

# MiR-126 Suppresses Colitis-Associated Colorectal Cancer by Regulating Crosstalk Between Epithelium Cells and Macrophages via CXCL12

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## Research

**Keywords:** miR-126, colitis-associated colorectal cancer, macrophages, CXCL12, IL-6

**Posted Date:** December 28th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-130875/v1>

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# Abstract

## Background

MicroRNAs (miRNAs) are small non-coding RNAs that play essential roles in cancer initiation and progression. Previously, we demonstrated that miR-126 acts as a tumor suppressor in colorectal cancer (CRC), and negatively associated with tumor progression, metastasis, and prognosis of patients with colon cancer. However, the role and mechanism of miR-126 in the progression of colitis-associated colorectal cancer (CAC) remain elusive.

## Methods

Mice were modeling CAC with Azoxymethane(AOM) and Dextran sulfate sodium(DSS). The expression of miR-126 throughout the progression of CAC was analyzed by in situ hybridization (ISH). The effects of miR-126 on CAC were investigated on intestinal epithelium cells (IECs) specific miR-126 deletion (miR-126<sup>ΔIEC</sup>, cKO) mice and wild type (WT) mice by flow cytometry assay, immunohistochemistry, and enzyme-linked immunosorbent assay. The underlying mechanism was explored through mRNA Immunoprecipitation assay, establishing co-culture systems of IECs and macrophages, cell migration assays, IECs proliferation assays, and immunofluorescence staining.

## Results

We found that miR-126 expression decreased in the colon tissues during the development of CAC in the mouse model and patients' tissue. Deletion of miR-126 exacerbated CAC in cKO mice. Then we identified CXCL12 as a direct and functional target of miR-126. By blocking the CXCL12/CXCR4 axis with AMD3100 in the CAC mouse model, the CAC tumorigenesis alleviated in mice from cKO+AMD3100 group compares to cKO mice. CXCL12, together with its receptor CXCR4 has been well studied in the effect on hematopoietic stem cells maintenance and homing, as well as being a potent chemokine attracting lymphocytes and macrophages. However, its function on CAC is still unclear. We found that miR-126 regulates the recruitment of macrophages via CXCL12, and down-regulated its IL-6 and IL-1 $\beta$  expression. Furthermore, we found that after co-cultured with miR-126 silenced cells, macrophages would promote colon cell migration, proliferation, and EMT activity, whereas anti-IL-6 antibodies dampened these phenotypes.

## Conclusions

Collectively, our studies indicate that miR-126 plays a protective role in the development and progression of CAC, and suggest that miR-126 may act as a novel molecular marker for early diagnosis of CAC in the future.

# Background

Colorectal cancer (CRC), one of the most commonly diagnosed malignant tumors, is the third most common cancer among all cancers and ranks second in terms of mortality[1]. Colitis-associated cancer (CAC) is a subtype of colorectal cancer that is commonly preceded by clinically detectable inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), which is characterized by relapsing and chronic inflammation of the intestinal tract with disorders of the gut. IBD is associated with an increased 1.5–2.4-fold higher risk of developing CAC[2], particularly in patients with ulcerative colitis (UC), although CAC only accounts for about 1-2% of CRC cases in the general population[3,4]. However, the initiation and progression of CAC is a complicated transformation process, which mechanisms remain largely unclear.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules containing 20-25 nucleotides. miRNAs regulate protein expression by affecting the stability and/or translation of their target mRNAs and play important roles in inflammatory diseases, cancer initiation, and progression[5]. miRNA dysregulation is also involved in the transition from colitis to CAC. It is reported that miRNAs function as potential links between inflammation and cancer by inducing their target mRNA degradation or blocking translation of key molecular targets involved in IBD[6]. He C et al found that miR-301A promotes intestinal inflammation and colitis-associated cancer development by inhibiting BTG1[7]. Mao R et al found that the loss of miR-139-5p promotes colitis-associated tumorigenesis by mediating PI3K/AKT/Wnt signaling[8].

We previously demonstrated that miR-126 acts as a tumor suppressor via the down-regulation of CXCR4 and the inhibition of the RhoA/ROCK pathway in colon cancer[9]. Other researchers reported that miR-126 exerts antitumor functions in various types of cancers, such as ovarian cancer, liver cancer, gastric cancer, and non-small cell lung cancer[10,11]. miR-126 is also involved in the regulation of inflammatory events[12]. Up-regulated miR-126 reduces inflammation in intestinal colitis and suppresses colorectal aberrant crypt foci [13–15]. However, other reports indicate that miR-126 increases in IBD and promotes the pathogenesis of ulcerative colitis [16]. The role and mechanism of miR-126 in the progression of CAC remains to be further defined.

In this study, we investigated the potential role of miR-126 in the initiation and progression of CAC. Here, we established a CAC mouse model by administering AOM/DSS to provide important insights into inflammation-associated tumorigenesis. We carried out a comprehensive analysis of miRNA expression profiles throughout the progression of CAC. The expression levels of miR-126 are down-regulated along the process as well as in CRC patients' colon tissues. We generated mice containing intestinal epithelium cells specific miR-126 deficiency (miR-126<sup>ΔIEC</sup>, cKO), which were more sensitive to the development of AOM/DSS-induced CAC. By RNA pull-down analysis, we identified CXCL12 as the direct and functional target of miR-126. The CAC tumorigenesis and progression were alleviated after blocking the CXCL12/CXCR4 axis with AMD3100 in the CAC mouse model. We determined that miR-126 regulates the recruitment of macrophages via CXCL12, inhibits its pro-inflammation factors expression and, consequently, tumorigenesis.

# Methods

## Generation of IEC specific miR-126 deficient mice

MiR-126 floxed mice were developed by the Shanghai Model Organisms Center, Inc (Shanghai, China). The Cre/loxP and Flp/FRT recombination systems were used to target exon1 of miR-126 by ES cell targeting methods to generate the miR-126<sup>flox</sup>-Neo/+ mice. The miR-126<sup>flox</sup>-Neo/+ mice were then crossed with Flp transgenic mice to delete the Neo cassette and obtain the miR-126<sup>fl</sup>/+ mice. To generate mice lacking miR-126 in the intestinal epithelium cells, the miR-126<sup>fl</sup>/+ mice were crossed with Villin-Cre (Tg(Vil1-Cre), The Jackson Laboratory). Genomic DNA was extracted from tail biopsies and used as a template for PCR analysis with the appropriate primers to identify mouse genotypes (primers are listed in Table 1). Routine genotyping of mice was carried out by PCR amplification (sFigure 1). The male and female cKO mice were fertile, developed normally, and displayed no gross morphological abnormalities.

Initially, all used mice in this study were 8–10 weeks old and bred in-house to generate comparable groups. And all mice were maintained under the pathogen-free condition and fed autoclaved food and water. All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The protocol was approved by the Animal Ethics Committee of Central South University for the protection of animals used for scientific purposes.

## Induction of CAC

Eight to ten-week-old C57 mice, cKO mice, and wild-type(WT) littermates were injected with an intraperitoneal administration of AOM (Sigma-Aldrich, St. Louis, MO, USA; 10 mg/kg) dissolved in physiological saline. Seven days later, the mice were given drinking water including 1.5% DSS (MP Biomedicals, Solon, OH, USA; MW, 36000–50000 Da) for the following seven consecutive days, followed by regular water for two weeks. These three weeks constituted one cycle, and additional two cycles were repeated. And then regular water was administered until the end of the experiment on the 18<sup>th</sup> weeks. The experiments were performed with at least 6–8 mice in each AOM-treated condition. During the treatment course, bodyweight, diarrhea, and haematochezia were recorded daily.

In order to interference with the CXCL12/CXCR4 axis, mice were treated daily with AMD3100 (5 mg/kg, i.p) or PBS for the control group. These mice were sacrificed at the indicated time intervals, and colon tissues were opened longitudinally, colon size and the number of tumors were measured. Colon sections were frozen in liquid nitrogen for total RNA and protein extraction or fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin for histological analyses. Serum collected from each mouse was prepared for inflammatory cytokines detection. All mice procedures were performed in accordance with institutional guidelines.

## Scoring of mouse disease activity index (DAI)

Scoring of the mouse disease activity index was performed as previously reported in a blinded fashion<sup>16</sup>. Briefly, DAI ranging from 0 to 4 was calculated as follows: stool consistency (normal, loose, diarrhea), presence or absence of haematochezia (guaiac paper test and macroscopic evaluation of the anus), and weight change.

### **Histopathological analysis**

Paraffin-embedded sections were then stained with hematoxylin and eosin (H&E) for microscopic examination. The slides were reviewed and scored for inflammation by two pathologists blinded to the treatment based on the criteria as follows: score 0, no inflammation; score 1, modest numbers of infiltrating cells in the proper layer; score 2, infiltration of inflammatory cells with mild mucosal hyperplasia; score 3, massive infiltration of immune cells with disrupted mucosal architecture, and marked mucosal hyperplasia; score 4, all of the earlier described plus crypt abscesses or ulceration.

