Original Article

miRNA-320 inhibits colitis-associated colorectal cancer by regulating the IL-6R/STAT3 pathway in mice

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Background: Colitis-associated colorectal cancer (CAC) is a serious complication of inflammatory bowel disease (IBD). microRNA-320 (miRNA-320) promotes intestinal mucosal barrier repair in IBD and inhibits tumor progression. However, the role of miRNA-320 in the progression of CAC remains to be defined. We studied the mechanisms of miRNA-320 in the progression of CAC in mice.

Methods: CAC was induced in mice (C57BL/B6) by the administration of azoxymethane (AOM) and dextran sulfate sodium (DSS), and the mice were given a lentiviral vector (LV) overexpressing mmu-miRNA-320. The level of miRNA-320 was analyzed by quantitative real-time polymerase chain reaction (qPCR). Colonic inflammation, histological analysis, and tumorigenesis were evaluated. Ki-67 in colonic tissues was examined by immunohistochemistry. B-cell lymphoma-extra large (BCL-xl) and proliferating cell nuclear antigen (PCNA) expression was examined by Western blot. Furthermore, the proliferation, migration, and invasion of colorectal cancer (CRC) cells were evaluated. The levels of interleukin-6 receptor (IL-6R), signal transducer and activator of transcription 3 (STAT3), and phosphorylated-signal transducer and activator of transcription 3 (p-STAT3) were examined by Western blot and qPCR.

Results: miRNA-320 was downregulated in CAC mice (0.57±0.13 *vs.* 1.00±0.12, t=−5.95, P<0.001). miRNA-320 decreased the disease activity index (DAI) scores, improved colonic inflammation, and inhibited tumor formation (tumor number: 8.00±2.90 *vs.* 13.67±2.73, t=−3.49, P<0.01) in mice with CAC. miRNA-320 suppressed the expression of BCL-xl, PCNA, and Ki-67 (0.38±0.07 *vs.* 0.69±0.08, t=−7.30, P<0.001). miRNA-320 inhibited colon cancer cell proliferation, migration, and invasion. miRNA-320 significantly inhibited the levels of IL-6R [colon tissue messenger RNA (mRNA): 4.06±1.44 *vs.* 10.05±1.55, t=−6.94, P<0.001], STAT3, and p-STAT3 *in vivo* and *in vitro*. Silencing IL-6R expression partially reversed the IL-6R/STAT3-suppressing and tumor-inhibiting effect of miRNA-320.

Conclusions: miRNA-320 inhibits tumorigenesis in mice with CAC by suppressing IL-6R/STAT3 expression, and IL-6R is a target gene of miRNA-320.

Keywords: Inflammatory bowel disease (IBD); colitis-associated colorectal cancer (CAC); microRNA-320 (miRNA-320); interleukin-6 receptor (IL-6R); signal transducer and activator of transcription 3 (STAT3)

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Introduction

In recent years, the incidence of inflammatory bowel disease (IBD) has steadily increased in Asian countries (1). Colitis-associated colorectal cancer (CAC) is one of the most serious complications of IBD, and the risk of CAC increases with the duration of IBD (2). Unlike sporadic colorectal cancer (CRC), CAC has a younger age of onset, multiple lesions, more difficult endoscopic detection, more aggressive tumors, and a relatively worse prognosis (3). CAC has attracted increasing attention, but its pathogenesis remains ambiguous. Therefore, further research on the pathogenesis of CAC has important clinical significance.

MicroRNAs (miRNAs) are short noncoding RNAs that participate in diverse biological processes in many species (4). The study has shown that miRNAs are involved in regulating the occurrence and development of IBD and CAC (5). miRNA-320 has been reported to be differentially expressed in IBD and cancerous diseases. For example, the expression level of miRNA-320 in the intestinal mucosal tissues of patients with ulcerative colitis (UC) and Crohn's disease is decreased significantly, and miRNA-320 is decreased more significantly in inflamed mucosal tissues that in noninflammatory mucosal tissues (6). Animal experiments have shown that interleukin 33 (IL-33) promotes intestinal mucosal repair in mice, mainly through upregulating the expression of miRNA-320 to promote intestinal epithelial cell (IEC) proliferation and repair (7). Similarly, *in vitro* experiments have shown that miRNA-320 plays a role in maintaining the normal permeability of the intestinal mucosal barrier by targeting and regulating the gene *PPP2R5B*, which encodes serine/threonine phosphoprotein phosphatase 2A (PP2A) (8). In addition, miRNA-320 expression levels have been found to be downregulated in a variety of tumors. In gastric cancer, miRNA-320 inhibits cancer cell invasion and promotes apoptosis by inhibiting cell cycle-related proteins (9). Likewise, miRNA-320 inhibits tumor cell migration and invasion in CRC, cervical cancer, prostate cancer, and other tumors (10-12). These studies have found that miRNA-320 promotes intestinal mucosal barrier repair in IBD and inhibits tumor progression. However, the roles and mechanisms of miRNA-320 in the progression of CAC remain to be defined. This study mainly explored the effect and mechanism of miRNA-320 on CAC progression.

The results of this study showed that miRNA-320 was downregulated in CAC mice. miRNA-320 improved inflammation and inhibited tumor formation in mice with CAC and inhibited cell proliferation, migration, and invasion *in vitro*. Furthermore, we demonstrated that miRNA-320 suppressed interleukin-6 receptor (IL-6R)/signal transducer and activator of transcription 3 (STAT3) expression in CAC colonic tissues and cells, and that IL-6R was a target gene of miRNA-320. These findings suggest that miRNA-320 reduces CAC tumorigenesis and could be a potential therapeutic target. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-237/rc).

Methods

Animals

Male C57BL/6N mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and bred and maintained in specific pathogen-free conditions at the Experimental Animal Center of the Second Hospital of Hebei Medical University (China). All mice were maintained under specific pathogen-free conditions with a 12-hour light/dark cycle and given a regular chow diet and water ad libitum. The mice were used at 6–8 weeks of age. Experiments were performed under a project license (No. 2021-AE025) granted by Research Ethics Committee of the Second Hospital of Hebei Medical University, in compliance with the Chinese Academy of Sciences Animal Care and Use Committee guidelines for the care and use of animals.

Azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced CAC in mice

CAC was induced as described in previous research with slight modifications (13). Briefly, on day 1, the mice were injected intraperitoneally (i.p.) with 10 mg/kg AOM (Sigma-Aldrich, St. Louis, MO, USA) and maintained on a regular diet and water for 7 days. After 7 days, the mice were administered 2% DSS (MP Biomedicals, Irvine, CA, USA) in their drinking water for 7 consecutive days, followed by 14 days of recovery. This cycle was repeated 3 times. At day 91, the mice were sacrificed and randomly divided into 4 groups as follows: control group, model group (AOM/DSS), negative control group [AOM/DSS + lentiviral vector (LV) negative control], and miRNA-320 group (AOM/DSS + mmu-miRNA-320 overexpression).

During the study, body weight (BW), diarrhea, and hematochezia were monitored and described as a disease activity index (DAI) score. The colon was removed and opened longitudinally. Macroscopic tumors were counted and measured with a caliper. Tissue samples were maintained in RNA stabilization solution for RNA analysis or fixed in 4% paraformaldehyde for histopathologic analysis.

Histological analysis

For the histopathological analysis, the intestinal tissues were fixed in 4.0% paraformaldehyde, embedded in paraffin, and sectioned into 4 μm thick slices. The sections were stained with hematoxylin and eosin (H&E). The extent of hyperplasia was measured and scored as described in previous research (14). For the immunohistochemical analysis, colon sections were incubated with a primary antibody against Ki-67 (Cell Signaling Technology, Danvers, MA, USA) at 4 ℃ overnight, followed by the appropriate secondary antibodies.

