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Research article

# Circular RNA FMN2 motivates colorectal cancer development by mediating tumor-associated macrophage polarization by controlling the microRNA-150-5p/PIK3R3 axis ☆

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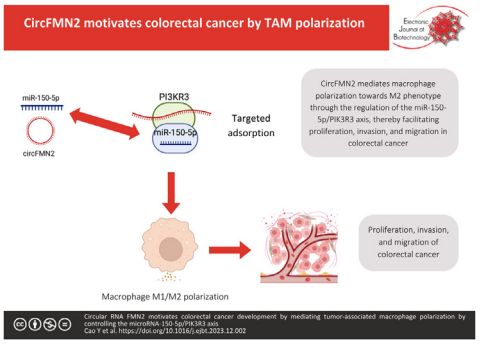
## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* It has been reported that circular RNA formin 2 (circFMN2) can motivate colorectal cancer (CRC) proliferation. However, the effect of circFMN2 on the polarization of tumor-associated macrophages (TAMs) in the CRC tumor microenvironment remains unclear. The study was to figure out the latent mechanism by which circFMN2 impacts TAM polarization to motivate the malignant behavior of CRC cells.

*Results:* circFMN2 and PIK3R3 levels were reduced in M1 macrophages but elevated in M2 macrophages, whereas miR-150-5p level was the opposite. circFMN2 knockdown downregulated M2 macrophage markers CD163, CCL22 and CD206 and upregulated M1 macrophage markers CD86, TNF-α and IL-1β in M2 macrophages. Co-culture with M2 macrophage-conditioned medium with circFMN2 knockdown reduced CRC proliferation, invasion, and migration, while knockdown of miR-150-5p had the opposite effect. CircFMN2 adsorbed miR-150-5p to mediate PIK3R3 in M2 macrophages. Overexpression of miR-150-5p can reverse the promoting effects of overexpression of circFMN2 on M2 polarization, CRC cell proliferation, invasion, and migration. Elevation of PIK3R3 could turn around the repressive effect of circFMN2 knockdown on M2 polarization and CRC cell proliferation, invasion, and migration. In an *in vivo* model, M2 macrophages expressing low or high circFMN2 were co-transplanted with CRC cells into nude mice, resulting in inhibition and promotion of tumor growth, respectively.

☆ Audio abstract available in Supplementary material.

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*Conclusions:* All in all, circFMN2 mediates TAM polarization to M2 type by controlling the miR-150-5p/PIK3R3 axis to motivate CRC development and may offer a latent molecular target for CRC treatment.
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## 1. Introduction

Colorectal cancer (CRC) is a malignant cancer of the digestive system. The prevalence of CRC worldwide is increasing with changes in dietary patterns [1]. Nowadays, CRC treatment mainly relies on radiotherapy, surgery, and chemotherapy [2]. Invasion and malignant metastasis are the major elements for CRC recurrence and unpleasing survival prognosis, and about 50% of patients die of distant tumor metastasis [3,4,5]. In the process of CRC tumor growth, changes in the tumor microenvironment play a key role in the distant metastasis of CRC [6,7].

Tumor-associated macrophages (TAMs) are crucial in controlling the tumor microenvironment [8,9]. According to functional differences, macrophages are generally divided into M1 type (classically activated macrophages) and M2 type (alternatively activated macrophages) [10]. M1 macrophages play an anti-tumor and pro-inflammatory role and secrete inflammatory factors like interleukin (IL)-1β and tumor necrosis factor (TNF)-α. M2 macrophages have the opposite effect to M1 macrophages. In the early stage of tumor, TAMs mainly manifest as M1 type. However, as tumors grow, TAMs gradually polarize toward the M2 type and can motivate malignant behaviors such as angiogenesis [11,12]. Therefore, seeking potential targets that affect the tumor microenvironment is of great significance for inhibiting tumor growth and metastasis.

Circular RNA (circRNA) is a kind of non-coding RNA, which is different from traditional linear RNA. Owing to its closed-loop structure, its expression in tissues and cells is very stable. circRNAs are crucial in controlling TAM polarization in the tumor microenvironment. For example, tumor exosome-derived circ00481117 performs as a sponge for miR-140 to motivate M2 macrophage polarization [13]. circ0110102 represses macrophage activation and hepatocellular carcinoma progression through the miR-580-5p/Peroxisome proliferator-activated receptor α/C—C motif ligand 2 pathway [14]. circRNA ankyrin repeat and PH domain 1 in liver cancer samples are positively associated with $CD68^+$ TAMs [15]. The mechanism by which circRNAs control TAM in the tumor microenvironment is not completely elucidated. circFMN2 can motivate tumor proliferation in CRC [16]. However, circFMN2′s function in the tumor microenvironment is uncertain.

The study was to figure out the latent mechanism by which circFMN2 controls TAM polarization to motivate malignant behavior in CRC. It was found that circFMN2 could motivate TAM polarization to M2 type and CRC cell growth by cross-talking with miR-150-5p/phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) axis.

## 2. Materials and methods

### 2.1. Clinical sample collection

From January 2022 to March 2023, 42 pairs of fresh CRC and adjacent normal tissues (>5 cm from the cancer tissues) were collected from patients undergoing surgery at the Third Affiliated Hospital of Anhui Medical University. No radiotherapy or chemotherapy was conducted in patients prior to surgical removal. The collected tissues were frozen at −80°C for subsequent studies. Histopathological diagnosis was made by 2 pathologists according to WHO criteria. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Anhui Medical University. Written informed consent was signed by all participants.

### 2.2. Cell culture

Human monocytic cell line (THP-1), normal intestinal epithelial cell line (NCM460), and CRC cell lines (SW480, SW620, HCT116) were purchased from the National Collection of Authenticated Cell Cultures. All cells were cultured in a humid environment at 37°C with 5% $CO_2$. THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, Logan), antibiotics (penicillin 100 U/mL and streptomycin 0.1 mg/mL), and 1% N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid. SW480, SW620, and HCT116 cells were cultured in RPMI-1640 medium containing 10% FBS and antibiotics. Cells in the logarithmic phase of growth were collected for subsequent experiments.