### **In situ hybridization (ISH) and immunohistochemical staining (IHC)**

In situ hybridization was used to detect miR-126 expression in the mouse colon tissue. The detection of miR-126 by ISH was performed utilizing an enhanced sensitive ISH detection kit (Boster, Wuhan, China) according to the manufacturer's instruction. Here, the image with brown or yellow was regarded as positive signals of miR-126 expression. Immunohistochemistry was carried out on 4µm-thick paraffin-embedded sections from the colorectums in mice. Here, the image with blue or purple was regarded as positive signals of miR-126 expression. The deparaffinized sections were incubated with primary antibody at 4°C overnight followed by incubation with biotinylated goat antirabbit or mouse IgG antibody (Vector Laboratories, Burlingame, CA) for 30 min. Primary antibodies were purchased from the listed vendors: p65(Cell Signaling Technology, Danvers, MA); CXCL12(Cell Signaling Technology, Danvers, MA); CXCR4 (Santa Cruz Biotechnology, Santa Cruz, CA); p-p65(Abcam, Cambridge, UK); and CD68 (Boster, Wuhan, China). The omission of the primary antibody was used as the negative control. The immunostained slides were observed under a microscope (OLYMPUS BX-51, Japan) by two independent pathologists blinded to clinicopathologic features and clinical courses. A semi-quantitative scoring criterion for immunohistochemistry was used, in which both staining intensity and positive areas were recorded. The intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of positive tumor cells was set as follows: 0 (< 5%), 1 (5–25%), 2 (26–50%), 3 (51–75%). And the final score = 0 was defined as negative expression, otherwise was defined as positive expression.

### **Cell culture and transfection**

Human CRC cell line(Caco2), HEK293 cell line, and the human monopoly cell line THP-1 were obtained from the Cancer Research Institute of Central South University (Changsha, Hunan, China). All cells were cultured at 37°C in RPMI 1640 medium (Hyclone) supplemented with 10% fetal calf serum, 100U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO<sub>2</sub>. THP-1 cells (4×10<sup>5</sup>/ml) were differentiated into macrophages using 150 nM phorbol 12-myristate 13-acetate (PMA,

Sigma-Aldrich) for 2d. miR-126 mimic, miR-126 inhibitor, and the corresponding scrambled negative control (NC) vectors and inhibitor negative control (iNC) vectors were synthesized by GenePharma (Suzhou, China). Cells were transfected with 50 nM miRNA mimics or 50nM miRNA inhibitors using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### **Generation of instantaneous transformants**

MiR-126 mimics, miR-126 inhibitors, the NC, and iNC vectors were purchased from GenePharma (Guangzhou, China). Cells were transfected with 50 nM miRNA mimics or 50 nM miRNA inhibitors using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### **Cell migration assays**

The impacts of CXCL12 and colon cells with different expression levels of miR-126 on the migration of macrophages were determined by transwell migration assays, respectively. Briefly, THP-1 induced macrophages ( $2 \times 10^5$  cells/well) in 200 $\mu$ l FBS-free medium were cultured in triplicate in the top chambers of 24-well transwell plates (8.0 $\mu$ m pore, Millipore). In the bottom chamber, 15% FBS medium (600 $\mu$ l /chamber) with 100ng/ml CXCL12, 100ng/ml AMD3100, or Caco2 cells transfected with miR-126 mimics, negative vector, inhibitor, or inhibitor negative vector was added as a chemoattractant. After being cultured for 24h, the macrophages remaining on the membrane surface of the top chamber were removed with a cotton swab. The migrated cells onto the bottom surface of the top chambers were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet solution, and imaged under a light microscope. The cells in 10 fields selected randomly were counted in a blinded manner.

### **RNA extraction and quantitative real-time PCR**

Total RNA was extracted from the cells and tissues using Trizol method. The purity and concentration of RNA were determined by ultraviolet spectrophotometry. RNA integrity was assessed by agarose gel electrophoresis. Total RNA was used to synthesize cDNA by reverse transcription with the SuperScript™ RT reagent Kit (Thermo Fisher Scientific, Waltham, USA) with a reaction system volume of 20 $\mu$ L. Expression of the mRNAs was evaluated using SYBR green qRT-PCR (Takara Biotechnology Ltd., Dalian, China) under the standard protocol. GAPDH was used as an internal control, and the relative quantitative method was applied to calculate the relative mRNA copy number (measured in triplicate)  $2^{-\Delta\Delta C_t}$  was used to express the ratio of target mRNA expression relative to the GAPDH mRNA expression. The miDETECT A Track™ miRNA qRT-PCR kit (RiboBio, Guangzhou, China) was used to synthesize the cDNA of miR-126 and analyze its expression. U6 small nuclear RNA was used as an internal control for assay of miRNA expression levels, and expression of each gene was quantified by measuring cycle threshold (Ct) values and normalized using the  $2^{-\Delta\Delta C_t}$  method relative to U6 small nuclear RNA<sup>16</sup>. The RT-PCR primers are listed in Supplementary Table 1.

### **Western blot**

In Western Blot analysis, total proteins were extracted from the cultured cells using RIPA buffer with both phosphatase and protease inhibitors (Roche, IN). Equal aliquots of 50µg total proteins were separated by an SDS-PAGE (10% acrylamide). Then they were transferred to a PVDF membrane (Merck Millipore, Germany). Membranes were blocked in TBST containing 5% skim milk and then incubated with primary antibodies overnight at 4°C followed by secondary antibodies for 1h at 37°C. Finally, an ECL detection system (Merck Millipore, Germany) was used for signal detection. Mouse monoclonal antibodies against IL-1β(3A6) and CXCL12 (D32F9) were purchased from Cell Signaling Technology. Rabbit polyclonal primary antibodies against IL-6 (ab6672) were purchased from Abcam. The mouse monoclonal antibody against GAPDH and the rabbit monoclonal antibody against β-Tubulin was purchased from Google biology (Wuhan, China).

### **miRNA: mRNA Immunoprecipitation (RIP)-Chip with Anti-pan-Ago**

For each sample, 2×10<sup>7</sup> cell equivalent HEK293 cells were subjected to immunoprecipitation using the miRNA Target IP kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, approximately 24h after transfection of 5µmol mimics-miR-126, cells were washed with ice-cold PBS, scraped off, and lysed in 150µL ice-cold complete lysis buffer (provided in the kit). Approximately 100µL of the whole-cell extract was incubated with 900µL Immunoprecipitation Buffer containing magnetic beads conjugated with human anti-pan-Ago antibody or negative control normal mouse IgG (provided in the kit) and rotated overnight at 4 °C. Samples were washed 7 times with 1× Wash Buffer. The samples were then incubated with Proteinase K buffer at 55°C for 30 min with shaking to digest the protein. Co-immunoprecipitated RNA including miRNA:mRNA complexes were subjected to qRT-PCR analysis. According to the manufacturer's protocol, the enrichment of miRNA was calculated as follows: Fold Enrichment = AE (Neg IgG CT–Ago CT), where AE = Amplification Efficiency = 10<sup>(-1/slope)</sup>=10<sup>(-1/s-3.508)</sup>. Representative bar diagram from three independent experiments, each set of experiment was done in triplicates.

### **Gut lamina propria lymphocytes (LPLs) isolation**

The intestines were opened longitudinally and cut into 0.5cm sections, shook twice with HBSS containing 10mM HEPES, 25mM NaHCO<sub>3</sub>, 1mM DTT, and 1mM EDTA (all from Sigma-Aldrich), and 2% fetal bovine serum (Gibco, Portland, OR) at 37°C for 20 min. IECs were then washed using HBSS to remove away. For the isolation of LPLs, intestinal pieces were digested for 40 min with complete RPMI-1640 containing 1mg/ml collagenase (Roche Applied Science, Upper Bavaria, Germany), 40ul/ml dispase (Sigma-Aldrich), and 4ul/ml DNase I (Sigma-Aldrich) at 37 C. The samples were then passed through a 70 µm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), and the resulting cell suspension was centrifuged at 500g for 5 min, and subsequently resuspended in 1× PBS.

### **Flow cytometry and antibodies.**

The anti-mouse antibodies F4/80-PE, CD11b-FITC, and their respective fluorochrome-conjugated isotypes were purchased from BD Biosciences (San Jose, CA). All of the antibodies were used according to the

manufacturer's instructions. Cells were acquired on a BD FACS CANTO II flow cytometer (BD Biosciences, San Diego, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

### **Enzyme-linked immunosorbent assay**

The mouse serum protein levels of IL-1 $\beta$  and IL-6 were measured at the 0<sup>th</sup>, 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> week using Sandwich ELISA kits (4A Biotech, Beijing, China) According to the manufacturer's instructions.

### **Statistics**

Data were presented as mean  $\pm$  standard error of the mean (SEM) or mean  $\pm$  standard error of at least three independent experiments. Graphical analyses, statistical analysis, and nonlinear regression analysis of the data were performed using GraphPad Prism Software (La Jolla, CA, USA). Differences between groups were determined using 1-way analysis of variance (ANOVA), 2-way ANOVA, or unpaired Student's 2-tailed t-test. P-value of <0.05 was considered statistically significant.