Western blot

The colon tissue and cell proteins were homogenized with lysis buffer solution. The protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Solarbio, Beijing, China). Equal amounts of total proteins were fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated with primary antibody overnight at 4 ℃ after blocking with 5% skim milk for 1 hour. The membranes were then washed in Tris-buffered saline with Tween 20 (TBST) 3 times and incubated with secondary antibodies for 1 hour at room temperature. Protein bands were quantified using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were applied in this study: anti-PCNA, anti-BCL-xL (Wanlei Biology, Shenyang, China), anti-STAT3, anti-phospho-STAT3 (Tyr705; Abways Technology, Shanghai, China), anti-IL-6R, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Affinity, Changzhou, China).

Cell culture

HT29, HCT116, DLD1, and SW480 cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HT29 cells and HCT116 cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA), while DLD1 cells and SW480 cells were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA). Both were supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 IU/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 ℃ in a 5% CO_2 incubator.

Lentivirus construction and transfection

The LVs used in this study to investigate miRNA-320 were synthesized by and purchased from GeneChem (Shanghai, China). All transfections were carried out according to the manufacturer's instructions. The corresponding sequences were amplified by polymerase chain reaction (PCR) and identified by PCR and sequencing. LVs overexpressing mmu-mir-320 (primer sequences 5'-GGAAAGGACGAAACACCGGGGCGGAAGTCTGCGTGGCAAGG-3' and 5'-TGTCTCGAGGTCGAGAATTAAAAAAGCCCCTCCACCTCCGACCCCGAG-3'), were harvested (7×10^8 TU/mL) after ligation into hU6-MCS-ubiquitin-EGFP-IRES puromycin (GV309). Then, 150 μL of mmu-miRNA-320 overexpression lentivirus or negative control lentivirus was resuspended in polybrene and enhanced infection solution. Finally, viral vector solution was injected intraperitoneally into AOM/DSS-induced CAC mice at the beginning of the 3rd DSS drinking. LVs overexpressing hsa-mir-320 (primer sequences 5'-GGAAAGGACGAAACACCGGGGCGGAAGTCTGCGTGGCAGG-3' and 5'-TGTCTCGAGGTCGAGAATTAAAAAACGGGACCCTGATCTTGGCGCC-3'), were harvested (7×10^8 TU/mL) after ligation into hU6-MCS-ubiquitin-EGFP-IRES puromycin (GV309). LVs overexpressing hsa-miR-320 sponges (primer sequence TCGCCCTCTCGACCAGCTTTTCTTCTCGCCCTCTCGACCAGCTTTTCTTCTCGCCCTCTCGACCAGCTTTTATCCGTATCGCCCTCTCGACCAGCTTTTCTTCTCGCCCTCTCGACCAGCTTTTCTTCTCGCCCTCTCGACCAGCTTTTCTTC), were harvested (1×10^9 TU/mL) after ligation into Ubi-MCS-SV40-EGFP-IRES-puromycin (GV369). To generate a genetically stable gene cell line, an LV overexpressing or inhibiting miRNA-320 was used for cellular genetic manipulation. Briefly, human CRC cells were cultured to 30–50% confluence and then transfected with LVs overexpressing or inhibiting miRNA-320 and their negative control vectors. Puromycin was used to select

stable cell strains overexpressing or inhibiting miRNA-320. PCR was performed to evaluate the levels of miRNA-320.

SiRNA transfection

Small-interfering RNAs (siRNAs) targeting IL-6R (si-IL-6R) were synthesized by GenePharma (Suzhou, China), and the sequences were as follows: sense CCUUUCAGGGUUGUGGAAUTT and antisense AUUCCACAACCCUGAAAGGTT. When cells reached 30–50% confluence, they were transfected with si-IL-6R or with control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The efficiency of transfection was tested by PCR.

Cell Counting Kit-8 (CCK-8)

Cell proliferation was evaluated using a CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, the cells were seeded in 96-well plates at a density of 5×10^4 per well and incubated at 37 ℃ with 5% CO_2 for 24 hours. Then, recombinant cytokine IL-6 (PeproTech, Princeton, NJ, USA) at different concentrations (0, 20, 50, 100, and 150 ng/mL) was added to each well, and the cells were incubated with serum-free medium at a final volume of 100 μL for 24 hours. For the CCK-8 assay, 10 μL of CCK-8 solution was added to the wells, and the cells were incubated at 37 ℃ for 3 hours. The absorbance was measured at 450 nm using a microplate reader.

Colony formation assay

For the cell proliferation assay, the cells were seeded in 6-well plates at 2,000 cells per well. The plates were incubated until visible colonies formed. Then, the colonies were stained with Giemsa (Solarbio, Beijing, China), imaged, and counted.

Xenograft model

We chose 4- to 6-week-old male BALB/c nude mice (n=6/group) for tumor xenograft experiments. For the tumorigenicity assay, HT29 cells (1×10^7) stably transfected with LVs overexpressing miRNA-320 or an empty vector were suspended in 1.0 mL phosphate buffered saline (PBS). Then, the cells were injected subcutaneously into the right dorsal flanks of the nude mice. The tumor size was measured using a digital caliper every 3 days for 3 weeks. On the last day, the mice were sacrificed, and the tumor nodules were excised and detected for tumor weight and volume. Tumor volumes were calculated using the following equation: tumor volume (mm^3) = (longer diameter × shorter diameter^2)/2.

Wound healing and transwell assays

Cells were seeded in 6-well plates and cultured in the presence of IL-6 (50 ng/mL) until the cells reached 80% confluence. A straight wound was made with a 200 mL sterile pipette tip. The cells were incubated in medium without FBS for 48 hours. Images were taken at 0, 24, and 48 hours after wounding. To analyze the invasion, a BD BioCoat Matrigel invasion chamber (BD Biosciences, Franklin Lakes, NJ, USA) was used. Cells were incubated with serum-free medium for 12 hours and then seeded in the top of the insert (Corning, Corning, NY, USA) without serum. Medium with 10% FBS was added to the lower chamber to stimulate cell invasion. Forty-eight hours later, cells in the bottom chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The invasive cells were counted using immunofluorescence microscopy.

RNA isolation and qPCR

Total RNA was extracted using a miRcute miRNA isolation kit (Tiangen, Beijing, China). For the miRNA-320 quantitative analysis, reverse transcription was carried out using a miRcute Plus miRNA First-Strand cDNA Kit (Tiangen). Quantitative real-time PCR (qPCR) was carried out using a miRcute Plus miRNA qPCR Kit (Tiangen) according to the manufacturer's instructions. miRNA expression was normalized using the detection of U6. The primer sequences for miRNA-320 and U6 amplification were (forward) 5'-ACACTCCAGCTG GGAAAAGCTGGGTTGAGAG-3' and (forward) 5'-CTCGCTTCGGCAGCACATA-3', respectively. A reverse transcription kit (Aidlab, Beijing, China) was used to synthesize first-strand complementary DNA (cDNA) from IL-6R and STAT3 messenger RNA (mRNA). qPCR amplification was performed with SYBR Green (Tiangen). mRNA expression was normalized against GAPDH. The primers used are shown in *Table 1*. The relative expression was calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method.

Table 1 Oligonucleotide sequences used for the qPCR analysis

Transcript	Sequence (5'-3' direction)
IL-6R (mouse)	Forward: GCCGGATCCACCTGCCAACCTT
	Reverse: GGGCCACCGGGAGCAGCAACAC
IL-6R (human)	Forward: GGGGGTAGAGCCGGAAGACAAT
	Reverse: CGGCCCGGTAGCATGAATAGTT
STAT3 (mouse)	Forward: CCGGAAGCGAGTGCAGGATCTAG
	Reverse: CCCGCCAGCTCACTCACAATG
STAT3 (human)	Forward: CACCGGCCGATGCTGGAGGAGA
	Reverse: GGGGCCGGTCAGGATGCATGG
GAPDH (mouse)	Forward: TCGTCCCGTAGACAAAATGG
	Reverse: TTGAGGTCAATGAAGGGGTC
GAPDH (human)	Forward: GGCGCTGAGTACGTCGTGGAGTC
	Reverse: CAGCAGAGGGGGCAGAGATGATGA

qPCR, quantitative real-time polymerase chain reaction; IL-6R, interleukin-6 receptor; STAT3, signal transducer and activator of transcription 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). All data are presented as means ± standard deviation (SD). Differences between 2 groups were determined using two-tailed Student's *t*-test. Differences among multiple groups were evaluated by one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls (SNK) *post hoc* test. P<0.05 was considered statistically significant.