### 2.3. Induction of M1 and M2 macrophages

Macrophages were induced and polarized into M1 and M2 macrophages. THP-1 cells were seeded at $15 \times 10^5$ cells/well in the six-well plates and treated with 200 ng/mL Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h to differentiate into M0 macrophages. To induce M1 macrophages, M0 macrophages were treated with 50 ng/mL interferon (IFN)-γ (PeproTech, Princeton, NJ, USA) and 100 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. To induce M2 macrophages, M0 macrophages were treated with 20 ng/mL IL-4 (PeproTech, Princeton, NJ, USA) for 24 h.

### 2.4. Cell transfection

miR-150-5p mimic/inhibitor and negative control (NC) were obtained from GenePharma (Shanghai, China). Small interfering RNAs targeting circFMN2 and PIK3R3 (si-circFMN2/PIK3R3) and overexpression plasmids (oe-circFMN2) and corresponding NCs were designed and produced by RIBOBIO (Guangzhou, China). The above oligonucleotides and plasmids were transfected into M1 macrophages or M2 macrophages using Lipofectamine 3000 (Invitrogen, Eugene, OR, USA). After transfection for 48 h, macrophages were collected.

### 2.5. Macrophage-conditioned medium

M1 or M2 macrophages were cultured in FBS-free RPMI-1640 medium for 24 h, and the supernatant was collected by centrifugation at 2000 × g as the conditioned medium for subsequent experiments.

### 2.6. Colony formation assay

SW480 cells were evenly distributed in 6-well plates (300 cells per well) and cultured in an FBS-free conditioned medium for 24 h. Subsequently, cells were fixed with cold methanol (Melone, Dalian, China) and stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA). The number of colonies was counted under a light microscope (Olympus, Japan).

### 2.7. Transwell

SW480 cells were cultured in FBS-free conditioned medium for 24 h, and $5 \times 10^4$ cells diluted in serum-free medium were seeded into the upper chamber (8.0 μm, Millipore, Burlington, NJ, USA). Transwell invasion and migration tests were performed with or without Matrigel coating (Sigma). The lower chamber contained a medium supplemented with 10% FBS. After 24 h, cells in the lower chamber were fixed for 30 min and stained with 0.05% crystal violet for 2 h. Images were taken in five random areas under a microscope.

### 2.8. Flow cytometry

SW480 cells were detached with trypsin and resuspened in phosphate-buffered saline (PBS). Afterward, cells were treated with 1 μg/mL fluorescein isothiocyanate-conjugated human anti-CD206 (BioLegend, San Diego, CA, USA) or algal hemoglobin-conjugated human anti-CD86 (BioLegend, San Diego, CA, USA) at 4°C for 30 min. Analysis was performed in a BD FACSCalibur™ (BD Bioscience, USA) flow cytometry system.

### 2.9. RT-qPCR

Extraction of total RNA was implemented with TRIzol (Invitrogen, Eugene, OR, USA). mRNA was reverse-transcribed into cDNA using kit (TaKaRa), and miRNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (TaKaRa). mRNA and miRNA were determined with SYBR Green PCR Master Mix Kit (TaKaRa) and quantitative real-time PCR instrument (Applied Biosystems, USA). The reaction condition was set (95°C for 30 s, 56°C for 45 s, 72°C for 20 s in 40 cycles of amplification). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 served as endogenous controls to quantify mRNA and miRNA, respectively. The $2^{-\Delta\Delta Ct}$ method was utilized for analyzing relative fold change. Table 1 shows primer sequences.

### 2.10. Western blot

Extraction of proteins from cells or tissues was conducted with Radio-Immunoprecipitation assay lysis buffer (Beyotime, China), and protein concentrations were measured with Bradford assay (BioRad, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8–12%) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% nonfat milk at room temperature and incubated with the following primary antibodies PIK343 (AP8025a, Abgent) and GAPDH (ab8245, Abcam) at 4°C overnight. After antibody incubation, PVDF membranes were washed with Tris-buffered saline with 0.1% Tween 20 twice and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobin G antibody (ab6721, Abcam). After incubation, membranes were developed by chemiluminescence and analyzed by Image-Pro Plus 6.0 software.

### 2.11. Xenotransplantation experiment

Ten BALB/c athymic nude mice (male, 4–6 weeks old, 16–20 g) were purchased from the Third Affiliated Hospital of Anhui Medical University. All mice were fed at the Third Affiliated Hospital of Anhui Medical University in line with the guidelines for the Care and Use of Laboratory Animals. Animal experiments were performed in the light of the requirements of the Ethics Committee of Third Affiliated Hospital of Anhui Medical University and the approval was gained. SW480 cells ($1 \times 10^6$/ mouse) and M1 macrophages ($1 \times 10^5$/mouse) transfected with si-circFMN2 were injected subcutaneously into nude mice. Tumors were measured with calipers every 7 d to observe the growth of the tumor, and tumor volume was calculated by the formula (volume = length × width$^2$ × 0.5 cm$^3$). Four weeks after inoculation, mice were euthanized, and tumors were weighed.

### 2.12. Dual luciferase reporter experiment

The circFMN2 and PIK3R3 3′-UTR primers containing presumed miR-150-5p target sites were amplified by PCR from human genomic DNA, and the DNA fragments were cloned into the pmiR-RB-REPORT vector (RiboBio) and named WT-circFMN2 and WT-PIK3R3. For the pmiR-RB-circFMN2-MUT and pmiR-RB-PIK3R3-MUT vectors, miR-150-5p targets in circFMN2 and PI3KR3 '-UTR were mutated by PCR-based methods. According to the manufacturer's method, the luciferase reporter vectors and miR-150-5p mimic and mimic NC (GenePharma) were co-transfected into SW480 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured 24 h after transfection, and reporter gene assays were performed according to the manufacturer's protocol (Promega, E2920). The percentage of renilla luciferase activity normalized to firefly luciferase activity and was reported as a control.

### 2.13. Data analysis

All experiments were repeated at least 3 times. Data were shown as mean ± standard deviation. Statistical analysis and graphing were done with GraphPad Prism 7 software (GraphPad Software, USA). Two-tailed Student's t-test was applied to compare two-group data. $P < 0.05$ was considered statistically significant.