## **Results**

### **MiR-126 is down-regulated in the progression of colitis-associated colorectal cancer**

Previously, we demonstrated that miR-126 acts as a tumor suppressor in colon cancer[9]. Chronic colitis increased the risk of developing CAC in IBD patients[17]. MiR-126 is reported to be involved in the regulation of inflammatory events. However, the biological function of miR-126 in CAC has not been fully elucidated.

Therefore, we established a CAC mouse model by administrating AOM/DSS and carried out a comprehensive analysis of miR-126 expression profiles in the progression of inflammation-associated tumorigenesis. We detected miR-126 mRNA express level in colon tissues of normal, acute inflammation, chronic inflammation, dysplasias, and colorectal cancer five representative time points (at the end of the 0<sup>th</sup>, 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> week, respectively) by ISH and qRT-PCR (Figure1A, B), which showed that miR-126 was down-regulated at least 2-folds in inflammation, dysplasia, and cancerous tissues compared to normal tissues. We then analyzed the expression level of miR-126 in the colon and rectal human tissues from GEO database GSE115513[18] (Figure 1C). Respectively four comparison groups in colon and rectal reveal similar tendencies as mouse CAC model in expression pattern. The mean expression levels of miR-126 in the colon and rectal normal mucosal are 1.5-4 folds of that in pre-cancerous tissues, such as adenoma, serrated sessile polyp as well as high-grade dysplasia. We also assessed the expression of miR-126 in the colon tissues of normal, UC, CD, and colon cancer patients. We found that miR-126 decreased in inflammation (UC, CD), and cancer tissues when compared that with normal colon tissues (Figure1D). These findings suggest that miR-126 may inhibit the process of colorectal cancer tumorigenesis.

### **Deletion of miR-126 in the intestinal epithelium promotes CAC tumorigenesis**

To illuminate the function of miR-126 in CAC, we used WT mice and cKO mice received an intraperitoneal injection of AOM (10 mg/kg) followed by three cycles of DSS treatment (1.5% in drinking water) (Figure 2A). Daily monitoring revealed more weight loss, severer diarrhea, and hematochezia in cKO mice than in their control littermates. In both male and female cohorts of mice receiving AOM/DSS treatment, mice lost weight during DSS cycles, while they recovered during the inter-cycles. The cKO mice showed higher increased disease indexes as evaluated by the severe level of rectal bleeding and diarrhea scores (figure 2B).

In the present study, cKO mice and WT mice were euthanized at the end of 0<sup>th</sup>, 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> weeks and the colons were resected to measure the tumor number and colon length. Results showed that the histopathology measurement of the samples collected at the 0<sup>th</sup> week were normal, at the 2<sup>nd</sup> week were pure inflamed mucosa, at the 6<sup>th</sup> week were dysplasias, at the 10<sup>th</sup> week were grade dysplasias with moderate-to-severe inflammation, and at the 18<sup>th</sup> week were adenocarcinoma. The cKO mice displayed decreased colon length (figure 2B) and a noticeable increase in the number of macroscopically visible tumors compared with WT mice (figure 2C). Histological analysis showed increased inflammation and tissue injury indices in cKO mice than that in WT mice at the 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> week (figure 2D, E, F). However, samples collected at the 0<sup>th</sup> week of WT mice and cKO mice showed no differences. Collectively, these data suggested miR-126 in the intestinal epithelium protects against colitis-associated tumorigenesis.

### **CXCL12 is a functional target of miR-126**

To further demonstrate the mechanism of miR-126 inhibiting the process of CAC tumorigenesis, a functional target is needed. To this end, we look through miRTargetLinks database and found that CXCL12 is a potential target of miR-126 with strong evidence (figure 3A left). We further conducted miRNA-target interaction prediction in miRanda and Targetscan, the algorithms indicated that human miR-126 has 3 potential binding sites (could target 3 evolutionarily conserved sequences) in CXCL12 whereas murine miR-126 has one binding site (figure 3A right). As a first step in the binding analysis, following transfection of miR-126-3p into HEK293 cells, cell lysates were exposed to protein G magnetic beads and pan-Ago antibodies to collect RNA induced silencing complex (RISC) and further purified into RNA to detect downstream targets. As shown in figure 4B, the expression level of miR-126 in the miR-126 mimic group is significantly higher than that in the Non-targeting group, indicating the successful transfection of miR-126, the effective targeting of protein G magnetic beads, and pan-Ago antibodies. The levels of CXCL12 mRNA in the pull-down material isolated from HEK293 cells transfected with miR-126-3p were 10 times more than that in the control group (figure 3B), suggesting that CXCL12 is a direct target of miR-126.

We further established miR-126 over-expressed and miR-126 suppressed Caco2 cells (figure 3C) and detect its expression level of CXCL12. Real-time and Western Blot results revealed that miR-126 suppressed the expression of CXCL12 (figure 3C, D). We also detected the expression of CXCL12 in colon tissue of CAC by IHC, we found that CXCL12 is more obvious in cKO mice than that of WT mice at five-

time points (figure 3E). Collectively, our results showed that CXCL12 is a direct and functional target of miR-126.

### **AMD3100 dampened the anti-tumor effects of miR-126 in CAC**

A previous study demonstrated that blocking of the CXCL12/ CXCR4 axis significantly ameliorates murine experimental colitis[19], indicating a possible role of CXCL12 in the intestinal inflammatory response. To further determine the function of miR-126 and CXCL12 in the progression of CAC, we constructed AOM/DSS-induced CAC mouse model in cKO mice and injected AMD3100 daily (5 mg/kg, i.p) to block the CXCL12/CXCR4 axis. The cKO mice showed an increased DAI, while AMD3100 could alleviate the severity of symptoms and reduce the DAI (figure 4A). The cKO mice displayed decreased colon length, while treated with AMD3100 could decrease the degree of colon length shortness at all the time points (figure 4B). The number of macroscopically visible tumors in the colon of cKO mice was more than WT mice, while AMD3100 can partially reverse this result (figure 4C). Additionally, there are more mice with rectal prolapse in cKO group than the other two groups. Histological analysis also showed increased inflammatory and tissue injury indices in cKO mice than that in WT mice, while AMD3100 would partly dampen the damage (figure 4E). After one week of DSS treatment (week 2), mice of cKO group showed severe structure damage including goblet cell loss, erosion, and cryptitis with massive inflammatory cell infiltration whereas the other two groups largely remain the epithelial barrier. Only 6 weeks after treatment, mice of cKO group showed obvious crypt hyperplasia, which then became high-grade dysplasia large polyps, and carcinoma. AMD3100 dampened the damage and delayed the onset of tumorigenesis. Collectively, these data suggested that CXCL12 is an important target of miR-126 to mediate the process of CAC.

### **MiR-126 regulates the recruitment of macrophages via CXCL12**

The inflammatory and tumor microenvironment plays an essential role in the regulation of the development of colitis-associated colorectal cancer, among which tumor-associated macrophages (TAMs) are a major component and play pivotal roles[20]. CXCL12 is a potent chemotactic factor for recruiting TAMs[21].

In the present studies, we induced a human macrophage cell line by stimulating monocyte cell line THP-1 with 150ng/ml PMA for 2 days. We also build a co-culture system of macrophages and miR-126 overexpression/silenced colon cancer cells to detect the recruitment and secretion of macrophages, in which AMD3100 or CXCL12 was used to block or stimulate the CXCL12/CXCR4 axis. The transwell assays result showed that miR-126 over-expression decreased the number of migrated TAMs, however, the number of migrated macrophages significantly increased when adding CXCL12. On the contrary, miR-126 suppression increased the number of migrated macrophages. Interestingly, when blocking the CXCL12/CXCR4 axis with AMD3100, the number of migrated macrophages significantly decreased (Figure 5A).

To further elucidate the mechanism by which miR-126 regulates the recruitment of macrophages, FACS, and IHC analyses were performed to detect CD68+ cells and macrophages in the colon tissue on the 0<sup>th</sup>, 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> week. FACS showed that the number of macrophages was significantly increased in the colon of cKO mice compared to that in WT mice at 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> week, but when treated with AMD3100, the number of macrophages significantly decreased (Figure 5B, C). The IHC staining showed that CD68+ macrophages were significantly increased in the colon of cKO mice compared with that in WT mice at the 2<sup>nd</sup>, 10<sup>th</sup>, and 18<sup>th</sup> weeks. However, when treated with AMD3100, the number of CD68+ macrophages significantly decreased at the 10<sup>th</sup> and 18<sup>th</sup> weeks (Figure 5D, E). These results suggested that miR-126 regulates the recruitment of macrophages via CXCL12.

### **MiR-126 regulates the secretion of macrophages via CXCL12**

The inflammatory microenvironment plays an important role in regulating the development of carcinogenesis[22]. As an important part of the inflammatory microenvironment, macrophages are critical mediators of inflammation, and chemokines also involved in the inflammatory microenvironment by stimulating macrophages recruitment and secretion[20]. It is reported that the contribution of macrophages to CAC development may be through cytokine production and therefore suppressing the immune response and promoting a nurturing niche for cancer stem cells[23].