Results

miRNA-320 reduces CAC tumorigenesis

To identify the regulatory effect of miRNA-320, we successfully constructed a CAC model in mice. During the experiment, the DAI (weight loss, stool status, and fecal blood) was assessed. Compared with those in the model group and the negative control group, the mice in the miRNA-320 group experienced distinctly less BW loss (*Figure 1A*), and their DAI was evidently decreased (*Figure 1B*).

We isolated the colonic tissue of the mice and measured the colon length (*Figure 1C*). The mice in the model group had shorter colons (7.51±0.58 *vs.* 8.80±0.63 cm, *t*=–3.66, P<0.01) and higher morphology scores (3.67±0.52 *vs.* 0.00±0.00, *t*=17.39, P<0.001) than the mice in the control group. The mice in the miRNA-320 group had longer colons (8.35±0.53 *vs.* 7.51±0.58 cm, *t*=2.58, P<0.05) and lower morphology scores (2.00±0.63 *vs.* 3.67±0.52, *t*=–5.00, P<0.001) than the mice in the model group (*Figure 1D*,*1E*).

The intestinal tract was opened longitudinally and unequally sized nodules were observed (*Figure 1F*). The number of macroscopically visible tumors (8.00±2.90 *vs.* 13.67±2.73, *t*=–3.49, P<0.01) was significantly decreased in the miRNA-320 group compared with that in the model group (*Figure 1G*). Moreover, the size of the tumors in the miRNA-320 group was much smaller than that in the model group (*Figure 1H*). These results suggested that miRNA-320 improved inflammation and inhibited tumor formation in mice with CAC.

miRNA-320 regulates the survival and proliferation of IECs in mice

We performed a histological analysis to explore whether miRNA-320 regulates the proliferation of IECs in CAC mice. In the model group and the negative control group, histological analysis of the colon sections showed a series of atypical hyperplasia manifestations, including disordered glands, distortion, and the disappearance of polarity. The mice from the miRNA-320 group developed better-differentiated adenocarcinomas than the mice from the model group, and their hyperplasia scores (1.67±0.82 *vs.* 2.67±0.52, *t*=–2.54, P<0.05) were markedly decreased (*Figure 2A*,*2B*). Immunohistochemistry showed that expression of Ki-67 (0.38±0.07 *vs.* 0.69±0.08, *t*=–7.30, P<0.001) was downregulated in the miRNA-320 group compared with that in the model group (*Figure 2C*,*2D*).

The expression of B-cell lymphoma-extra large (BCL-xl) and proliferating cell nuclear antigen (PCNA) in the colonic tissues of the mice was examined by Western blot. Compared with that in the model group, the expression of proliferation-related proteins, including BCL-xl and PCNA, was significantly decreased in the miRNA-320 group (*Figure 2E-2G*). In addition, we detected the level of miRNA-320 in the colonic tissues using qPCR. Compared with that in the control group, miRNA-320 expression was decreased in the model group (0.57±0.13 *vs.* 1.00±0.12, *t*=–5.95, P<0.001) and in the negative control group (0.58±0.13 *vs.* 1.00±0.12, *t*=–5.70, P<0.001). Compared with that in the model group, miRNA-320 expression was significantly increased in the miRNA-320 group (7.57±1.21

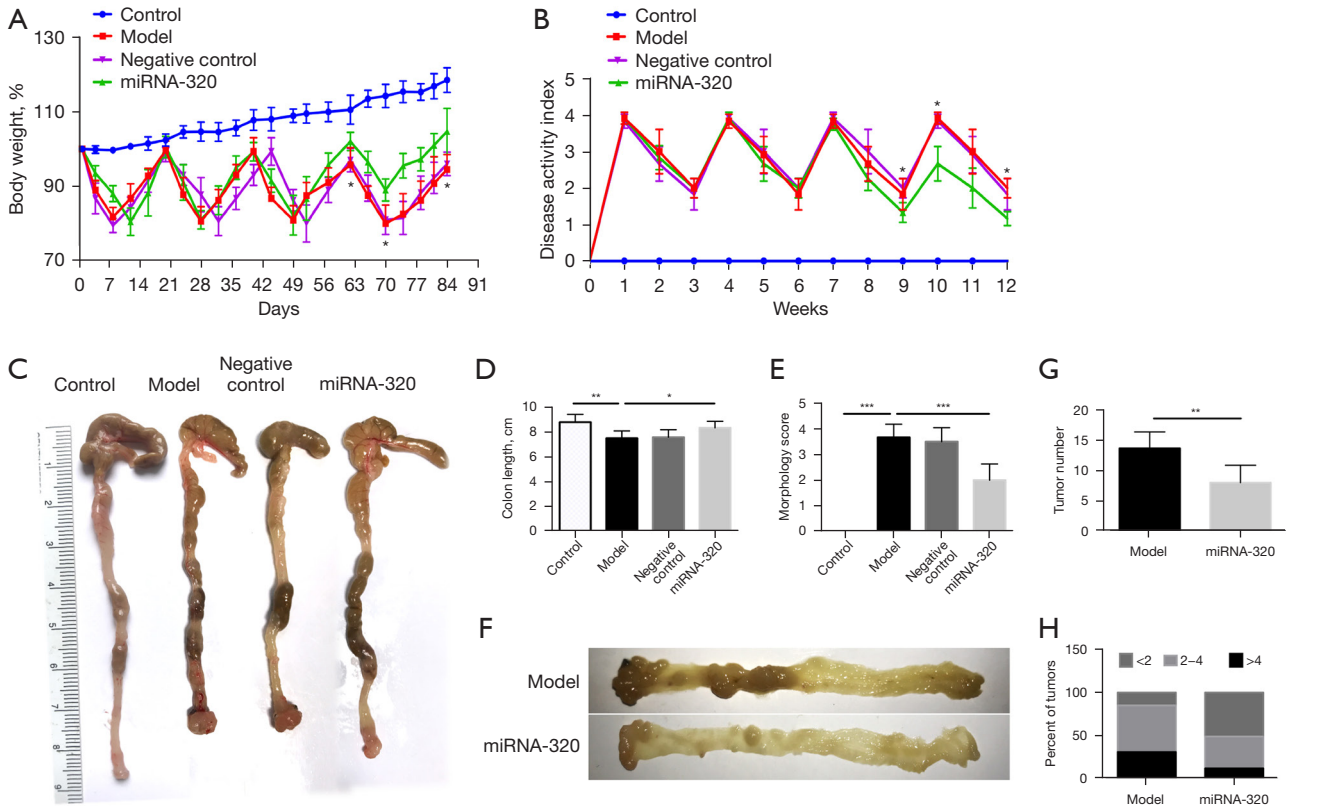

Figure 1 miRNA-320 reduces CAC tumorigenesis. (A) The body weight was assessed daily and expressed as the percentage of basal body weight. (B) Disease activity index, consisting of weight loss, stool consistency, and stool blood, was measured daily. (C) Gross morphology of the colon on day 91. (D) Colon length. (E) Morphology score. (F) Colons of the model group and miRNA-320 group were opened longitudinally, and the number of tumors was measured. (G) Number of tumors in the whole colon. (H) Tumor size distribution according to the tumor numbers. Data are expressed as the mean ± SD. *, P<0.05; **, P<0.01; ***, P<0.001. miRNA, microRNA; CAC, colitis-associated colorectal cancer; SD, standard deviation.

vs. 0.57±0.13, *t*=14.06, P<0.001; *Figure 2H*). These data indicated that miRNA-320 inhibited the proliferation of IECs in mice with CAC.

miRNA-320 inhibits cell proliferation

To evaluate the functional role of miRNA-320 in CRC cells, we overexpressed miRNA-320 by transfection of LV-miR-320 in CRC cell lines with lower miRNA-320 expression (HT29 and SW480) and inhibited miRNA-320 by transfection of LV-anti-miRNA-320 in CRC cell lines with higher miRNA-320 expression (HCT116 and DLD1; Figure S1A). Compared with the negative control, overexpression of miRNA-320 led to a significant increase in miRNA-320 expression in HT29 and SW480 cells (Figure S1B). However, LV-anti-miR-320 did not decrease the miRNA-320 expression in HCT116 and DLD1 cells (Figure S1C), because sponges inhibit the function of miRNA-320 by adsorbing rather than degrading miRNA.