**Table 1**
Primers used for qRT-PCR.

| Gene | Forward | Reverse |
|---|---|---|
| TNF-α | 5′-ATGGGCTCCCTCTCATCAGT-3′ | 5′-AAATGGCAAATCGGCTGACG-3′ |
| IL-1β | 5′-ACAAAAATGCCTCGTGCTGTC-3′ | 5′-GTGCCGTCTTTCATCACACAG-3′ |
| CD163 | 5′-CTTGGGGCAGCGTTGGCAGGAATAG-3′ | 5′-ATGCAGGGCTGATGTCCCCTCTGTC-3′ |
| CCL22 | 5′-TCAGACTCCTGCCGGAGGCCTG-3′ | 5′-CAGGGCAGTCTGGGGTCAGCAC-3′ |
| U6 | 5′-CTCGCTTCGGCAGCACA-3′ | 5′-AACGCTTCACGAATTTGCGT-3′ |
| GAPDH | 5′-GCACCGTCAAGGCTGAGAAC-3′ | 5′-TGGTGAAGACGCCAGTGGA-3′ |

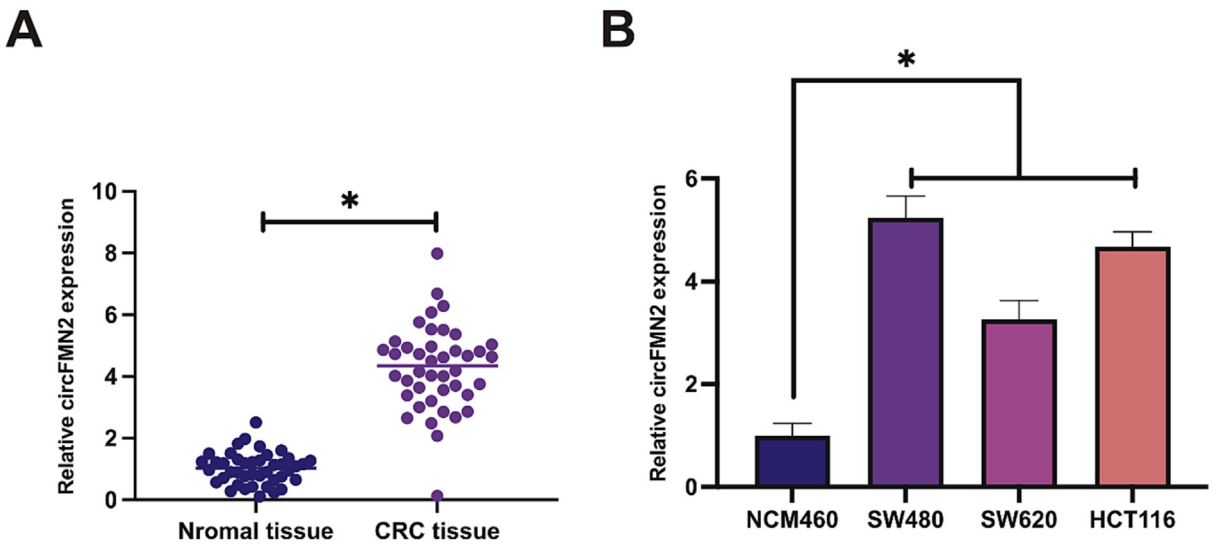


**Fig. 1. CircFMN2 was elevated in CRC.** (A-B) RT-qPCR detection of circFMN2 in CRC tissues and adjacent normal tissues, in normal intestinal epithelial cells NCM460 and CRC cell lines (SW480, SW620, HCT116). The values were clarified as mean ± standard deviation (B, n = 3); *$P$ < 0.05.

## 3. Results

### 3.1. CircFMN2 was elevated in CRC

RT-qPCR analyzed circFMN2 in CRC. CircFMN2 was elevated in CRC tissues compared to adjacent normal tissues (Fig. 1A). Meanwhile, circFMN2 in the three CRC cell lines was elevated versus NCM460 cell line (Fig. 1B).

### 3.2. CircFMN2 was elevated in M2 macrophages

To explore circFMN2′s impacts on macrophage polarization in the tumor microenvironment, THP-1 cells were first induced into M0 macrophages by PMA, and M0 macrophage surface marker CD11b was detected by flow cytometry. As shown in Fig. 2A, after PMA induction, CD11b was positive, indicating that THP-1 cells were successfully induced into M0 macrophages. Then, M0 macro-

