Circ_0067835 acts as an oncogenic factor in colorectal cancer by increasing MAPK1 expression via sponging miR-873-5p

Shouchao Li and Feifei Sun*

Abstract

Many circular RNAs (circRNAs) were recognized to affect the development of colorectal cancer (CRC). Herein, we investigated the functions and mechanisms of circ_0067835 in CRC progression. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay was performed to examine the expression of circ_0067835, microRNA-873-5p (miR-873-5p) and Mitogen-Activated Protein Kinase 1 (MAPK1). Cell counting kit (CCK)-8 assay and colony formation assay were used to assess cell proliferation, and flow cytometry was conducted to monitor cell apoptosis. Transwell assay was applied to detect cell migration and invasion. Western blot assay was implemented to determine the protein levels of MAPK1 and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway-related proteins. Interaction between miR-873-5p and circ_0067835 or MAPK1 was validated by dual-luciferase reporter and RNA immunoprecipitation (RIP) assays. Moreover, the role of circ_0067835 in vivo was evaluated via xenograft model assay. Circ_0067835 and MAPK1 were highly expressed in CRC tissues and cells, while miR-873-5p expression was downregulated. Depletion of circ_0067835 suppressed cell viability, clonogenicity, migration, invasion, survival, and the activity of PI3K/AKT pathway in CRC cells, while these effects were reversed by miR-873-5p inhibition. Circ_0067835 was mainly located at the cytoplasm, and it could sponge miR-873-5p. MiR-873-5p targeted MAPK1 to hamper cell proliferation and metastasis, and inactivated PI3K/AKT pathway. Additionally, circ_0067835 deficiency exerted an inhibitory effect on tumor growth in vivo. Circ_0067835 played an oncogenic role in CRC development through the miR-873-5p-MAPK1-PI3K/AKT pathway, offering a possible molecular strategy for CRC therapy.

Keywords: CRC, circ_0067835, miR-873-5p, MAPK1, PI3K/AKT pathway

Introduction

Colorectal cancer (CRC) is a major cause of tumor-related mortality worldwide [1, 2]. Due to the recurrence and metastasis, CRC patients usually share a bad prognosis [3, 4], especially those of distant metastasis with 5-year survival rate of 14% [5, 6]. Therefore, more studies on the mechanisms associated with CRC progression are required.

Circular RNAs (circRNAs) are primarily generated from back-splicing and form circular structures, with favorable stability and conservative property in eukaryotic cells [7]. With diverse biological functions, circRNAs have a close connection with the oncogenesis and progression of multiple human cancers, including CRC [8]. For example, circERBIN expression was increased in CRC, and circERBIN exerted oncogenic properties in CRC development [9].
CircBANP was overexpressed in CRC and had a positive impact on CRC cell proliferation [10]. Additionally, circITGA7 blocked CRC cell proliferation and metastasis via inactivating the Ras pathway [11]. Hsa_circ_0067835, generated from the exons of intraflagellar transport 80 (IFT80) mRNA, was previously manifested to be highly expressed in CRC tissue samples [12]. Circ_0067835 was also shown to provoke CRC cell growth and in vivo tumorigenesis [12]. The data hinted the oncogenic property of circ_0067835 in CRC. However, the role of circ_0067835 in CRC and its action mechanism have been fully elucidated and need further investigation.

Mechanistically, circRNAs can function in the development of a variety of cancers via splicing microRNAs (miRNAs) [13]. MiRNAs are a cohort of short non-coding RNAs, with important regulatory potency in animal development and disease [14]. As reported, miRNAs play dual roles in CRC progression [15], working as oncogenic factors (like miR-21 [16], miR-31 [17], miR-96 [18], etc.) or tumor suppressors (miR-126 [19], miR-101 [20], miR-302a [21], etc.), which was attributed to different cellular environments. As for miR-873-5p, its expression level was discovered to be decreased in CRC tissues and cells [22]. By the prediction of Circular RNA Interactome, miR-873-5p is a possible target of circ_0067835, and their interactions have not been confirmed in other diseases. It is meaningful to investigate the interaction between miR-873-5p and circ_0067835 in CRC.

MiRNAs affect the expression of targeted genes by pairing to the seed region in a post-transcriptional way [23]. TargetScan recognizes that the 3′ untranslated region (3’UTR) of Mitogen-Activated Protein Kinase 1 (MAPK1) endows a complementary sequence with miR-873-5p. MAPK1 was reported to be upregulated in CRC, and it was involved in miR-422a-induced CRC cell growth inhibition [24]. It is unclear whether the involvement of MAPK1 in CRC development is associated with miR-873-5p. Furthermore, the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway is strongly associated with tumor pathogenesis, making it a potential therapeutic way [25].