To choose the most suitable concentration of IL-6 to stimulate CRC cell growth, we examined the effect of IL-6 at different concentrations on cell proliferation using the CCK-8 assay. We found that the proliferation of HT29, SW480, HCT116, and DLD1 cells was significantly enhanced by IL-6 at a concentration of 50 ng/mL (Figure S1D).

Next, we evaluated the effect of miRNA-320 on proliferation *in vitro* using the CCK-8 assay and plate colony formation assay. The results showed that the upregulation of miRNA-320 significantly inhibited cell viability and clonogenicity. The viability and colony formation number were significantly decreased by LV-miRNA-320 in HT29 (cell viability: 0.74±0.08 *vs.*

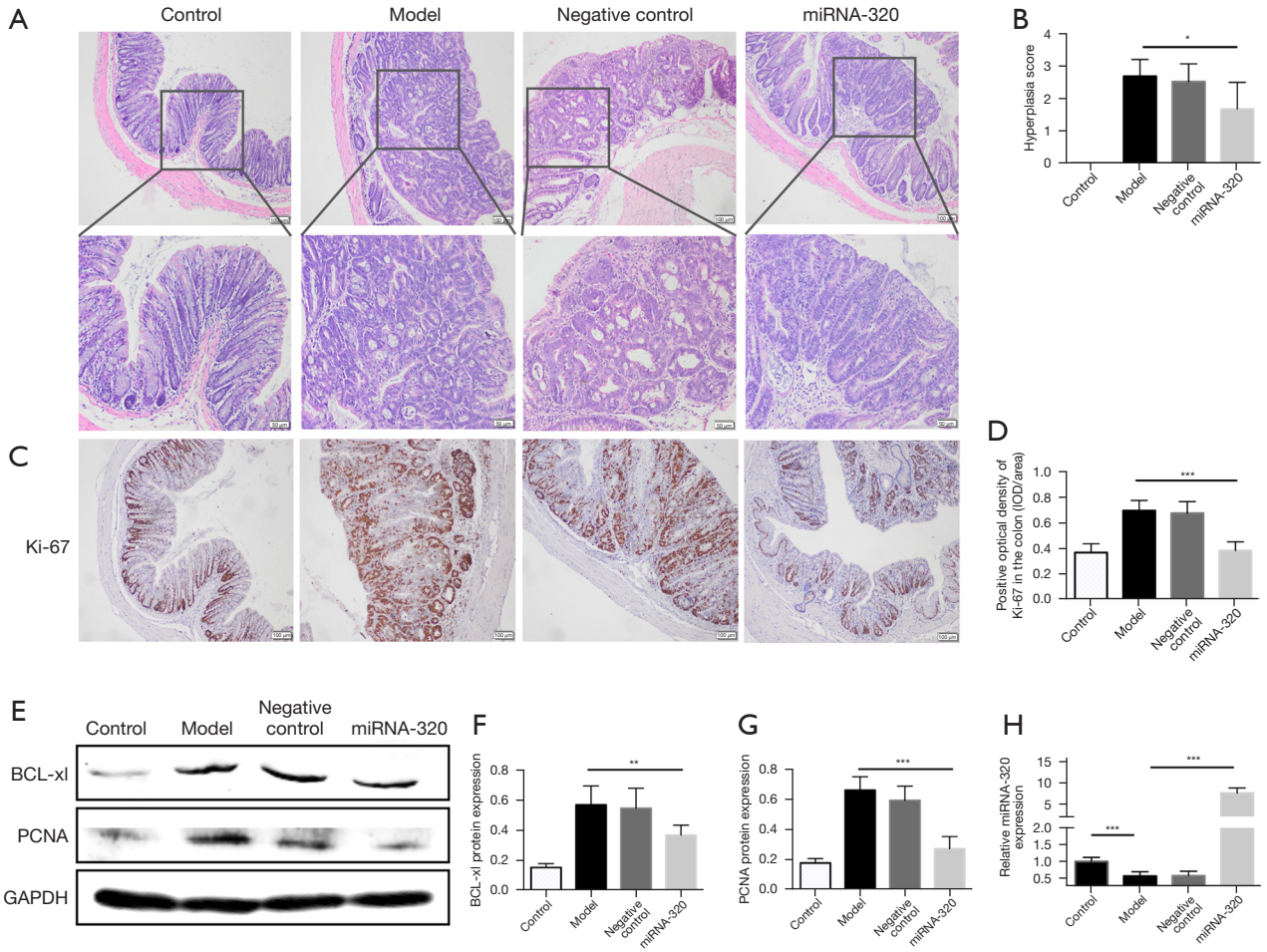

Figure 2 miRNA-320 inhibits the survival and proliferation of IECs in mice. (A) Representative images of colonic tumor tissues of H&E stained (×100, ×200). (B) Hyperplasia score. (C,D) Expression of Ki-67 protein in colon tissue was analyzed by immunohistochemistry. (E-G) Expression of BCL-xl and PCNA proteins in colon tissue was analyzed by Western blot. The same column was GAPDH as an internal control. (H) miRNA-320 expression in the intestine detected by qPCR in mice. Data are expressed as the mean ± SD. *, P<0.05; **, P<0.01; ***, P<0.001. miRNA, microRNA; IECs, intestinal epithelial cells; H&E, hematoxylin and eosin; Ki-67, marker of proliferation Ki-67; BCL-xl, B-cell lymphoma-extra large; PCNA, proliferating cell nuclear antigen; qPCR, quantitative real-time polymerase chain reaction; GAPDH, glyseraldehyde-3-phosphate dehydrogenase; SD, standard deviation; IOD, integrated optical density.

1.50±0.08, t=–15.86, P<0.001; clonogenicity: 0.44±0.12 *vs.* 1.00±0.28, t=–4.58, P<0.01) and SW480 cells (cell viability: 1.81±0.08 *vs.* 2.36±0.10, t=–10.02, P<0.001; clonogenicity: 0.25±0.06 *vs.* 1.00±0.24, t=–7.39, P<0.001) and increased by LV-anti-miRNA-320 in HCT116 (cell viability: 3.16±0.13 *vs.* 2.16±0.16, t=11.65, P<0.001; clonogenicity: 2.27±0.58 *vs.* 1.00±0.25, t=4.94, P<0.001) and DLD1 cells (cell viability: 3.00±0.13 *vs.* 1.82±0.14, t=15.31, P<0.001; clonogenicity: 2.70±0.35 *vs.* 1.00±0.19, t=10.34, P<0.001; *Figure 3A,3B*). The suppressive effect of miRNA-320 on cell proliferation was further verified by the Western blot method, which showed that ectopic expression of miRNA-320 in HT29 and SW480 cells significantly inhibited the expression of BCL-xl and PCNA, and LV-anti-miRNA-320 transfected in HCT116 and DLD1 cells increased the levels of these proteins (*Figure 3C*). To further investigate the tumorigenic ability of miRNA-320, HT29 cells stably transfected with an LV overexpressing miRNA-320 or a control vector were subcutaneously injected into BALB/c nude mice. The results showed that overexpression of miRNA-320 markedly reduced the tumor size (202.50±110.43 *vs.* 599.42±158.51 mm^3, t=–5.03,