Our previous study indicated that IL-6 and IL-1 $\beta$  was up-regulated in CAC, and combined with our present studies[24], we speculate that macrophages involved and promoted the CAC development may be through secreting IL-1 $\beta$  and IL-6. We used Western Blot assay to detect the expression of IL-1 $\beta$  and IL-6 in macrophages in the co-culture system. The result shows that miR-126 over-expression cells down-regulated the expression of IL-1 $\beta$  and IL-6, however, their expression increased when stimulated with CXCL12. And miR-126 silenced cells up-regulated the expression of IL-1 $\beta$  and IL-6, while this effect can be reversed by blocking the CXCL12/CXCR4 axis with AMD3100 (Figure 6A).

We further examined the expression of IL-1 $\beta$  and IL-6 in CAC mice by ELISA and IHC analyses. ELISA assays showed that IL-1 $\beta$  and IL-6 were significantly highly expressed in serum in CAC in cKO mice, while AMD3100 can inhibit the up-regulation induced by the deletion of miR-126 in IECs (Figure 6B). IHC analyses showed that IL-1 $\beta$  and IL-6 were increased in the progress of CAC. And the expression of IL-1 $\beta$  and IL-6 were significantly increased in the colon tissues of cKO mice compared to that in WT mice at the 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup>, and 18<sup>th</sup> weeks (Figure 6C-F). However, AMD3100 could dampen the up-regulation. Collectively, these data suggested that miR-126 may regulate the recruitment and secretion of macrophages via CXCL12.

### **IL-6 is the key molecular for miR-126 dampen colitis carcinogenesis**

“Educated” macrophages by miR-126 through CXCL12 have shown increased secretion of a famous pro-inflammatory factor: IL-6. IL-6 signaling is generally considered a bad guy that supports tumor initiation and progression in numerous mouse models as well as in human tumorigenesis[25]. We measured cell

migration, invasion and epithelial-mesenchymal transition (EMT) in IL-6 stimulated colon cell. With no surprise, we found that after stimulation, cells expressed a lower level of E-cadherin and a higher level of Vimentin which indicated an increased EMT activity, and shown significantly increased capacity of migration (sFigure2).

We then established a two-step co-culture system of TAMs and epithelial cells as shown in Figure7B. The TAMs were first co-cultured with miR-126 over-expressed or silenced cancer cells for 2 days, then the co-cultured TAMs were taken away from the previous system and put into a new co-culture system with untreated cells for another 2 days, and IL6 neutralizing antibody was added. Interestingly, after co-culture with “educated” macrophages which have experienced several steps of co-culture, the “un-treated” colon cells still shown different phenotypes, showing the long-term influence of miR-126 on macrophages through CXCL12 stimulation. “Educated” macrophages by over-expressed miR-126 cells dampened migration, proliferation, and decreased EMT activity (figure7A, C, D). On the contrary, after co-cultured with anti-miR-126 cells, macrophages would reversely promote migration, proliferation in colon cells, and increase its EMT activity, whereas IL-6 dampened these phenotypes, suggests that IL-6 is the key molecular for miR-126 dampen colitis carcinogenesis by targeting CXCL12 and recruiting TAMs.

## Discussion

MiR-126 exerts tumor suppressive-functions in various cancers, such as gastric carcinoma[26], breast carcinoma[27], and prostate cancer[28]. Consistent with them, we found that miR-126 was significantly down-regulated in CRC and miR-126 inhibits proliferation, invasion, and metastasis in CRC[9,29,30]. Although CAC account for only about 1-2% of CRC cases in the general population, it accounts for approximately 15% of all-causes mortality among IBD patients[4]. CAC is one of the most serious complications of long-duration IBD, particularly UC. CAC risk increases by around 0.5–1% each year following UC diagnosis and reach to 18.4% at 30 years after UC onset[31].

In the colon tissues from UC and CD patients, which have scattered neoplasia sites within the inflammatory lesion, we found an obviously decrease of miR-126 in inflammation (UC, CD), and cancer tissues when compared that with normal colon tissues. Other researchers have reported an increase of miR-126 in IBD patients’ colon tissues unidentified with or without neoplasia[32,33]. The difference of the miR-126 expression level in IBD tissues between their and our data indicates it might because of the disruption of downstream tumorigenic pathways. The mouse model of AOM/DSS-induced CAC mimics the overall “inflammation-dysplasia-carcinoma” process, providing important insights into inflammation-associated tumorigenesis. By analyzing the CAC mouse, we found that miR-126 expression decreased in inflammation, dysplasia, and cancerous tissues compared to normal tissues.

However, the pathogenic role of miR-126 in the progression of CAC remains unclear. Hyemee Kim et al reported that mango polyphenols attenuated inflammatory response by modulating the PI3K/AKT/mTOR pathway partly through upregulation of miRNA-126 expression both *in vitro* and *in vivo*[14]. Nivedita Banerjee et al showed that Pomegranate polyphenolics suppressed AOM-induced colorectal aberrant

crypt foci and inflammation through up-regulation of miR-126[13]. Gabriela Angel-Morales et al found that Red wine polyphenolics reduce the expression of inflammation markers in human colon-derived CCD-18Co myofibroblast cells by increasing the expression of miR-126[15]. While other reports indicated that miR-126 levels are increased in IBD and miR-126 promotes the pathogenesis of ulcerative colitis. Feng X et al reported that up-regulation of microRNA-126 may contribute to pathogenesis of ulcerative colitis via regulating NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ [34]. However, the biological function of miR-126 in CAC remained to be further claimed.

Intestinal epithelial cells are considered one of the most important components of innate immunity and play key roles in maintaining intestinal barrier function. It has been reported that miRNAs have been implicated in regulating intestinal homeostasis. Chong He et al demonstrated that miR-301A promoted intestinal inflammation in IEC through facilitating NF- $\kappa$ B signaling, compromised intestinal barrier integrity, and miR-301A also induced colon tumorigenesis by enhancing tumor cell proliferation[7]. In previous studies, we confirmed the decreased expression of miR-126 in CAC. To investigate the role of miR-126 in CAC, we established a miR-126 IEC specific deletion mouse model. We chose the AOM/DSS-induced inflammation model in which to monitor the difference between WT and cKO mice. The loss of miR-126 in IEC aggravated the intestinal injury and the development of CAC indicates that miR-126 deficiency enhanced tumorigenicity.

CXCL12 is firstly characterized as a pre-B cell growth-stimulating factor and its specific receptor is CXCR4, which also functions as an entry receptor for human immunodeficiency virus[35]. The CXCL12/CXCR4 chemokine axis has an important role in the development of cardiovascular, hematopoietic, and central nervous systems, and is also involved in several inflammatory diseases such as rheumatoid arthritis, acute lung injury, and sepsis[36–39]. Recent studies demonstrated that CXCL12 and CXCR4 are constitutively expressed in IECs, lamina propria T cells, and peripheral blood T cells of control patients, and the expression is increased in UC patients[40]. Also, CXCL12 and relative CXCL12-CXCR4 expression are independent prognostic factors for 5-year disease-free survival in colon cancer[41]. A previous study demonstrated that blocking of CXCR4 significantly ameliorates murine experimental colitis, indicating a possible role of this chemokine axis in intestinal inflammatory response[19].

Previously, we found that CXCR4 is a direct target of miR-126 in human colon cancer cells. MiR-126 inhibits colon cancer cell proliferation and invasion by negatively target CXCR4[29]. Besides, we found that the serum CXCL12 up-regulated during different phases of the inflammation-cancer process[24]. In this study, we found that CXCL12 is a direct and functional target of miR-126. MiR-126 inhibits CXCL12 inhibition in colon cancer cells. CXCL12 increased during the "inflammation-dysplasia-cancer" process in the colon tissues of cKO mice, whereas AMD3100 can invert this process. Combined with these results, we demonstrated that miR-126 may inhibit colitis-associated colon cancer by CXCL12.

The inflammatory microenvironment consists of inflammatory cells, such as macrophages, inflammatory factors, cytokines, and inflammation-related signal. Macrophage recruitment into tissues initiates inflammation[42]. The tumor microenvironment is a complex ecology of cells that evolves with and

provides support to tumor cells during the transition to malignancy. Among the innate and adaptive immune cells recruited to the tumor site, macrophages are particularly abundant and are present at all stages of tumor progression. Macrophages are innate immune cells that play a broad role in host defense and the maintenance of tissue homeostasis. Within the tumor, macrophages are a major stromal component, where they are commonly called TAMs. In the previous study, TAMs gradually increased in colonic mucosa during the "inflammation-dysplasia-carcinoma" process[43]. Kim D. et al. found that CXCL12 induces monocyte migration via its receptor chemokine CXCR4 in mice[44]. Rigo, et al., reported that macrophages may promote cancer growth via a GM-CSF/HB-EGF paracrine loop that is enhanced by CXCL12[45]. Increased amounts of TAMs correlate with shortened survival in cancers. CXCL12 may trigger events driving both CXCR4-positive cancer cells and macrophages migration and promote molecular crosstalk between them. Consistent with them, in the present study, we observed the CXCL12 stimulation of macrophage recruitment in vitro and we also observed an enhanced infiltration of macrophages in CAC and the cKO mice exhibited markedly increased macrophages in CAC compared with the WT mice, suggesting the CXCL12/CXCR4 stimulation of macrophage recruitment in CAC.