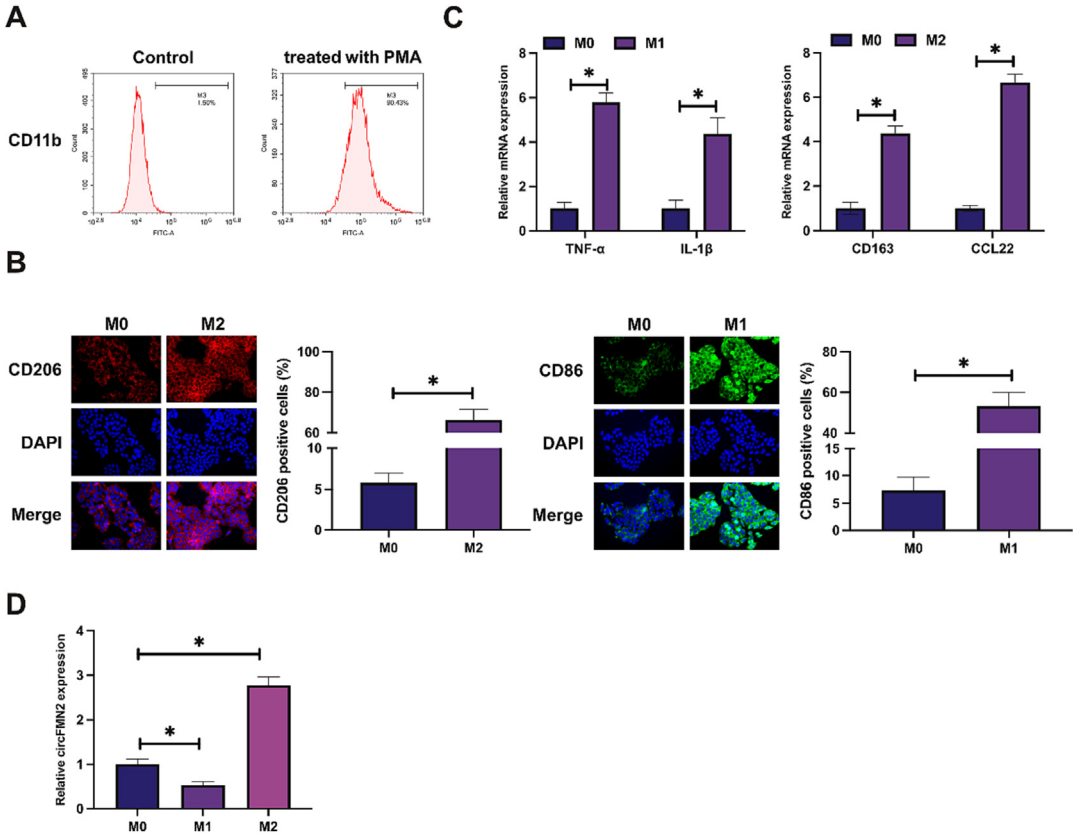


**Fig. 2. CircFMN2 was elevated in M2 macrophages.** (A) Flow cytometry to detect M0 macrophage surface marker CD11b before and after PMA induction; (B) Immunofluorescence to detect M1 macrophage surface marker CD86 and M2 macrophage surface marker CD206; (C) RT-qPCR detection of M1 macrophage markers IL-1β and TNF-α and M2 macrophage markers CD163 and CCL22; (D) RT-qPCR detection of circFMN2 in M1 and M2 macrophages. Values were clarified as mean ± standard deviation (n = 3); *$P$ < 0.05.

phages were induced to polarize toward M1 macrophages using LPS and IFN-γ. M0 macrophages were polarized to M2 macrophages using IL-4. M1 macrophage surface marker CD86 and M2 macrophage surface marker CD206 were analyzed, and the results illustrated that CD86 and CD206 were positive in M1 and M2 macrophages, respectively (Fig. 2B). Meanwhile, M1 macrophage markers IL-1β and TNF-α were also found to be elevated after LPS and IFN-γ treatment, and M2 macrophage markers CD163 and CCL22 were enhanced after IL-4 treatment (Fig. 2C). Moreover, circFMN2 was reduced after macrophage polarization to M1 type, while circFMN2 was elevated after polarization to M2 type (Fig. 2D). These results showed that M1 and M2 macrophages were successfully induced, and circFMN2 expression was increased during M2 polarization.

### 3.3. Knockdown circFMN2 repressed M2 polarization of macrophages

To verify the hypothesis that circFMN2 may regulate the polarization of macrophages, siRNA targeting circFMN2 was transfected into M2 macrophages. circFMN2 in M2 macrophages was suppressed after transfection of siRNA (Fig. 3A). Flow cytometry indicated that CD86 was elevated but CD206 was reduced in M2 macrophages after the knockdown of circFMN2 (Fig. 3B). Furthermore, knockdown of circFMN2 suppressed M2 macrophage markers CD163 and CCL22 but elevated M1 macrophage markers IL-1β and TNF-α (Fig. 3C). This suggested that knockdown of circFMN2 polarized M2 macrophages toward M1 macrophages. Subsequently, SW480 cells were cultured with the conditioned medium of M2 macrophages transfected with si-circFMN2. Colony formation assay suggested that the proliferation capacity of SW480 cells was reduced by M2 macrophage-conditioned medium with circFMN2 knockdown (Fig. 3D). Transwell results showed that the invasion and migration of SW480 cells were inhibited by M2 macrophage-conditioned medium with circFMN2 knockdown (Fig. 3E). This suggested that knockdown of circFNM2 prevented CRC malignant behavior by refraining M2 polarization.

### 3.4. Knockdown of miR-150-5p mediated M2 polarization t0000000000001o motivate CRC cell growth

miR-150-5p performs as a tumor suppressor gene to prevent CRC growth and metastasis [17]. In the present study, miR-150-5p expression was reduced in CRC tissues and cells (Fig. 4A,B). It was found that miR-150-5p was elevated in M1 macrophages and reduced in M2 macrophages after induction of M0 macrophage polarization (Fig. 4C). Subsequently, miR-150-5p inhibitor was transfected into M2 macrophages (Fig. 4D). The results illustrated that knockdown of miR-150-5p elevated CD206 but suppressed CD86 expressions (Fig. 4E). Furthermore, repressing miR-150-5p suppressed TNF-α and IL-1β but elevated CD163 and CCL22 levels (Fig. 4F). Subsequently, the conditioned medium of M2 macrophages with miR-150-5p knockdown was cultured with SW480 cells, which led to promotion of colony formation ability, proliferation, invasion, and migration (Fig. 4G–H). These results suggested that the knockdown of miR-150-5p could mediate macrophage M2 polarization to motivate the malignant behavior of CRC cells.

### 3.5. MiR-150-5p was competitively bound by circFMN2 and controlled TAM polarization and malignant behavior of CRC

Whether miR-150-5p is the downstream crosstalk miRNA of circFMN2 was examined. RT-qPCR indicated that miR-150-5p expression was elevated in M2 macrophages after the knockdown of circFMN2 (Fig. 5A). Meanwhile, on the bioinformatics website https://starbase.sysu.edu.cn/, circFMN2 and miR-150-5p had potential binding sites (Fig. 5B). Subsequently, the targeting relationship was examined by a dual luciferase reporting assay. Co-transfection of WT-circFMN2 and miR-150-5p mimic reduced luciferase activity, but co-transfection of MUT-circFMN2 and miR-150-5p mimic did not affect luciferase activity (Fig. 5C). This indicated that circFMN2 performed as a sponge for miR-150-5p. Next, oe-circFMN2 and miR-150-5p mimic were transfected into M2 macrophages and it was found that miR-150-5p downregulation by overexpression of circFMN2 was recovered by co-transfecting miR-150-5p mimic (Fig. 5D). Further, the elevation of circFMN2 could motivate CD206, CD163, and CCL22 and repress CD86, TNF-α, and IL-1β in M2 macrophages, but this phenomenon was reversed by co-transfection of miR-150-5p mimic (Fig. 5E, F). The conditioned medium of M2 macrophages with circFMN2 overexpression enhanced SW480 cell proliferation, invasion, and migration, but these effects were turned around by co-transfection of miR-150-5p mimic (Fig. 5G–H). These results suggested that circFMN2 mediated macrophage M2 polarization by adsorbing miR-150-5p to motivate CRC cell malignant behaviors.