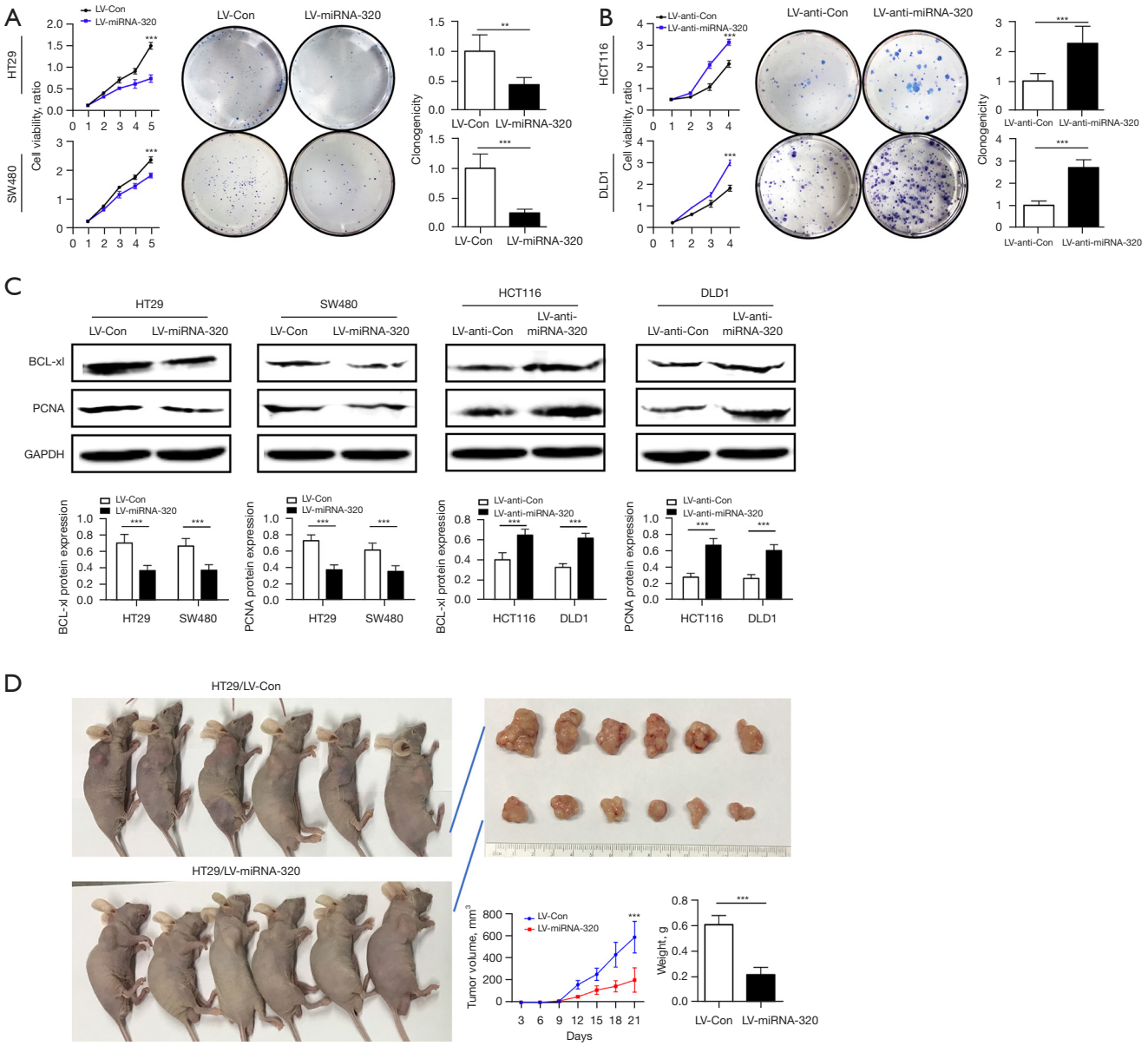

Figure 3 miRNA-320 inhibits cell proliferation. (A,B) Cell proliferation was measured by CCK-8 assays and plate colony formation assays (stained with Giemsa) in HT29 and SW480 cells transfected with LV-miRNA-320 or control (A) and HCT116 and DLD1 cells transfected with LV-anti-miRNA-320 or control (B). (C) The protein expression of BCL-xl and PCNA in HT29, SW480 cells, HCT116, and DLD1 cells. The same column was GAPDH as an internal control. (D) Image of subcutaneous xenograft tumors. Nude mice were injected with 1×10^7 HT29 cells transfected with LV-miRNA-320 (n=6/group). The tumors were extracted after 21 days. Analysis of the tumor volume measured every 3 days. Tumor weight in each group at the end of the experiment. Data are expressed as the mean ± SD. **, P<0.01; ***, P<0.001. miRNA, microRNA; CCK-8, Cell Counting Kit-8; BCL-xl, B-cell lymphoma-extra large; LV, lentiviral vector; PCNA, proliferating cell nuclear antigen; GAPDH, glyseraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