A higher density of TAMs infiltration is related to poorer prognosis in cancer patients. TAMs play important tumorigenic roles by secreting inflammatory factors such as IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and ICAM, which can activate signaling pathways associated with proliferation, metastasis, and angiogenesis[46]. Our previous studies showed that both IL-6, and IL-1 $\beta$ , dynamically, and persistently increase during "inflammation-dysplasia-carcinoma", especially in the early inflammatory and neoplastic phases[24,47,48]. In this study, we found that CXCL12 can stimulate macrophage recruitment and CXCL12 can up-regulate the expression of IL-1 $\beta$ , IL-6 in vitro. And we found that CD68+ macrophages, IL-1 $\beta$ , IL-6 were upregulated in the CAC progression, especially significantly increased in cKO mice. Collectively, these results indicated that miR-126 may inhibit the CXCL12 and then inhibit the recruitment and secretion of macrophages. Proinflammatory cytokine IL-6 has been reported to activate STAT3 pathways and promote tumor growth and metastatic colonization in colorectal cancer[49,50]. Our previous studies found that STAT3 signaling pathways were persistently activated during the "inflammation-dysplasia-carcinoma" process[24]. Here, our data showed that IL-6 stimulation increased colon cells' EMT activity and the capacity of migration. Through the two-step co-culture system of TAMs and epithelial cells, we found that after co-cultured with miR-126 silenced cells, macrophages would promote colon cell migration, proliferation, and EMT activity, whereas IL-6 dampened these phenotypes.

## Conclusions

In summary, our studies indicate that miR-126 plays a protective role in the development and progression of CAC. We determined that miR-126 regulates the recruitment of macrophages via CXCL12, inhibits its pro-inflammation factors expression and, consequently, tumorigenesis. MiR-126 may act as a novel molecular marker for early diagnosis of CAC and function as an effective therapeutic for colorectal cancers or other inflammation-associated diseases in the future.

# Abbreviations

AOM, Azoxymethane; CRC, colorectal cancer; CAC, colitis-associated colorectal cancer; CD, Crohn's disease; cKO, miR-126<sup>ΔIEC</sup>, intestinal epithelium cells specific miR-126 deletion mice; DSS, Dextran sulfate sodium; DAI, disease activity index; ELISA, Enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; IECs, intestinal epithelium cells; IBD, inflammatory bowel disease; iNC, inhibitor negative control; ISH, *in situ* hybridization; IHC, immunohistochemical staining; LPL, lamina propria lymphocytes; miRNAs, MicroRNAs; NC, negative control; PMA, phorbol 12-myristate 13-acetate; RISC, RNA induced silencing complex; RIP, mRNA Immunoprecipitation; TAMs, tumor-associated macrophages; UC, ulcerative colitis; WT, wild type.

# Declarations

## Ethics approval and consent to participate

Paraffin-embedded intestinal specimens from patients with IBD, CAC, and CRC were obtained from the Institute of Pathology of the Third Xiangya Hospital of Central South University (Changsha, China). The samples were gathered with informed consent according to the Institutional Review Board of Ethical Committee–approved protocol.

## Consent for publication

Not applicable.

## Availability of data and materials

The GEO database we analyzed in this study is included in this published article [Slattery ML, Herrick JS, Pellatt DF, Stevens JR, Mullany LE, Wolff E, et al. MicroRNA profiles in colorectal carcinomas, adenomas and normal colonic mucosa: variations in miRNA expression and disease progression. *Carcinogenesis*. 2016;37:245–61. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115513>].

## Competing interests

The authors declare that they have no competing interests

## Funding

This work was funded by grants from the National Natural Science Foundation of China (No. 81272736, No. 81670504 and No. 81472287).

## Authors' contributions

XW and SW conceived and designed the experiments. SW and WY performed the experiments. SW, KN, and WL analyzed the data. SW and ZS performed the statistical analysis. SW and WY wrote the

manuscript. All authors read and approved the final manuscript.

## Acknowledgments

Not applicable

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## Figures

Figure 1.

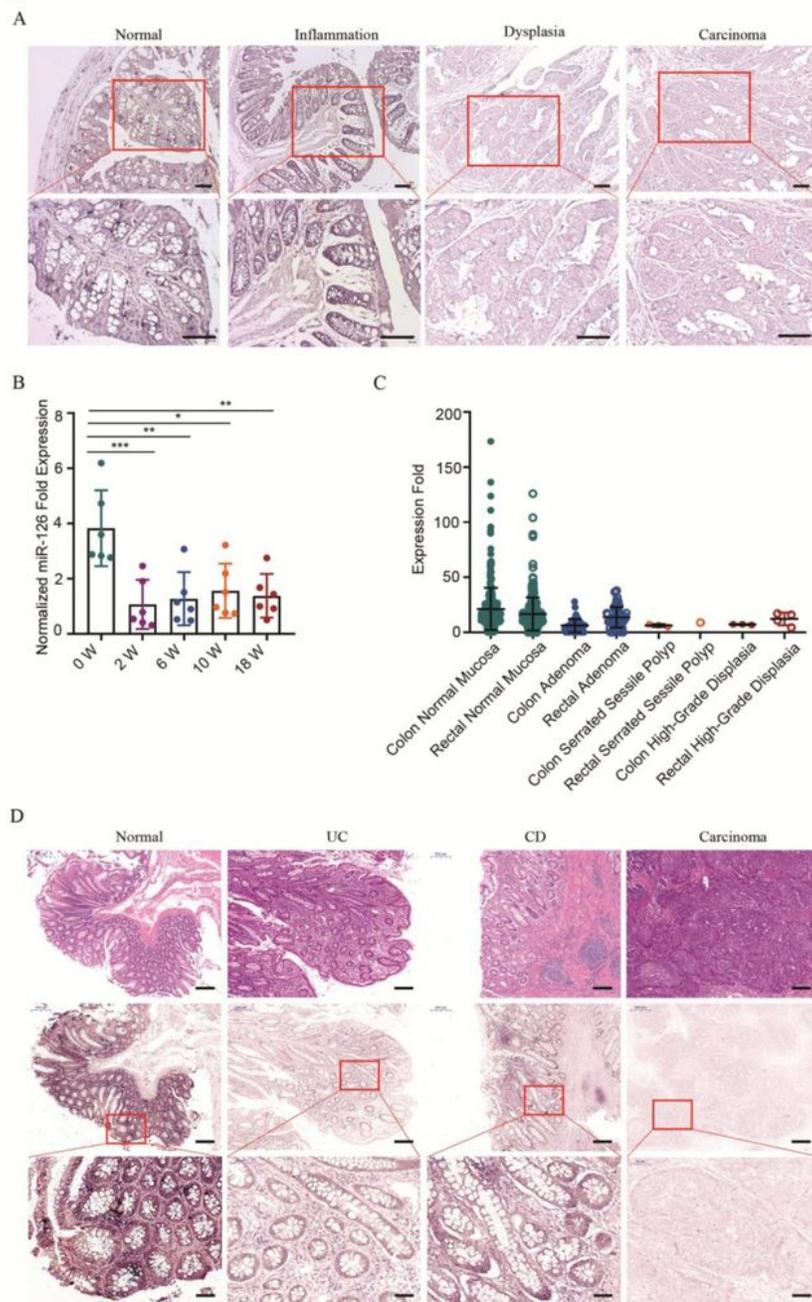


Figure 1

miR-126 is down-regulated in the progression of colitis-associated colorectal cancer. (A) Representative images of ISH analysis of miR-126 in normal, inflammation, dysplasia, and carcinoma colon tissues from AOM/DSS induced CAC mouse model. (B) Real-time RT-PCR detects the expression of miR-126 in colon tissues at different phases (the 0th, 2nd, 6th, and 18th weeks after AOM/DSS treatment) of CRC (n=6). (C) The expression level of miR-126 in the colon and rectal tissues at different phages of CRC from

dataset GSE115513. (D) ISH detects the expression of miR-126 in the colon tissue from normal, UC, CD, and CRC patients. Data are representative of at least five samples in each group with similar results. Data were shown as the mean  $\pm$  SD (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.0001).

Figure 2.