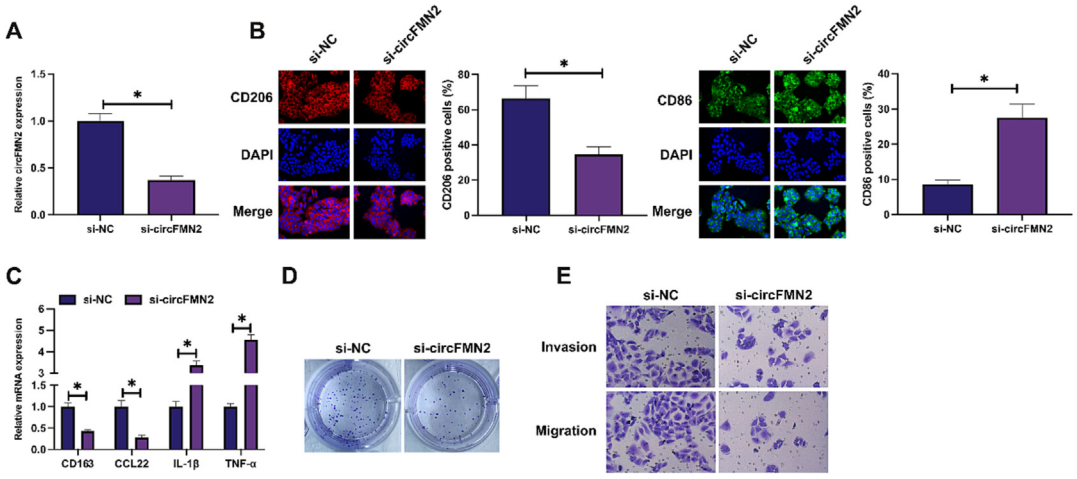


**Fig. 3. Knockdown circFMN2 repressed M2 polarization of macrophages.** (A) RT-qPCR to detect circFMN2 in M2 macrophages after transfection with si-circFMN2; (B) Immunofluorescence to detect CD86 and CD206 in M2 macrophages after transfection with si-circFMN2; (C) RT-qPCR detection of CD163, CCL22, IL-1β and TNF-α in M2 macrophages after transfection with si-circFMN2; (D) Colony formation assay detection of SW480 cell colony formation ability; (E) Transwell detection of the invasion and migration abilities of SW480 cells. Values were clarified as the mean ± standard deviation (n = 3); *$P < 0.05$.

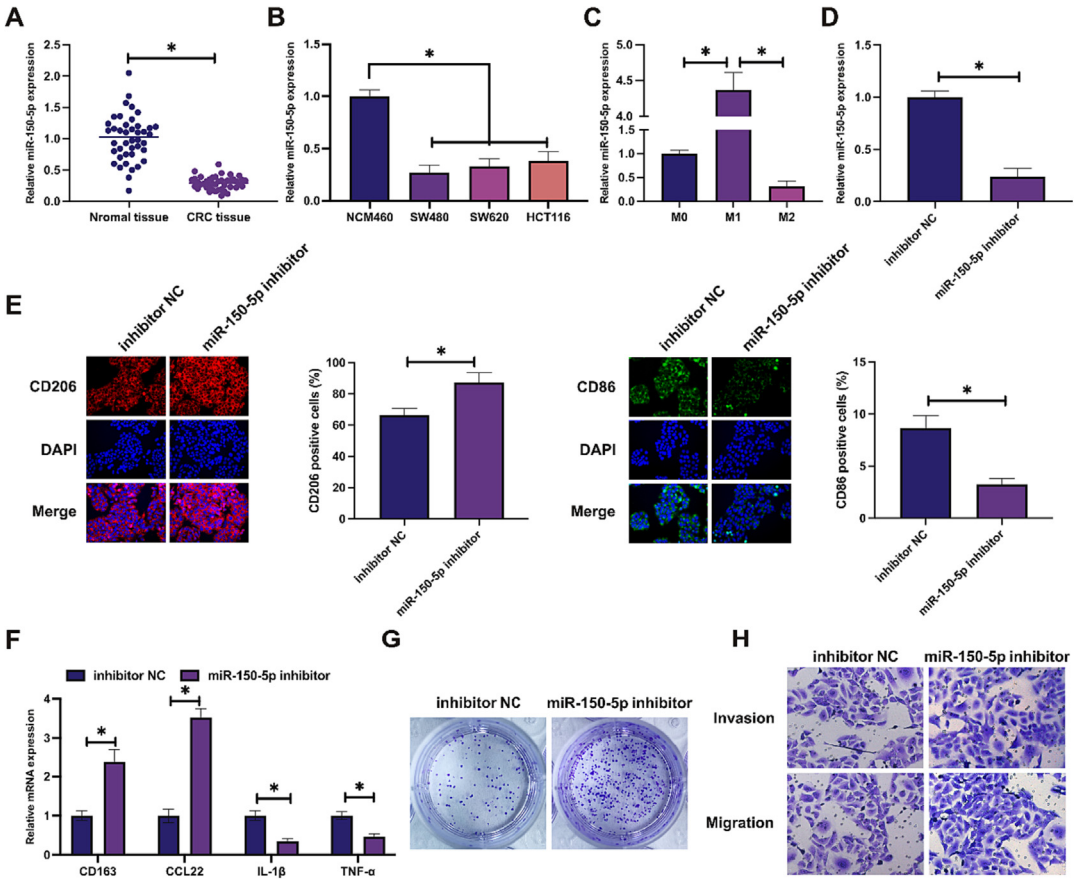


**Fig. 4. Knockdown miR-150-5p mediated macrophage M2 polarization to motivate CRC malignancy.** (A-C) RT-qPCR to detect miR-150-5p in CRC tissues and adjacent normal tissues, in normal intestinal epithelial cells NCM460 and CRC cell lines (SW480, SW620, HCT116), in M1 and M2 macrophages; (D) RT-qPCR to detect miR-150-5p in M2 macrophages after transfection with miR-150-5p inhibitor; (E) Immunofluorescence detection of CD86 and CD206 in M2 macrophages transfected with miR-150-5p inhibitor; (F) RT-qPCR detection of TNF-α, IL-1β, CD163 and CCL22 in M2 macrophages after transfection with miR-150-5p inhibitor; (G) Colony formation assay to detect the colony formation ability of SW480 cells; (H) Transwell detection of invasion and migration abilities of SW480 cells; Values were clarified as mean ± standard deviation (n = 3); $^{*}P < 0.05$.