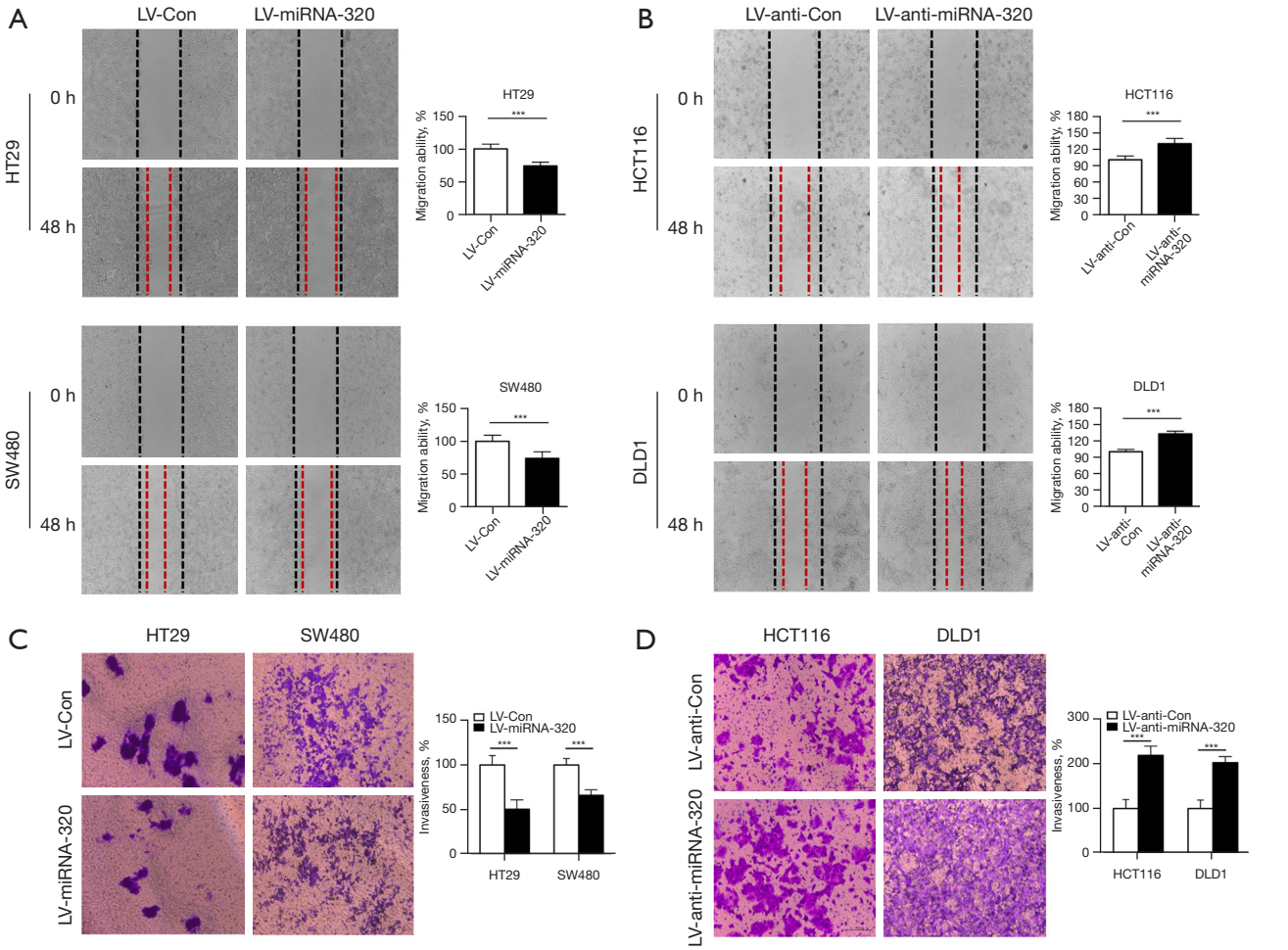

Figure 4 miRNA-320 inhibits cell migration and invasion. (A,B) Representative images of the wound healing assay revealed that ectopic expression of miRNA-320 inhibited cell migration in HT29 and SW480 cells (A), while downregulation of miRNA-320 promoted cell migration in HCT116 and DLD1 cells (B). Observe and take pictures under an inverted microscope. Magnification, 40×. The width of black dashed lines indicates the initial wound area. The width of red dashed lines indicates the wound area after 48 hours (C,D) Representative images of the transwell assay (stained with 0.1% crystal violet) revealed that ectopic expression of miRNA-320 inhibited cell invasion of HT29 and SW480 cells (C), while downregulation of miRNA-320 promoted cell invasion of HCT116 and DLD1 cells (D). Magnification, 40×. Data are expressed as the mean ± SD. ***, P<0.001. LV, lentiviral vector; miRNA, microRNA; SD, standard deviation.

P<0.001) and weight (0.22±0.06 *vs.* 0.61±0.07 g, *t*=−10.37, P<0.001) of the HT29 xenografts (*Figure 3D*). Our *in vivo* and *in vitro* results further supported the tumor-inhibiting role of miRNA-320 in CAC.

miRNA-320 inhibits cell migration and invasion

To examine the effect of miRNA-320 on CRC cell migration and invasion, wound healing and Matrigel invasion assays were carried out. Ectopic expression of miRNA-320 significantly suppressed the migration abilities of HT29 (74.12%±5.68% *vs.* 100.00%±7.14%, *t*=−6.95, P<0.001) and SW480 cells (73.96%±10.12% *vs.* 100.00%±9.32%, *t*=−4.64, P<0.001; *Figure 4A*), while suppression of miRNA-320 markedly promoted the migration abilities of HCT116 (129.06%±9.58% *vs.* 100.00%±6.73%, *t*=6.08, P<0.001) and DLD1 cells (132.15%±4.91% *vs.* 100.00%±4.01%, *t*=12.43, P<0.001; *Figure 4B*). Ectopic expression of miRNA-320 significantly suppressed the invasion abilities of HT29 (50.49%±10.56% *vs.* 100.00%±10.57%, *t*=−8.12, P<0.001) and SW480 cells (66.07%±6.15% *vs.* 100.00%±7.20%, *t*=−8.77, P<0.001; *Figure 4C*),

while suppression of miRNA-320 markedly promoted the invasion abilities of HCT116 (218.99%±20.73% *vs.* 100.00%±20.18%, t=10.07, P<0.001) and DLD1 cells (202.41%±13.89% *vs.* 100.00%±18.83%, t=10.72, P<0.001; *Figure 4D*). These experiments suggested that miRNA-320 inhibited the migration and invasion of CRC cells.

miRNA-320 suppresses IL-6R/STAT3 expression in CAC colonic tissues and cells

Although miRNA-320 expression in the model group was substantially decreased and overexpression of miRNA-320 protected the CAC mice, the target of miRNA-320 in CAC mice was unclear. The predicted targets of miRNA-320 were analyzed using TargetScan. According to this online analysis software, there is a specific binding area in the IL-6R 3'UTR for miRNA-320, which suggests that IL-6R is a potential target gene of miRNA-320. IL-6R is usually correlated with the process of CAC, and miRNAs usually suppress target genes. We demonstrated that miRNA-320 inhibited the expression of IL-6R. The miRNA-320 expression level of colon tissues was significantly increased in the miRNA-320 group compared with that in the model group, and the IL-6R (mRNA: 4.06±1.44 *vs.* 10.05±1.55, t=−6.94, P<0.001) level was significantly decreased (*Figure 5A,5B*). Overexpression of miRNA-320 markedly inhibited the protein level of IL-6R in HT29 and SW480 cells (*Figure 5C*). In contrast, inhibition of miRNA-320 markedly promoted IL-6R protein level in HCT116 and DLD1 cells (*Figure 5D*). Overexpression of miRNA-320 markedly inhibited the mRNA level of IL-6R in HT29 (0.50±0.16 *vs.* 1.00±0.13, t=−5.72, P<0.001) and SW480 (0.49±0.10 *vs.* 1.00±0.13, t=−7.53, P<0.001) cells (*Figure 5E*). In contrast, inhibition of miRNA-320 markedly promoted the mRNA level of IL-6R expression in HCT116 (3.20±0.55 *vs.* 1.00±0.17, t=9.33, P<0.001) and DLD1 cells (3.52±0.44 *vs.* 1.00±0.08, t=13.82, P<0.001; *Figure 5F*). In addition, the results of STAT3 and phosphorylated-signal transducer and activator of transcription 3 (p-STAT3) were consistent with the changes in IL-6R *in vivo* and *in vitro*. These results suggested that miRNA-320 downregulated IL-6R and that IL-6R might be a target gene of miRNA-320.

Silencing IL-6R attenuates the regulation of IL-6R/STAT3 by miRNA-320

We then assessed whether the tumor-inhibiting function of miRNA-320 depends on IL-6R. We used LV-inhibited miRNA-320 to transfect DLD1 cells and knocked down IL-6R by RNA interference. The results showed that knockdown of IL-6R by siRNA markedly decreased the expression of IL-6R (mRNA: 0.26±0.09 *vs.* 1.00±0.18, t=−8.82, P<0.001), STAT3 (mRNA: 0.37±0.13 *vs.* 1.00±0.13, t=−8.14, P<0.001), and p-STAT3 compared with knockdown by control RNA interference (*Figure 6A,6B*). As expected, the expression levels of BCL-xl and PCNA were reduced in response to the loss of IL-6R (*Figure 6C*). Taken together, this evidence suggested that silencing IL-6R expression partially reversed the effect of miRNA-320 in suppressing IL-6R/STAT3 and inhibiting tumorigenesis.

Discussion

The risk of CRC is significantly higher in IBD patients than in the general population, and CRC is one of the main causes of death in IBD patients (15,16). It has been widely accepted that miRNAs play an important role in cancer. In our present study, we investigated whether miRNA-320 has a potentially protective effect on CAC. To clarify the effect of miRNA-320 on CAC, we generated AOM- and DSS-induced colitis-associated colon cancer mouse models. Our experiment showed that miRNA-320 improved the general condition of CAC mice, inhibited the proliferation of IECs, and inhibited the proliferation, migration, and invasion of CRC cells. Importantly, we also found that miRNA-320 played a role in CAC by downregulating the IL-6R/STAT3 pathway. Our results showed that overexpressed miRNA-320 inhibited tumorigenesis in CAC. These observations provide evidence that miRNA-320 might serve as a novel regulator in the pathogenesis of CAC.

miRNA-320, a miRNA, is widely present in various tissues and inhibits the expression of its downstream target genes. Abnormal expression of miRNA-320 is closely related to many human diseases and is especially related to tumors. Studies have shown that miRNA-320 expression levels are downregulated in tumors such as cholangiocarcinoma, oral squamous cell carcinoma, and breast cancer (17-19). One study found that the expression level of miRNA-320 in 40 cases of CRC was significantly lower than in paired adjacent normal tissues (20). This provides a good theoretical basis for studying the influence of miRNA-320 on CAC.