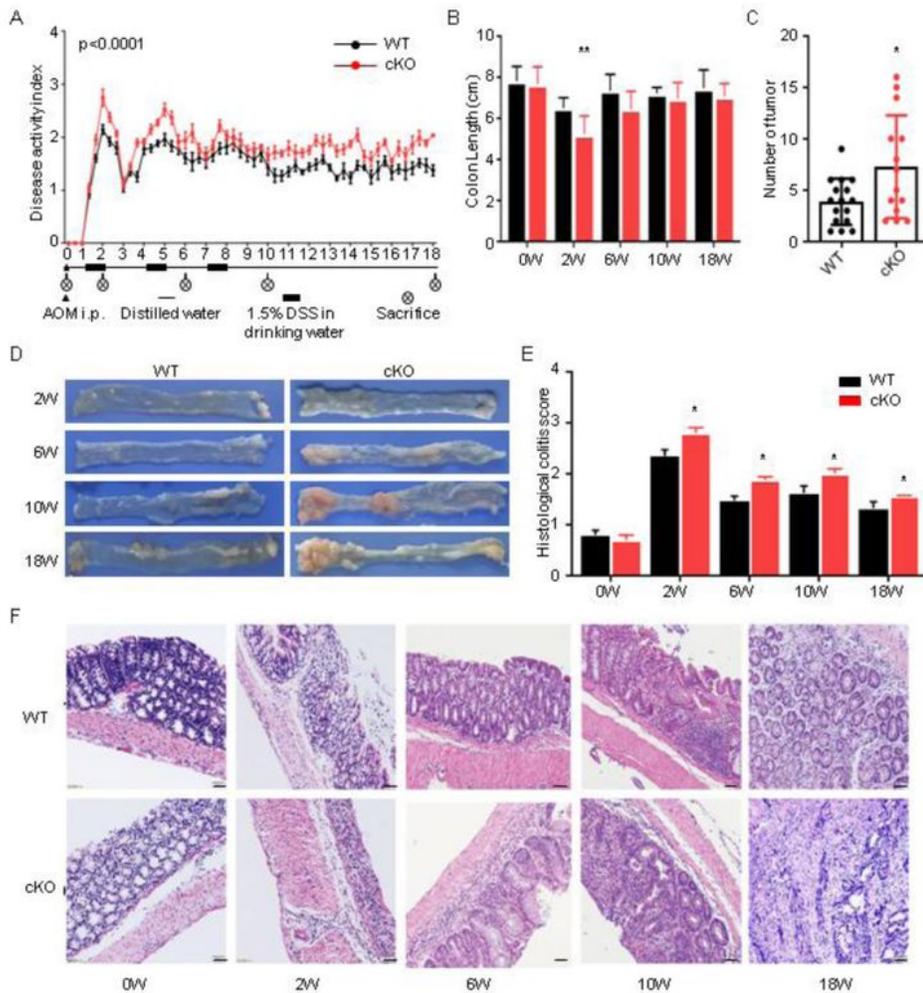


Figure 2

Deletion of miR-126 in the intestinal epithelium promotes CAC tumorigenesis. (A) During the treatment of AOM/DSS, WT mice and cKO mice were monitored and quantified by the DAI. DAI = (weight loss score +

stool characteristic score + hematochezia score)/3. (B) Colon length of WT and cKO mice were measured ( $n \geq 6$ ). (C) Intestinal lesion numbers of the intestinal surface area of each mouse were calculated ( $n \geq 13$ ). (D) Representative images of morphological observation of the distal colon and rectum cut longitudinally at different phases (0th, 2nd, 6th, and 18th weeks after AOM/DSS treatment) of CRC ( $n = 6$ ). (E, F) Histopathology score of colonic mucosa from WT and cKO mice by H&E staining (magnification,  $\times 200$ ), histological colitis score displays increased colitis and injury on cKO mice ( $n \geq 6$ ). Data are representative of six mice in each group with similar results. Data were shown as the mean  $\pm$  SD (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

Figure3

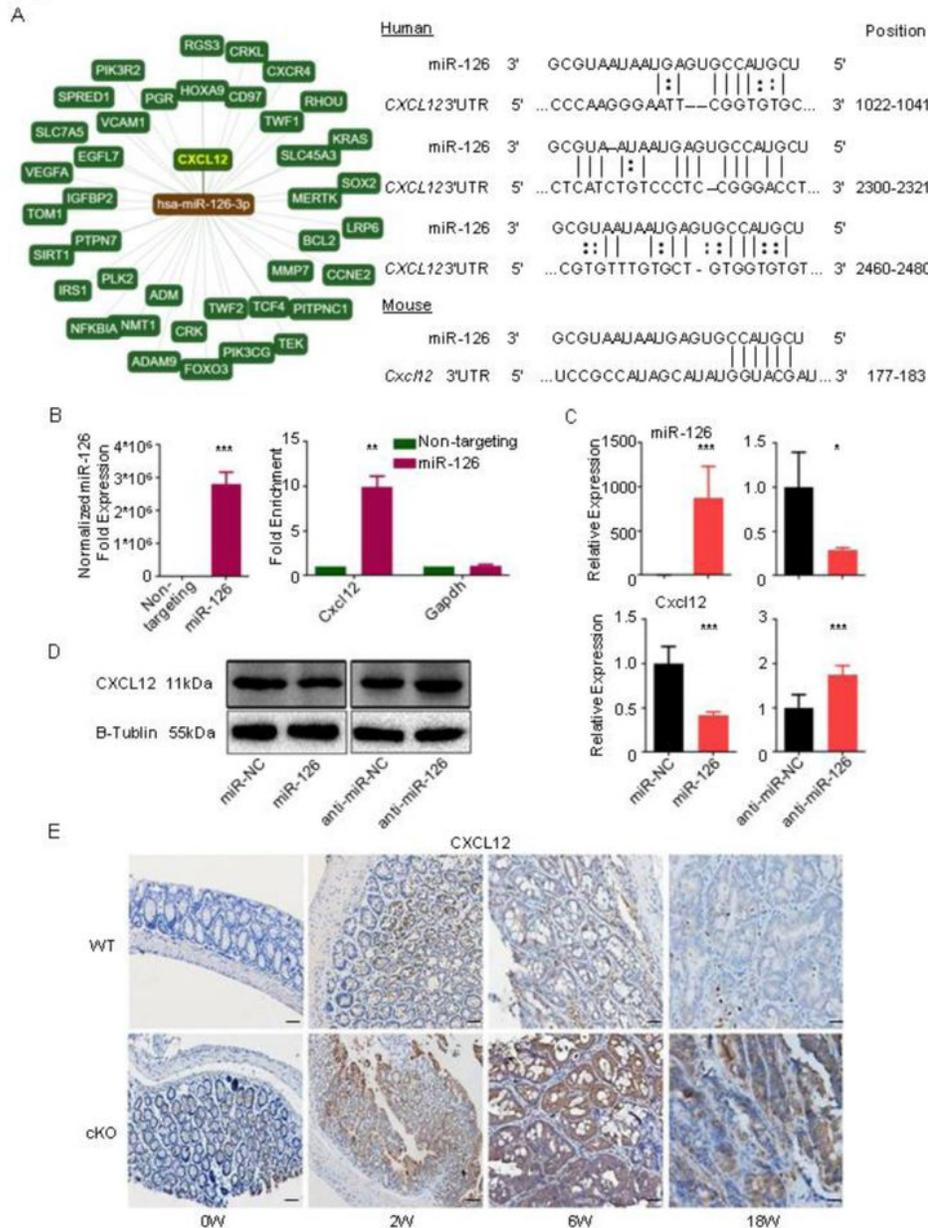


Figure 3

CXCL12 is a functional target of miR-126. (A) The miRTargetLinks database shows predicted downstream target molecules of miR-126 and binding sites between CXCL12 3'UTR and miR-126 by miRanda and TargetScan. (B) RNA Pull Down method analyzed the targeting regulation of miR-126 on CXCL12 (n≥3). (C) Establishment of miR-126 over-expressed and silenced Caco2 cell lines and the relative expression of miR-126 in these cell lines were analyzed by qRT-PCR. U6 small nuclear RNA served as the internal qRT-

PCR control (n≥3). (D, E) Relative expression of CXCL12 in miR-126 over-expressed and silenced Caco2 cell lines were analyzed by Western Blot and qRT-PCR (n≥3). (F) The expression of CXCL12 was assessed by IHC in WT and cKO mice colon tissues (n≥6). Data were shown as the mean ± SD (\*p<0.05, \*\*p<0.01, \*\*\*p<0.0001).

Figure4.