### *3.6. CircFMN2 mediated M2 polarization to motivate CRC malignant behavior by controlling the miR-150-5p/PIK3R3 pathway*

MiRNAs need to combine with the 3′UTR end of proteins to mediate post-transcriptional gene expression. PIK3R3 performs as a proto-oncogene in CRC and can motivate the malignant behavior of CRC [18,19]. In the present study, it was found that circFMN2 knockdown inhibited PIK3R3 expression in M2 macrophages, but miR-150-5p knockdown increased PIK3R3 expression in M2 macrophages (Fig. 6A). Bioinformatics website https://starbase.sysu.edu.cn/ found latent binding sites between PI3KR3 and miR-150-5p (Fig. 6B). Co-transfection with WT-PIK3R3 and miR-150-5p mimic reduced luciferase activity, while co-transfection with MUT-PIK3R3 and miR-150-5p mimic did not affect luciferase activity (Fig. 6C). This indicated that PIK3R3 was a downstream target gene of miR-150-5p. Subsequently, after co-transfection of si-circFMN2 and oe-PIK3R3 into M2 macrophages, it was found that si-circFMN2 reduced PIK3R3 expression in M2 macrophages, but co-transfection of oe-PIK3R3 restored PIK3R3 expression (Fig. 6D). Furthermore, the inhibitory effect of si-circFMN2 on the expression of CD206, CD163 and CCL22 and the promoting effect of CD86, TNF-α, and IL-1β in M2 macrophages were reversed by oe-PIK3R3 (Fig. 6E, F). Functional experiments revealed that the repressive effects of the conditioned medium of M2 macrophages transfected with si-circFMN2 on SW480 cell proliferation, invasion, and migration were reversed by transfection of oe-PIK3R3 (Fig. 6G, H). These results suggested that circFMN2 mediated M2 polarization by controlling the miR-150-5p/PIK3R3 pathway to motivate CRC cell malignant behaviors.

### *3.7. CircFMN2 motivated tumor growth by modulating macrophage polarization*

Finally, nude mouse tumor implantation experiments were performed to validate the *in vitro* results. M2 macrophages transfected with si-circFMN2 and oe-circFMN2 and SW480 cells were inoculated subcutaneously into nude mice. Co-inoculation of circFMN2-knockdown M2 macrophages and SW480 cells reduced tumor volume and size, whereas circFMN2-overexpressing M2 macrophages had the opposite effect (Fig. 7A–C). PIK3R3 expression decreased and miR-150-5p expression increased after co-inoculation of circFMN2-down-regulated M2 macrophages with SW480 cells, but co-inoculation of circFMN2-overexpressing M2 macrophages and SW480 cells had the opposite effect (Fig. 7D). Taken together, this suggested that circFMN2 motivated tumor growth by controlling macrophage polarization.

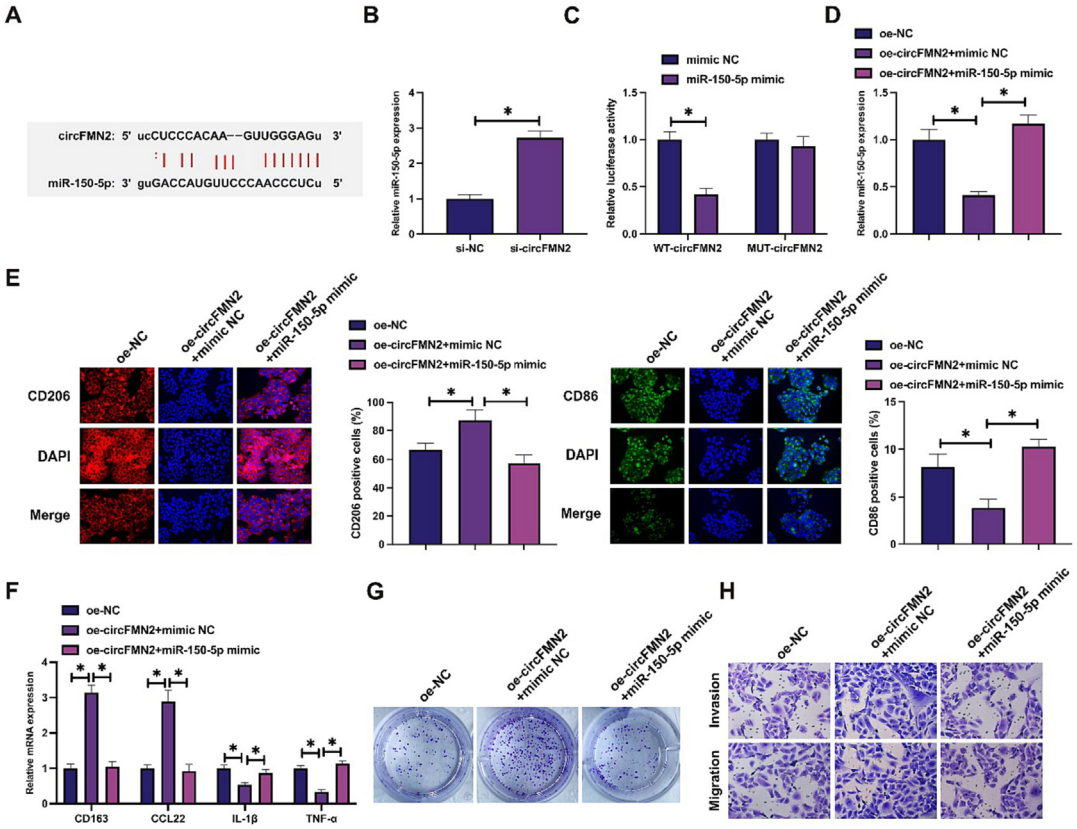


**Fig. 5. MiR-150-5p was competitively bound by circFMN2 and controls TAM polarization and malignant behavior of CRC.** (A) RT-qPCR detection of miR-150-5p in M2 macrophages after knockdown of circFMN2; (B) Bioinformatics website http://starbase.sysu.edu.cn/ to predict circFMN2 and miR-150-5p's binding site; (C) Luciferase activity assay to verify the targeting link between circFMN2 and miR-150-5p; D: RT-qPCR detection of miR-150-5p after co-transfection of oe-circFMN2 and miR-150-5p mimic in M2 macrophages; (E) Flow cytometry detection of CD86 and CD206 in M2 macrophages after co-transfection of oe-circFMN2 and miR-150-5p mimic; (F) RT-qPCR detection of CD1163, CCL22, TNF-α and IL-1β in M2 macrophages after co-transfection of oe-circFMN2 and miR-150-5p mimic; (G) Colony formation assay detection of colony formation ability of SW480 cells; (H) Transwell detection of SW480 cell invasion and migration capacities. Values were clarified as mean ± standard deviation (n = 3); $^{*}P < 0.05$.