In this study, it was found that the expression level of miRNA-320 was reduced in mice with CAC, which was consistent with previous studies of miRNA-320 levels in patients with IBD and colitis mice (6-8). Therefore,

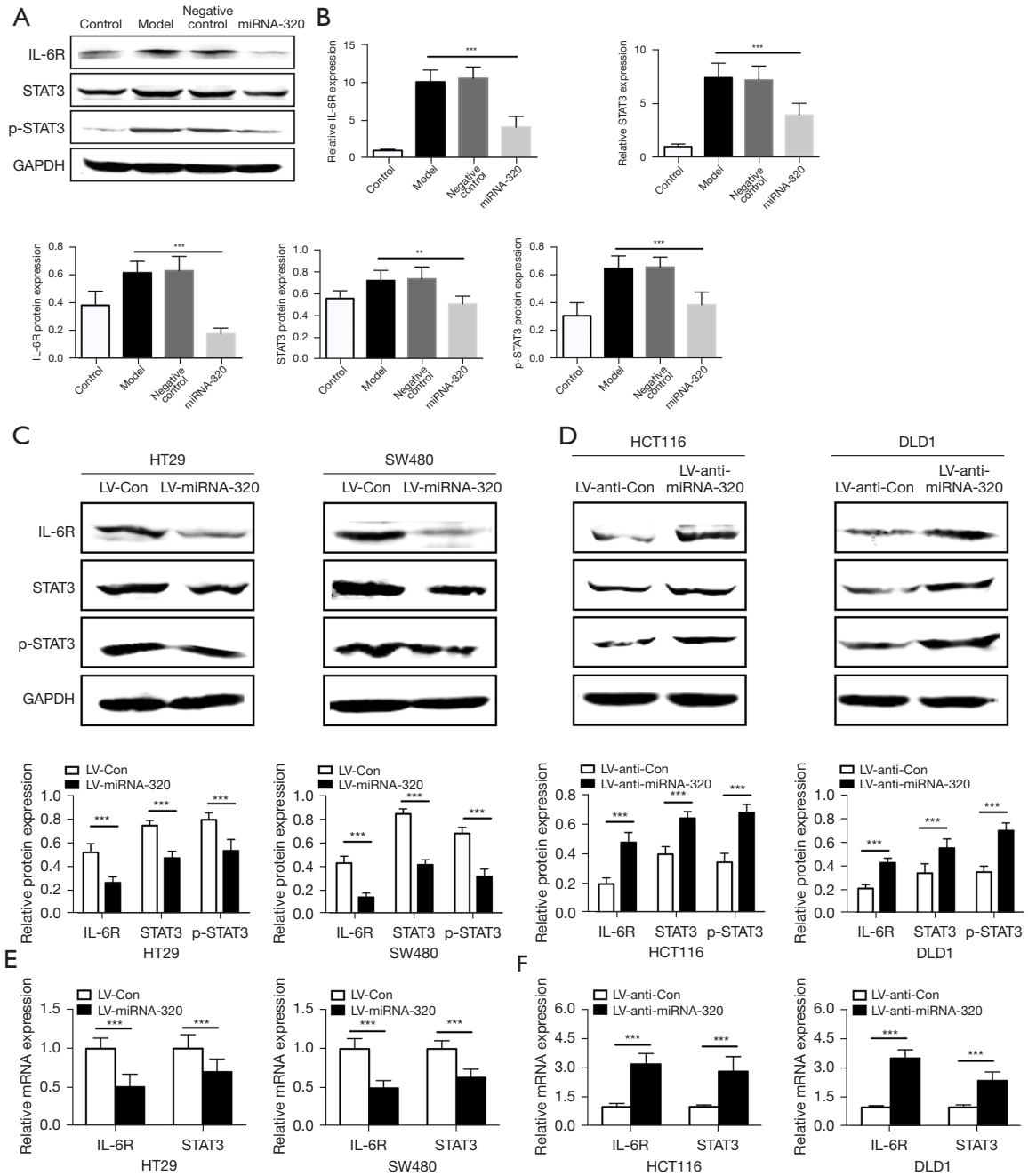

Figure 5 miRNA-320 suppresses IL-6R/STAT3 expression in CAC colonic tissues and cells. (A) Expression of IL-6R, STAT3, and p-STAT3 proteins in colon tissue was analyzed by Western blot. The same column was GAPDH as an internal control. (B) mRNA levels of IL-6R and STAT3 in colon tissue were evaluated by qPCR. (C,D) The protein expression levels of IL-6R, STAT3, and p-STAT3 were analyzed by Western blot in HT29 and SW480 cells transfected with LV-miRNA-320 or control (C) and HCT116 and DLD1 cells transfected with LV-anti-miRNA-320 or control (D). The same column was GAPDH as an internal control. (E,F) mRNA levels of IL-6R and STAT3 were evaluated by qPCR in HT29 and SW480 cells (E) and HCT116 and DLD1 cells (F). Data are expressed as the mean ± SD. **, P<0.01; ***, P<0.001. CAC, colitis-associated colorectal cancer; miRNA, microRNA; IL-6R, interleukin-6 receptor; LV, lentiviral vector; STAT3, signal transducer and activator of transcription 3; qPCR, quantitative real-time polymerase chain reaction; p-STAT3, phosphorylated-signal transducer and activator of transcription 3; mRNA, messenger RNA; GAPDH, glyseraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