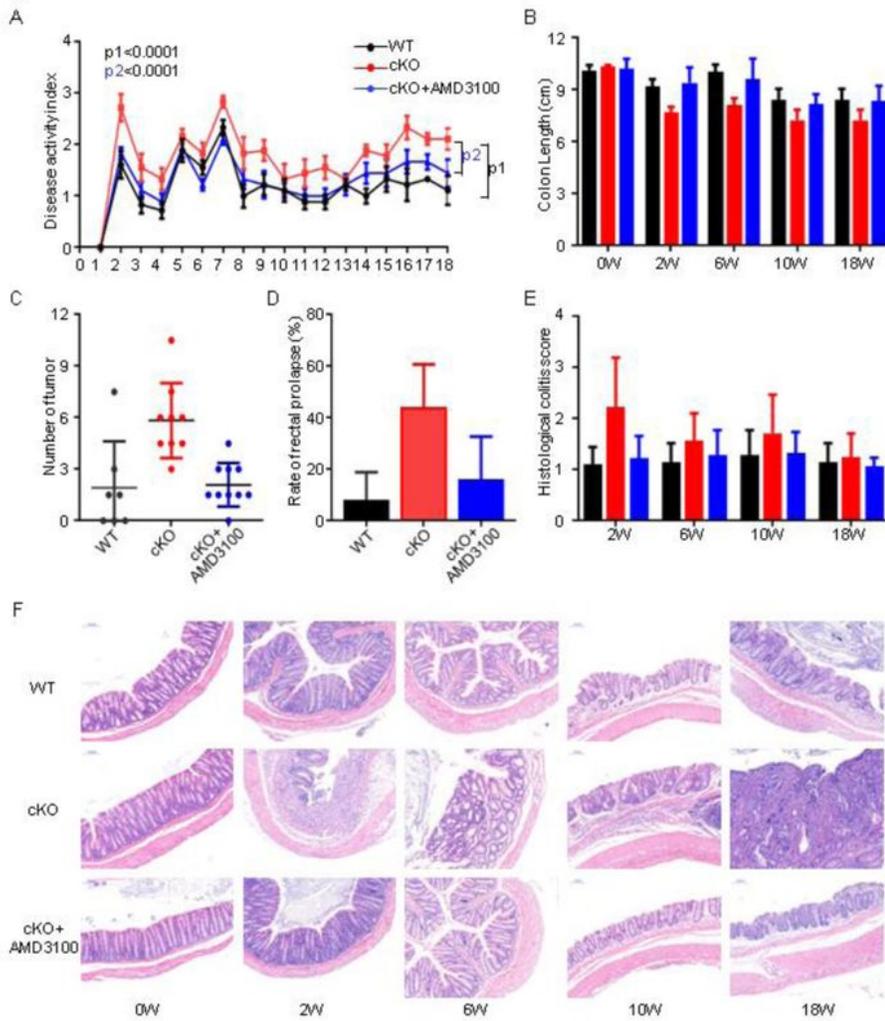
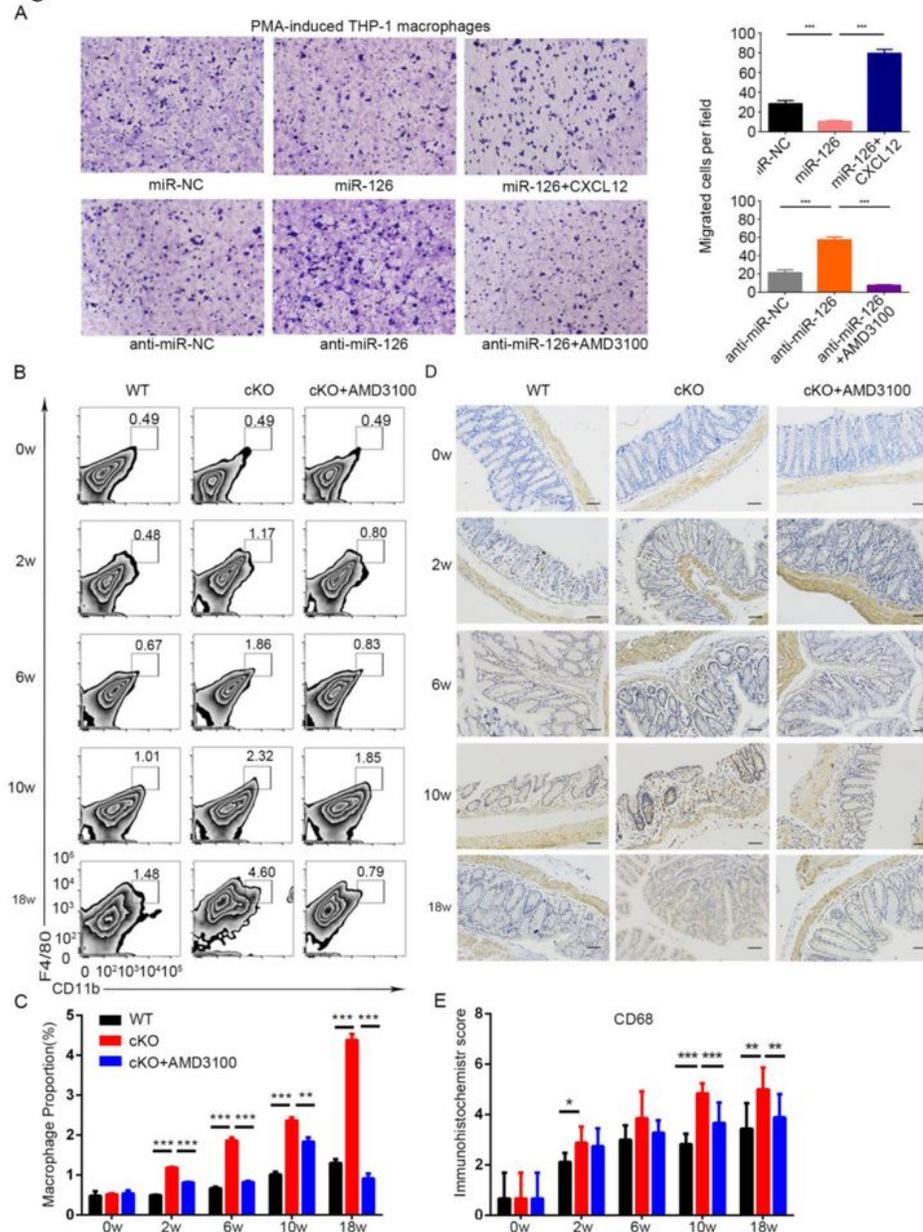


Figure 4

AMD3100 dampened the anti-tumor effects of miR-126 in CAC. (A) Measuring the DAI of mice from WT, cKO, and cKO+AMD3100 groups ( $n \geq 6$ ). (B) Colon lengths of mice from 3 groups were measured ( $n \geq 6$ ). (C) Intestinal lesion numbers of the intestinal surface area of each mouse were calculated ( $n \geq 6$ ). (D) Percentage of mice in each group with rectal prolapsed ( $n \geq 6$ ). (E, F) Histopathology of colonic mucosa from 3 groups by H&E staining and the histological score, scale bar=100nm (magnification  $\times 200$ ,  $n \geq 6$ ). Data are representative of six mice in each group with similar results. Data were shown as the mean  $\pm$  SD (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ ).

Figure 5.



## Figure 5

MiR-126 regulates the recruitment of macrophages via CXCL12. A. Transwell assay detected the chemotaxis of macrophages by colon cancer cells overexpressed or silenced of miR-126, and the recruitment of macrophages by miR-126 after AMD3100 (100 ng/ml) blocks CXCL12-CXCR4 axis (n=3). (B, C) Flow cytometric description of colorectal macrophages isolated from AOM/DSS mice at 0th, 2nd, 6th, and 18th-weeks post-induction. Two-parameter histograms F4/80+CD11b+ were recognized as macrophages and quantization table (n≥3). (D.E) The expression pattern of macrophages was assessed by IHC using an anti-CD68 antibody and the quantization table. Scale bar=50nm (magnification ×200, n≥6). Data are representative of at least three mice in each group with similar results. Data were shown as the mean ± SD (\*p<0.05, \*\*p<0.01, \*\*\*p<0.0001).

Figure6.