## 4. Discussion

Many studies have shown that non-coding RNA plays a role in the regulation of TAM polarization. However, the effect of circRNA on TAM polarization has not been completely reported. In this study, it was found that circFMN2 competitively combined with miR-150-5p to mediate PIK3R3 to motivate TAM polarization to M2 type, thereby accelerating CRC cell malignancy.

CircFMN2 is a new circRNA, which has not been studied much at present. Previous studies have revealed that circFMN2 acts as a proto-oncogene in cancers, including prostate cancer and CRC [16,20]. In the present study, it was found that circFMN2 was elevated in both CRC tissues and cells. Meanwhile, the knockdown of circFMN2 motivated the transformation of M2 macrophages into M1 macrophages. This suggested that circFMN2 could control macrophage polarization in the tumor microenvironment. Studies over the past few years have clarified that during the growth and metastasis of tumors, an immune-immune microenvironment is established to enable them to acquire immune escape function [21]. And TAM is a momentous part of this environment. The results of this study further support the crucial role of circRNAs in regulating the polarization of macrophages in the tumor microenvironment. Cancer immunotherapy has made advances in recent years. PD1/PDL1 is a crucial target of immunotherapy. Wang et al. [22] find that circ0001068 can induce PD1 expression in ovarian cancer. This suggests the potential of circRNAs in cancer immunotherapy. In the follow-up work, it is necessary to further figure out the latent mechanism by which circFMN2 impacts cancer immunity. circFMN2 motivates the proliferation and migration of CRC cells [16]. The present study further found that circFMN2 modulated CRC cell malignancy by affecting the polarization of M2 macrophages in the tumor microenvironment. Although the results of this study suggested that the knockdown of circFMN2 in an animal model could motivate macrophage M1 polarization to repress CRC tumor growth *in vivo*. However, the tumor microenvironment provides a material basis for distant tumor metastasis, and it is necessary to further figure out the effect of circFMN2 on liver metastasis of CRC *in vivo* in subsequent studies.

PIK3R3 is overexpressed in different types of human cancers and is a proto-oncogene. Many studies have demonstrated the mechanism of PIK3R3 regulation of cancer. For example, miR-367 directly targets PIK3R3 to repress the proliferation and invasion of oral cancer cells [23]. This study is the first to reveal the role of PIK3R3 in the polarization of M2 macrophages in the tumor microenvironment. circFMN2 competitively adsorbed miR-150-5p to regulate PIK3R3 expression in M2 macrophages. This effect affected the proliferation, invasion, and migration of CRC cells. Notably, PIK3R3 impacts multiple pathways or molecular targets in cancer including protein kinase B (Akt) and nuclear factor-kappaB (NF-κB) [18,24,25,26]. These findings suggest that PIK3R3 may play a carcinogenic role by regulating the Akt and NF-κB signaling pathways to regulate TAM polarization in the tumor microenvironment. In subsequent studies, further verification of the relationship between PIK3R3 and these signaling pathways is of great significance for understanding the immune escape process of CRC.

Given the potential of circRNA in cancer immunity, future studies could explore whether circFMN2 affects other immune