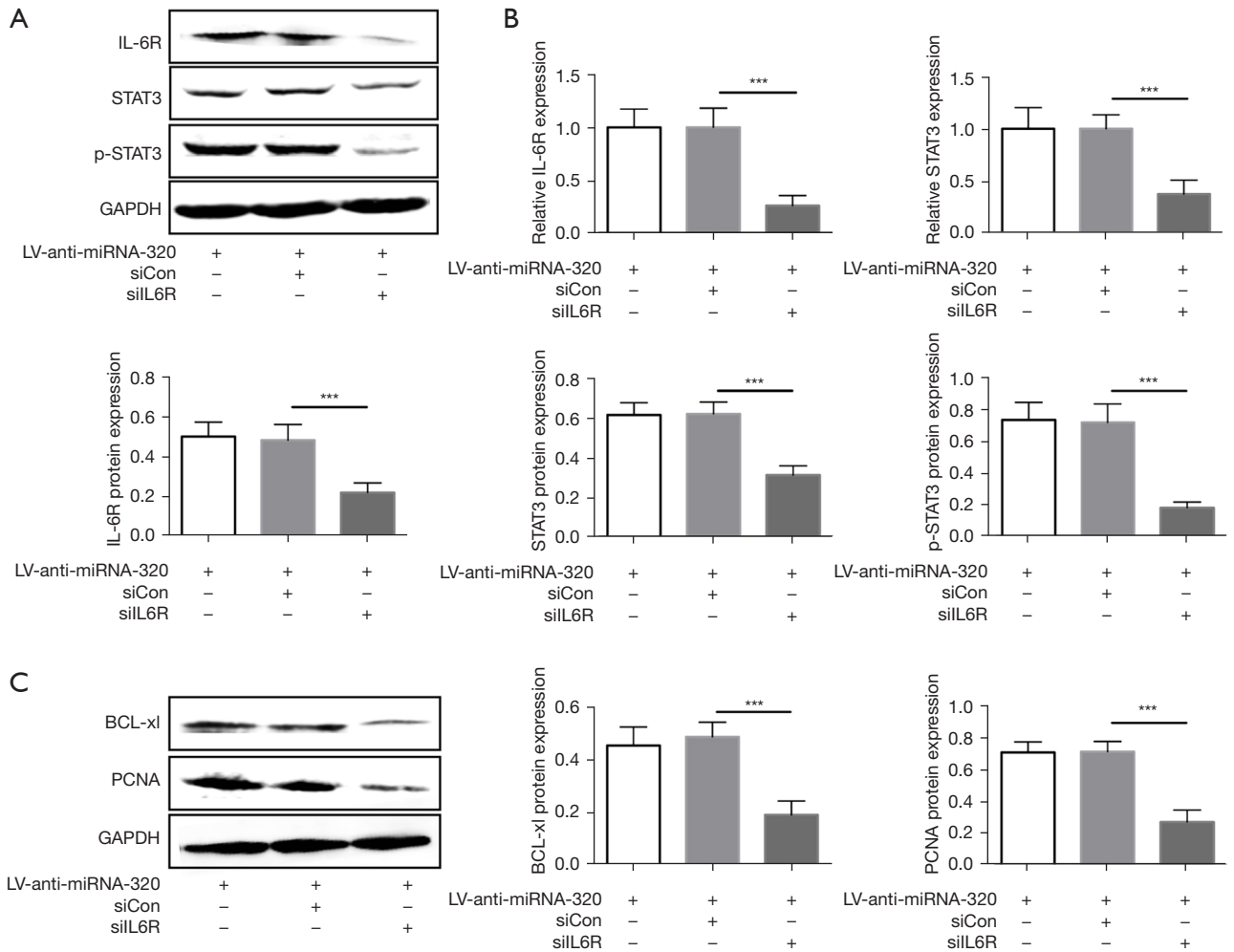

Figure 6 Silencing IL-6R attenuates the regulation of IL-6R/STAT3 by miRNA-320. (A) IL-6R, STAT3, and p-STAT3 protein expression in DLD1 cells transfected with LV-anti-miRNA-320 in the presence or absence of IL-6R siRNA was analyzed by Western blot. (B) mRNA levels of IL-6R and STAT3 were evaluated by qPCR in DLD1 cells transfected with LV-anti-miRNA-320 in the presence or absence of IL-6R. (C) Expression of BCL-xl and PCNA proteins in DLD1 cells transfected with LV-anti-miRNA-320 in the presence or absence of IL-6R siRNA was analyzed by Western blot. Data are expressed as the mean ± SD. A "+" means existence, and a "–" means not present. ***, P<0.001. IL-6R, interleukin-6 receptor; LV, lentiviral vector; STAT3, signal transducer and activator of transcription 3; miRNA, microRNA; p-STAT3, phosphorylated-signal transducer and activator of transcription 3; mRNA, messenger RNA; qPCR, quantitative real-time polymerase chain reaction; BCL-xl, B-cell lymphoma-extra large; PCNA, proliferating cell nuclear antigen; siRNA, small-interfering RNA; GAPDH, Glyseraldehyde-3-phosphate dehydrogenase; SD, standard deviation.

miRNA-320 has potential roles as non-invasive biomarkers for IBD disease monitoring and CAC pathogenesis screening. The most effective way to monitor and screen IBD and CAC is colonoscopy. If it can be matched with specific biomarker detection, the detection effect will be greatly improved (21). Compared with the model group, overexpressed miRNA-320 inhibited the number and size of tumors (*Figure 1F-1H*) and played a protective role in mice, such as improving the general condition of the mice and reducing their DAI scores. Improving inflammation is a key step in favoring mucosal healing. Similarly, miRNA-320 markedly reduced the tumor size and weight of HT29 xenografts in nude mice and colony formation *in vitro*. These results suggested that miRNA-320 improved inflammation and inhibited tumor formation in mice with CAC. We speculate that targeting miRNA-320 may have a

role in preventing IBD and CAC in the future.

miRNAs control the occurrence and development of CAC by regulating the balance of tumor suppressor genes and oncogenes in cells, and then participate in the regulation of biological behaviors such as tumor cell proliferation, apoptosis, migration and invasion. The study has shown that miRNA-320 is involved in the regulation of tumor cell proliferation. Sun *et al.* found that miR-320 can suppress colon cancer cell proliferation, induce cell cycle arrest in the G1 to S phase transition, and inhibit the expression of β-catenin in colon cancer cells, resulting in decreased levels of c-myc, cyclin D1, and survivin proteins of the Wnt pathway (20). Other studies found that miRNA-320 downregulation in gastric cancer cells and leukaemia cells can lead to a weakened inhibitory effect on the target gene survivin, resulting in a significant anti-apoptotic effect (22,23). Therefore, to further explore the effect of miRNA-320 on the proliferation of IECs in mice with CAC, we detected proliferation-related protein levels by Western blot. We found that miRNA-320 inhibited the expression of BCL-xl and PCNA *in vivo* and *in vitro*, suggesting that it does regulate cell proliferation.

In addition to tumor cell proliferation and apoptosis, miRNA-320 is closely related to tumor cell migration and invasion. Zhang *et al.* analyzed the clinical characteristics of 62 patients with colon cancer and showed that low levels of miR-320 were significantly associated with high liver and peritoneal metastasis rates and late clinical staging, suggesting that miR-320 can inhibit cell migration and invasion in the progression of tumors (24). In the present experiment, wound healing and Matrigel invasion assays were used to explore the effects of miRNA-320 on the migration and invasion of CRC cells. We found that miRNA-320 inhibited the migration and invasion of CRC cells.

Chronic inflammation is the primary cause of CAC. The study has shown that immune cells that produce pro-inflammatory factors and inflammation-related signaling pathways play an important role in the pathogenesis of IBD and CAC (25). IL-6 is an important inflammatory factor that activates the STAT3 signaling pathway by combining with IL-6R (26). The IL-6/STAT3 signaling pathway induces the production of inflammatory mediators to promote the progression of inflammation and tumorigenesis and the expression of antiapoptotic genes and cell proliferation (27). A lot of evidence has shown that the IL-6/IL-6R/STAT3 signaling pathway is one of the important pathways in the pathogenesis of CAC (28). The above studies provided evidence that miRNA-320 can inhibit the formation of tumors in mice with CAC by downregulating the expression of IL-6R/STAT3. Similarly, our research showed that the expression levels of IL-6R, STAT3, and p-STAT3 in the intestinal tissues of mice in the miRNA-320 group were significantly reduced when compared with those of the model group. We further explored the regulatory effect of miRNA-320 on IL-6R/STAT3 at the cellular level. The expression levels of IL-6R, STAT3, and p-STAT3 were significantly decreased by LV-miRNA-320 in HT29 and SW480 cells, which was consistent with the animal experiments, and increased by LV-anti-miRNA-320 in HCT116 and DLD1 cells. The above results indicated that overexpression or inhibition of miRNA-320 in colon cancer cells could significantly affect the expression levels of IL-6R, STAT3, and p-STAT3. When the target gene IL-6R was silenced, the expression levels of STAT3 and p-STAT3 were also decreased, which indirectly proved the negative regulation of IL-6R/STAT3 by mRNA-320. miRNAs regulate inflammatory responses in the tumor microenvironment by inhibiting inflammation-related target genes to regulate CAC progression (29).

In summary, this study identified the specific role of miRNA-320 in CAC. miRNA-320 inhibits CAC by downregulating the IL-6R/STAT3 pathway. This discovery provides new ideas for CAC treatment strategies targeting miRNA-320, although more extensive studies are necessary.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-22-237/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 2021-AE025) granted by Research Ethics Committee of the Second Hospital of Hebei Medical University, in compliance with the Chinese Academy of Sciences Animal Care and Use Committee guidelines for the care and use of animals.

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