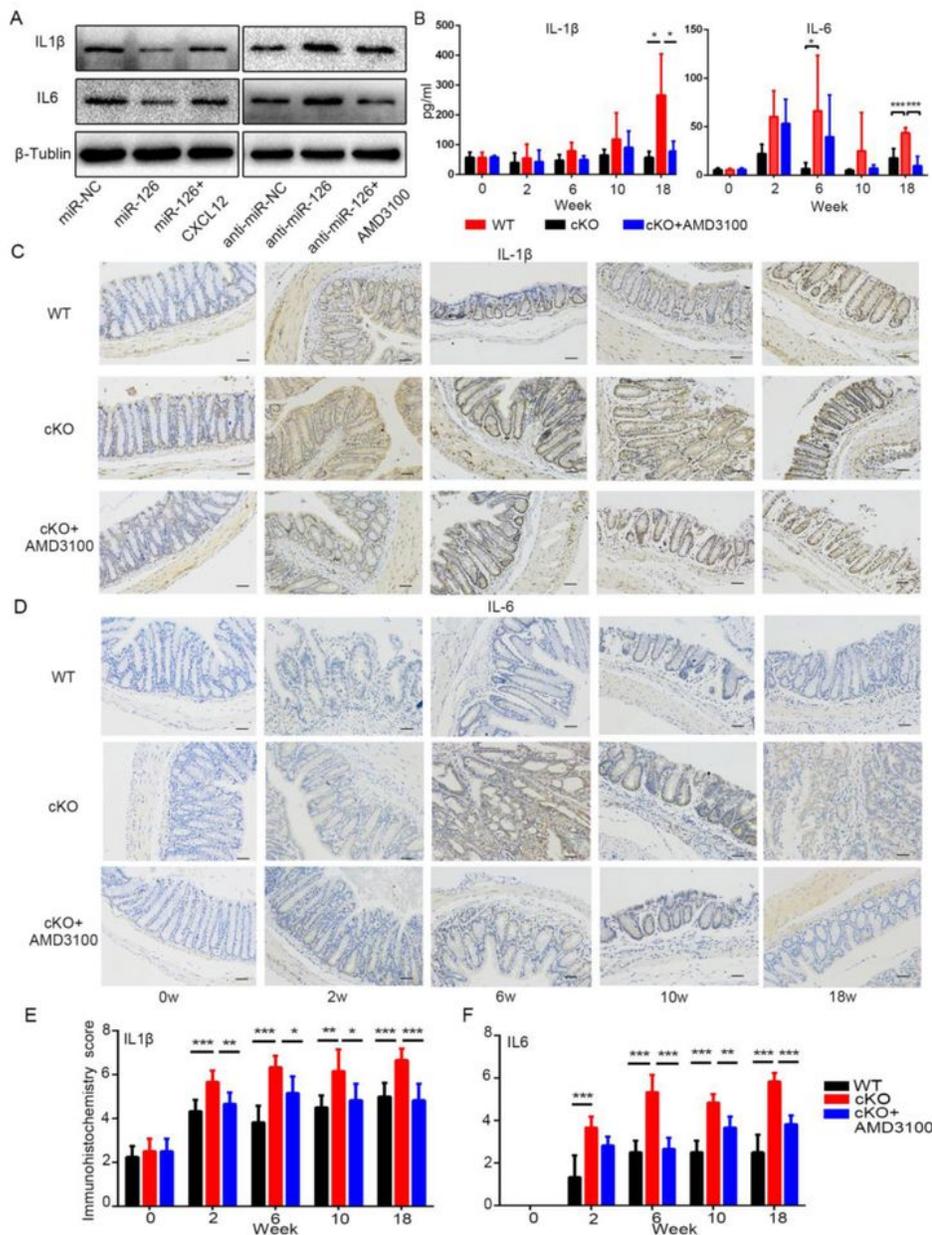


Figure 6

MiR-126 regulates the secretion of macrophages via CXCL12. (A) Western blot analysis of expression of IL-1 $\beta$  and IL-6 inflammatory factors in macrophages co-cultured with miR-126 over-expressed or silenced colon cancer cells. (B) The levels of IL-1 $\beta$  and IL-6 were measured in mice serum of 3 groups at 0th, 2nd, 6th, and 18th-weeks post-AOM/DSS treatment. (n $\geq$ 3) (C, E) The expression of IL-1 $\beta$  was assessed by IHC (magnification  $\times$ 200, n=6). Scale bar=50nm (D, F) The expression of IL-6 was assessed by IHC

(magnification  $\times 200$ ,  $n=6$ ). Scale bar=50nm. Data are representative of at least three mice in each group with similar results. Data were shown as the mean  $\pm$  SD (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.0001$ ).

Figure 7.

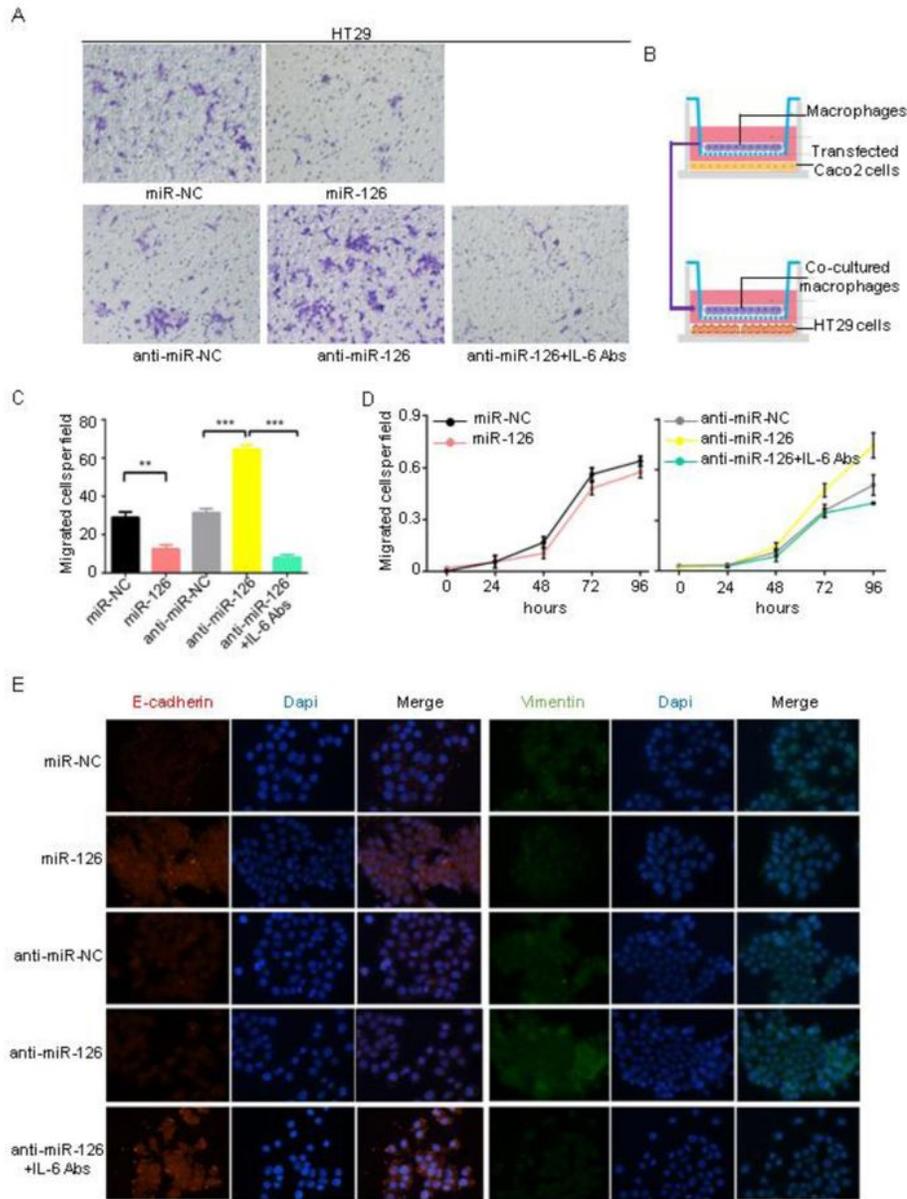


Figure 7

IL-6 is the key molecular for miR-126 dampen colitis carcinogenesis. (A, C) Transwell assays were used to measure cell recruitment capacity. Macrophages were co-cultured with miR-126 over-expressed or silenced colon cells, and AMD3100 (100 ng/ml) was added to block the CXCL12-CXCR4 axis. (B) Model

of the co-culture system of TAMs and epithelial cells. TAMs were first co-cultured with miR-126 over-expressed or silenced colon cells for 2 days, then the co-cultured TAMs were taken away from the previous system and put into a new co-culture system with untreated colon cells, and IL6 neutralizing antibodies were added in. (D, E) Detect cell growth and the expression of epithelial-mesenchymal transition (EMT) associated factors in untreated cells. Data were shown as the mean  $\pm$  SD (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.0001).

Figure8.

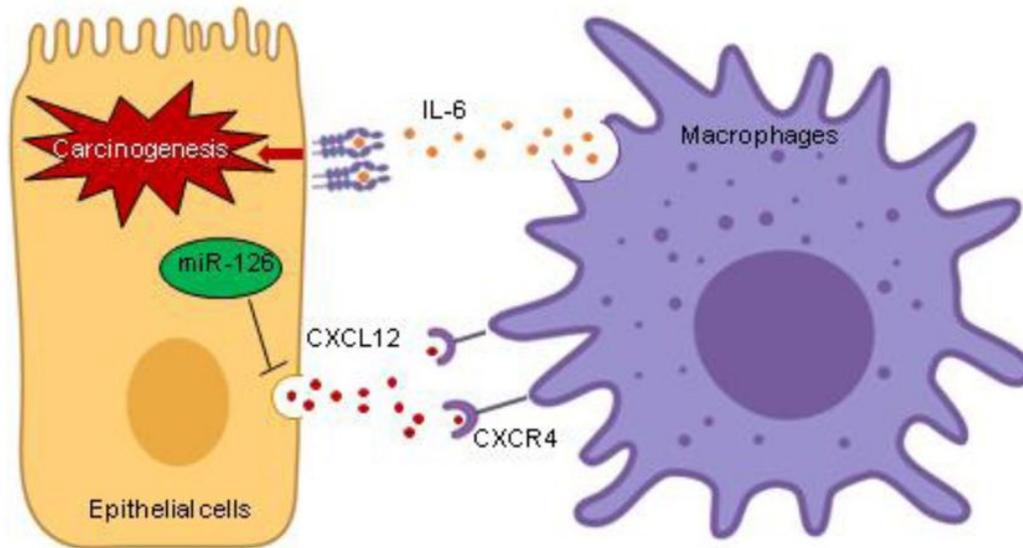


Figure 8

Mechanism model of miR-126 in CAC. Details are provided in the discussion section.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [sf1.jpg](#)
- [sf2.jpg](#)