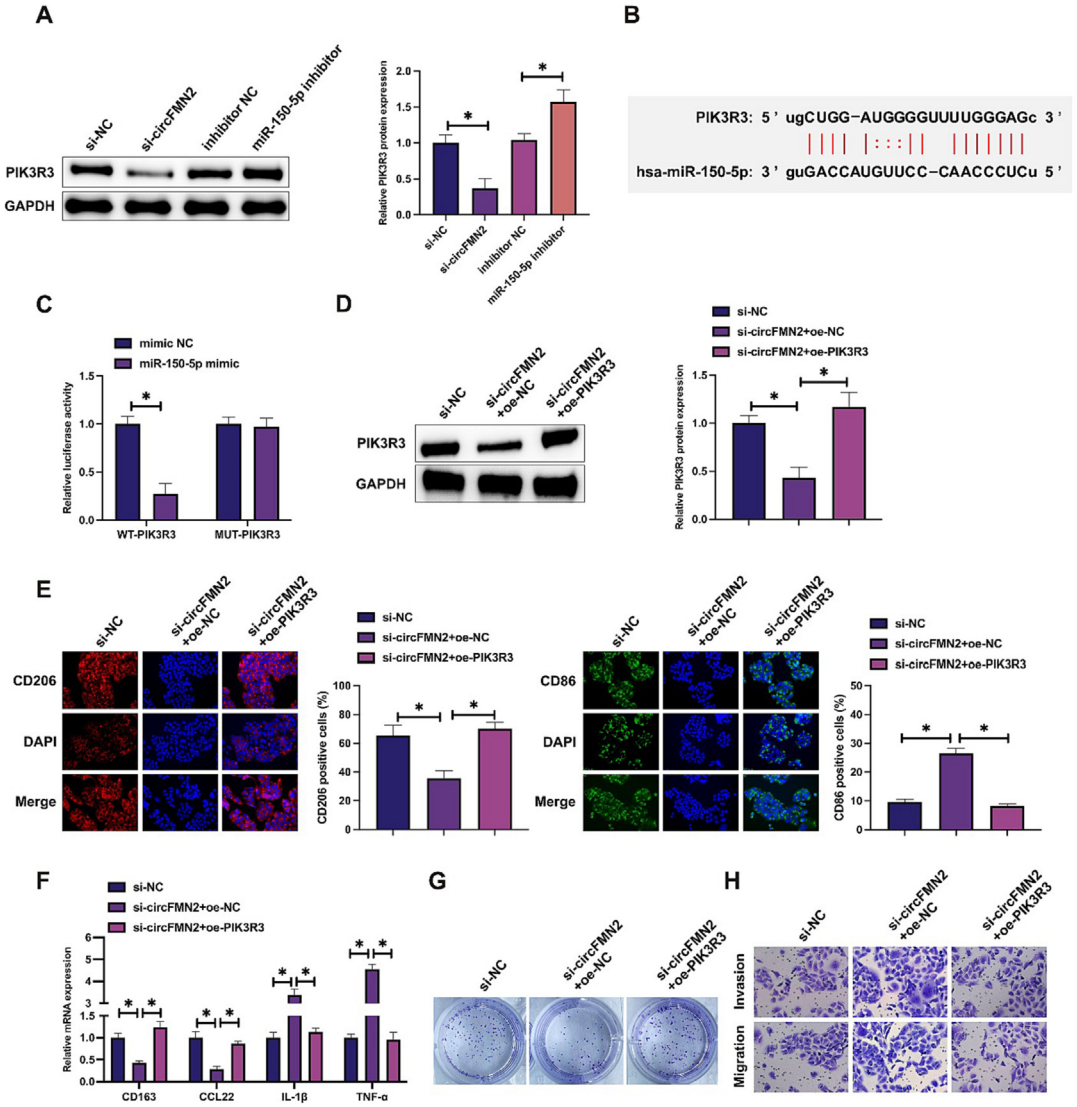


**Fig. 6. CircFMN2 mediated M2 polarization to motivate CRC malignant behavior by controlling the miR-150-5p/PIK3R3 pathway.** (A) Western blot detection of PIK3R3 in M2 macrophages after co-transfection of si-circFMN2 and oe-PIK3R3; (B) Bioinformatics website http://starbase.sysu.edu.cn/ to predict PIK3R3 and miR-150-5p's binding site; (C) Luciferase activity assay to verify the targeting link between miR-150-5p and PIK3R3; (D) Western blot detection of PIK3R3 in M2 macrophages after co-transfection of si-circFMN2 and oe-PIK3R3; (E) Flow cytometry detection of CD86 and CD206 in M2 macrophages after co-transfection of si-circFMN2 and oe-PIK3R3; (F) RT-qPCR detection of CD1163, CCL22, TNF-α and IL-1β in M2 macrophages after co-transfection of si-circFMN2 and oe-PIK3R3; (G) Colony formation assay detection of colony formation ability of SW480 cells; (H) Transwell detection of SW480 cell invasion and migration capacities. Values were clarified as mean ± standard deviation (n = 3); $^{*}P < 0.05$.

cells and investigate its potential as a target for immunotherapy. Through detailed biochemical analysis and functional experiments, we investigated the role of PIK3R3 in signaling pathways such as Akt and NF-κB, and how its interaction with circFMN2 affects these pathways and the progression of CRC.

All in all, circFMN2 promotes the polarization of TAM into M2 type by mediating the expression of PIK3R3 through the adsorption of miR-150-5p, thus promoting the proliferation, invasion, and migration of CRC cells. These results offer latent molecular targets for future treatment of CRC. However, the clinical efficacy of circFMN2 and its role in immune escape need to be further explored.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Third Affiliated Hospital of Anhui Medical University, and written informed consent was provided by all patients prior to the study start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and The Declaration of Helsinki, and its later amendments or comparable ethical standards. This study was approved by the Animal experiments were approved by Third Affiliated Hospital of Anhui Medical University Animal Experimental Ethics Committee, and all procedures complied with the National Institutes of Health Guide for the Use of Laboratory Animals.

## Author contributions

- Study conception and design: Y Cao.
- Data collection: D Cao.
- Analysis and interpretation of results: Y Cao; D Cao; T Zhu.
- Draft manuscript preparation: Y Cao.
- Revision of the results and approval of the final version of the manuscript: T Zhu.

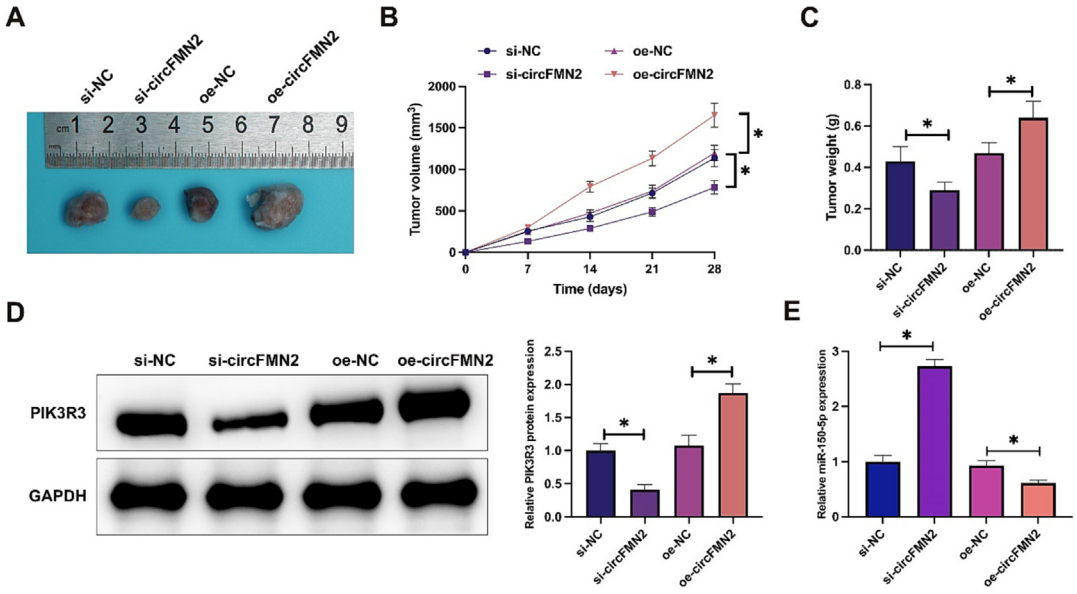


**Fig. 7. CircFMN2 motivated tumor growth by modulating macrophage polarization.** (A) Representative images of tumors in each group; (B) Tumor volume; (C) Tumor weight; (D) PIK3R3 in tumor detected by western blot; (E) qRT-PCR detection of miR-150-5p expression in tumors. Values were clarified as mean ± standard deviation (n = 6); *$P < 0.05$.

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## Conflict of interest

The authors have no conflicts of interest to declare.

## Supplementary material

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## Data availability

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

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