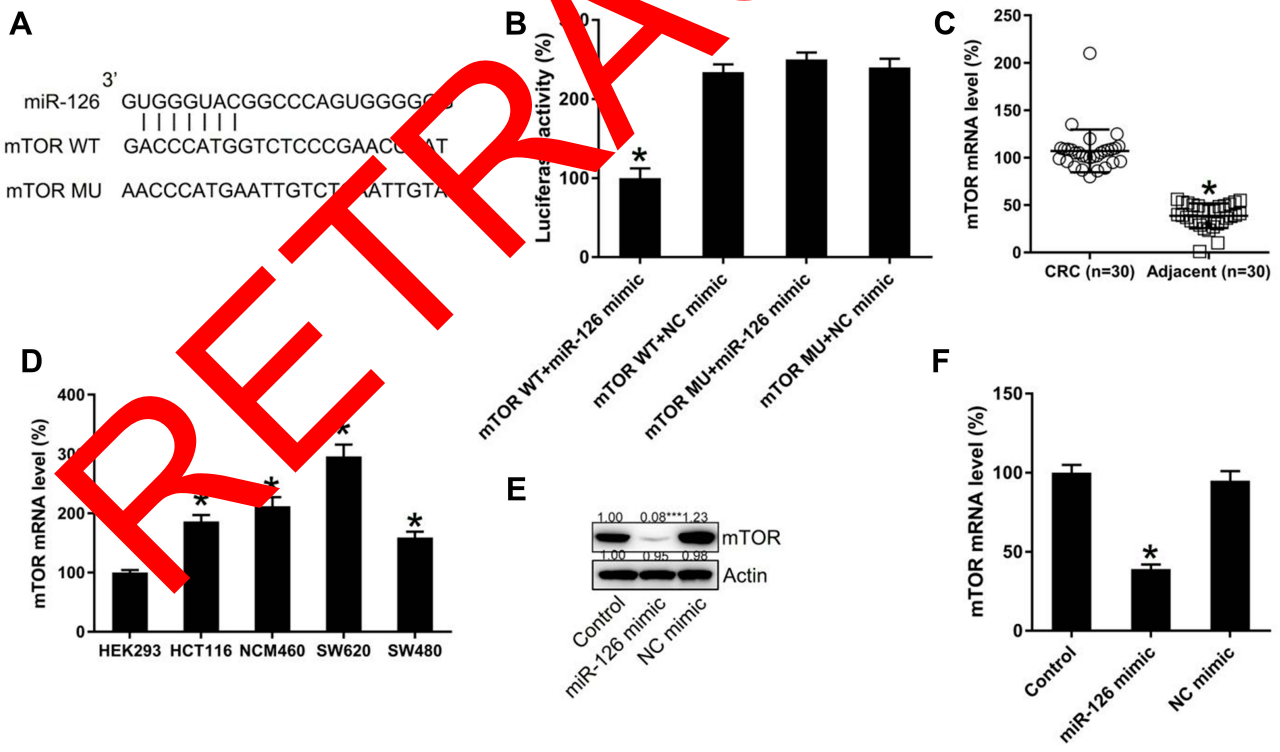

Figure 6 mTOR is a direct target of miR-126. (**A**) mTOR 3′-UTR conservative miR-126 binding motif diagram. (**B**) The luciferase reporter construct showed that DLRA contained a wild type (WT) or mutant (MU) version of human mTOR 3′-UTR after miR-126/NC mimic transfection. (**C**) Q-PCR was used to detect the relative expression of mTOR in the adjacent normal control group (n = 30) and CRC samples (n = 30). (**D**) The expression level of mTOR in HEK293T and CRC cell lines was analyzed by Q-PCR. WB (**E**) and Q-PCR (**F**) were used to assess the protein and mRNA levels of mTOR in HCT-116 cells after transfection with an miR-126 mimic and NC mimic. Results are expressed as mean ± SD. *P < 0.05 vs indicated group.

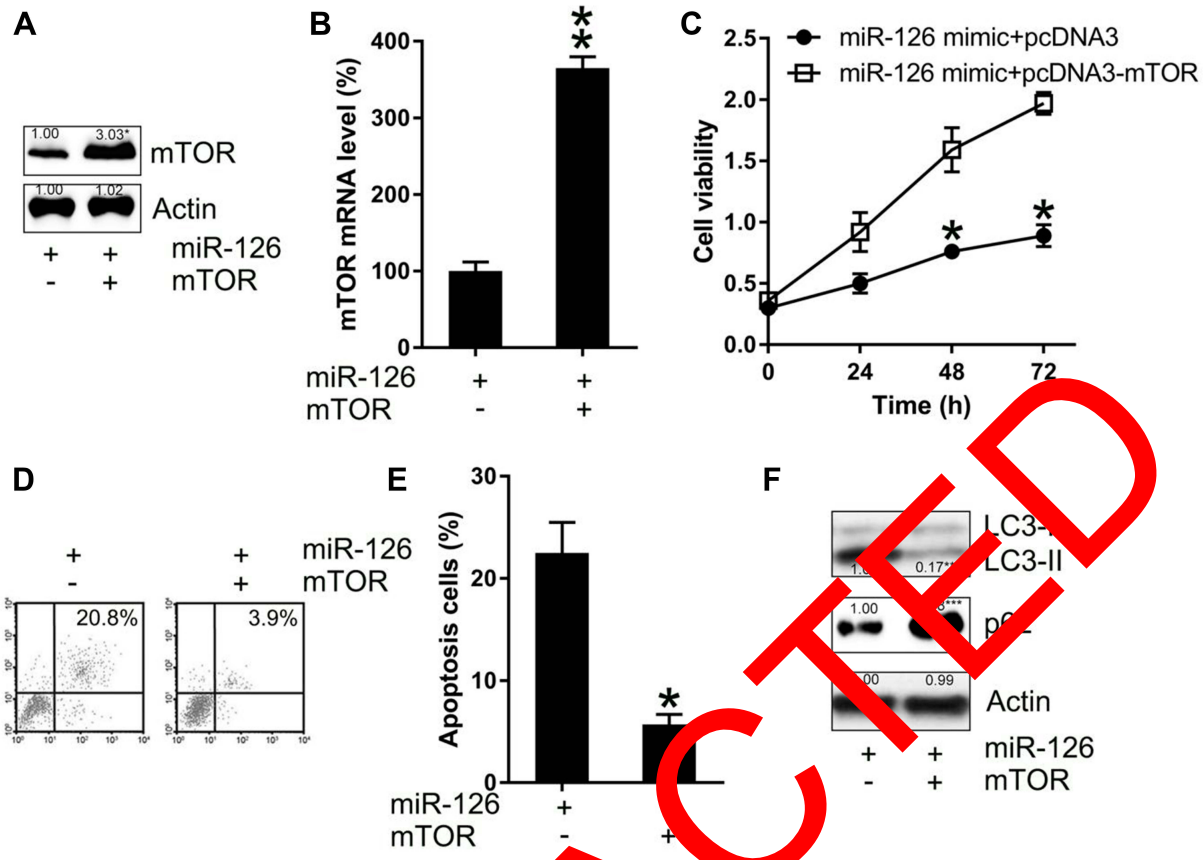

Figure 7 Overexpressed mTOR counteracted the effect of miR-126 on HCT-116 cell viabi... Cells were co-transfected with the miR-126 mimic/NC mimic using pcDNA-mTOR/NC. WB (**A**) and Q-PCR (**B**) were used to evaluate the protein and mRNA levels of mT... HCT-116 cells after transfection. (**C**) The proliferation rate of HCT-116 cells was measured at 24, 48, and 72 h after different transfectio... sing by MT... ay. (**D, E**) mTOR overexpression restored the apoptosis induced by the miR-126 mimic. Annexin V-FITC/PI and FC were used to assess the numb... of ea... apoptot... HCT-116 cells at 36 h post transfection. (**F**) WB was used to analyze analyzed the expression levels of LC3 and p62 in HCT-116 cells with diffe... transfec... ns. *P < 0.... **P < 0.01 vs indicated group.

have shown that miR-126 targets sites ... the promo... of p85β, a PI3K subunit that stabilizes and ...ropa...ates PI3K s...nal in CRC cells, which is involved i... the carcinog...esis of CRC.[9,22] Thus, the information abo... the role of miR-12... in the regulation of mTOR signalin... is not n...w, but authors have shown further proof of its importa... in CRC. A...though the potential role and mecha...sm o...ction o... miR...26 in CRC cell survival have been r...orted p...viously. In the present study, we have mainly studie... the effect of miR-126 on the autophagy and apoptosis of CRC ...lls in vitro and in vivo. After overexpression of miR-126 in... CRC cells, mTOR expression was decreased, and apoptosis and autophagy were induced. These results suggest that miR-126 is a tumor suppressor and that it considerably inhibits CRC cell proliferation by modulating the mTOR pathway.

The mTOR sensor has become a target for drug development; because it plays a vital role in cancer biology. In addition, the advent of the next generation mTOR inhibitors marks an exciting new stage in mTOR-related therapy. Indeed, the Pi3k/Akt/mTOR signal transduction axis is one of the main mechanisms for maintaining the growth and metastasis of tumors.[23] The PI3K/Akt/mTOR axis is involved in many biological events, such as apoptosis,[24,25] inflammation,[26,27] and autophagy.[28] Wu et al proved that the positive expression rate of Glut1 was 75% in CRC tissues and 0% in the adjacent normal tissues. Compared with the adjacent normal tissues, in CRC tissues, the expressions of Glut1, TGF-β1, PI3K, AKT, and mTOR were increased, whereas those of PTEN, Bax, cleaved caspase-3, and cleaved-PARP were decreased. These results revealed that Glut1 gene silencing can inhibit proliferation and promote apoptosis of CRC cells by inhibiting the TGF-β/PI3K-AKT-mTOR signaling pathway.[29] mTOR also plays an important role in the autophagy of CRC cells. Aspirin can inhibit mTOR effectors (S6K1 and 4E-BP1) by decreasing the mTOR signal transduction in CRC cells and can also induce autophagy, which is a characteristic of mTOR inhibition.[30] In the present study, after overexpression of mTOR in CRC

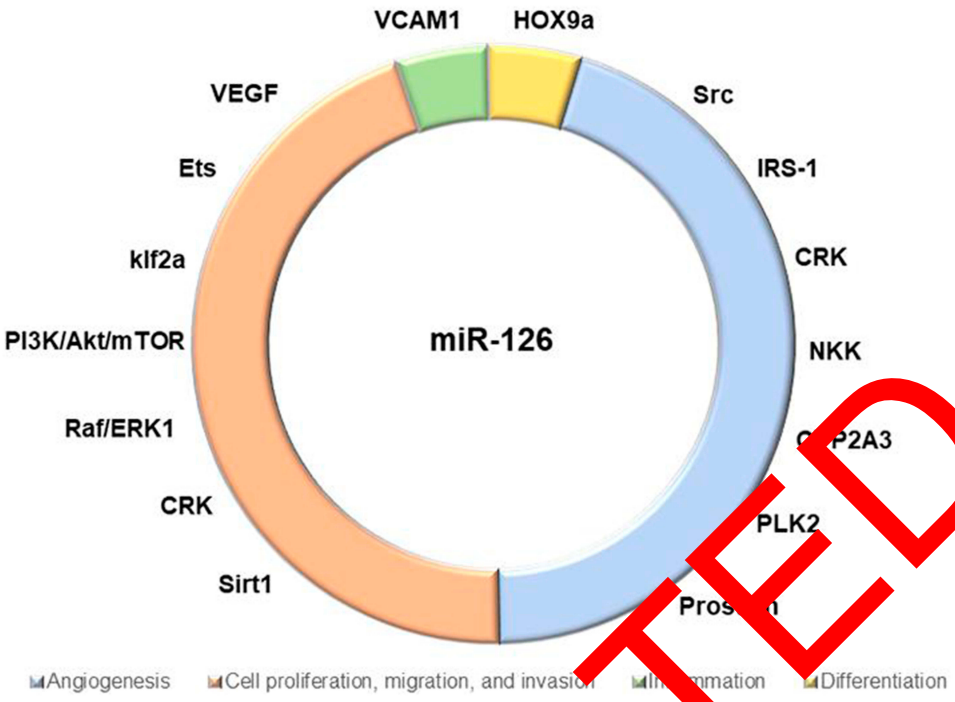

Figure 8 Schematic of the signal pathways and cell functions affected by miR-126. Multiple factors affect the expression of miR-126 during the processes of angiogenesis, vascular integrity disruption, inflammation, and differentiation. Additionally, miR-126 may act as tumor modulator via the inhibition of proliferation, migration, and invasion.

cells transfected with miR-126 mimic, we found that mTOR played an oncogenic role in CRC cells, as evidenced by the restored proliferation and reduced apoptosis and autophagy, which was also contrary to the function of miR-126.

In conclusion, we explored the role of miR-126 in the occurrence and development of CRC, and revealed the antitumor effect of miR-126 on CRC. A decrease in miR-126 expression in CRC tumors is related to impaired growth of cancer cells. Therefore, miR-126 can be further applied as a diagnostic and prognostic biomarker of CRC.

Ethics Approval and Informed Consent

This study has been approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. All study participants provided written informed consent before participating in the study.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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