In this research effort, the relative expression level of circ_0067835 in CRC tissues and cells was detected. The functional impact of circ_0067835 on the growth and metastasis of cells was investigated. Moreover, the crosstalk of circ_0067835, miR-873-5p and MAPK1 was clarified in CRC progression for the first time, so as to address a new molecular mechanism regarding circ_0067835 function in CRC.

Methods

Collection of clinical specimens

After the acquisition of the ethical permission from the Ethics Committee of Weifang People’s Hospital and handwritten informed consent from all participants, 33 cases of CRC tissues and homologous normal tissues were collected from 33 CRC patients through surgery operation at Weifang People’s Hospital. The clinical features of these patients were shown in Table 1. All specimens were kept in a refrigerator (−80°C). All these 33 participants were followed up from February 2015 (date of surgery) for 5 years or demise.

Cell culture and transfection

Normal human colon epithelial cells FHC (ATCC® CRL-1831), as well as CRC cells DLD1 (ATCC® CCL-221), SW480 (ATCC® CCL-228) and HCT116 (ATCC® CCL-247) were procured from American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) added with 10% (V/V) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) was utilized for culturing all cells, at 37°C with 5% CO2.

HCT116 and SW480 cells were transfected with specific small interfering RNAs (siRNAs) of circ_0067835 si-circ_0067835#1 (GACCCUUAAUCAUUGACACA; GenePharma, Shanghai, China), si-circ_0067835#2 (UUGACCCUUAAUCAUUGC; GenePharma, Shanghai, China), siRNA negative control si-NC (5’-AACGTCGGTGTGCGACTGG-3’; GenePharma, Shanghai, China), miR-873-5p mimic (miR-873-5p, 5’-GCAGGAAUCUGUGUCCU-3’; GenePharma, Shanghai, China), miR-873-5p inhibitor (anti-miR-873-5p, 5’-AGAGACUCAAGUUCUGCC-3’; GenePharma, Shanghai, China), miR-873-5p mimic (miR-NC, 5’-CGAUCGCAUCGCAUCUGACUG-3’; GenePharma, Shanghai, China), miR-873-5p inhibitor (anti-miR-873-5p, 5’-AGAGACUCAAGUUCUGCC-3’; GenePharma, Shanghai, China), inhibitor negative control (anti-miR-NC, 5’-CUAACGCAUCGCAUCUGACUG-3’; GenePharma, Shanghai, China), overexpression plasmid of MAPK1 (pcDNA-MAPK1; GenePharma, Shanghai, China) or pcDNA-con for 48 h utilizing Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) until 70% cell confluence. Moreover, HCT116 cells were infected with lentiviral-based small hairpin RNA (shRNA) of circ_0067835 (sh-circ_0067835; GenePharma, Shanghai, China) to establish cells stably expressing sh-circ_0067835, with sh-NC (GenePharma, Shanghai, China) as negative control.
Table 1. Correlation between circ_0067835 expression and clinical features of colorectal cancer patients

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Number (n = 33)</th>
<th>circ_0067835 expression</th>
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Notes: p < 0.05 by chi-square test.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was prepared by the use of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) complying with manufacturer’s guidelines, followed by reverse transcription with Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) or miRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) into complementary DNA (cDNA). qRT-PCR assay was executed with SYBR Green Mix (Applied Biosystems, Austin, TX, USA) or MiRNA Assay Kit (Applied Biosystems, Austin, TX, USA). Data were processed via 2-ΔΔCT method, with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, for circ_0067835, IFT80 and MAPK1) or U6 (for miR-873-5p) as intrinsic control. Primers involved in qRT-PCR assay were: circ_0067835-forward, 5′-GCATCCATTACTTCACTTGCA-GTGC-3′ and circ_0067835-reverse, 5′-GGATGGTCCAGCCACACAGC-3′; IFT80-forward, 5′-CTGATGCTGCCCTTGTTTCCACAT-3′ and IFT80-reverse, 5′-CTGCCATAGAGCTGACAAAGC-3′; miR-873-5p-forward, 5′-GAGGCAGGAACCTGTGAG-3′ and miR-873-5p-reverse, 5′-CTCACTGAGTCTGCTGA-3′; MAPK1-forward, 5′-ACACCAACCTCCTGCTACATCGG-3′ and MAPK1-reverse, 5′-TGGCAGTAGGTCTGGTGCTCAA-3′; GAPDH-forward, 5′-CAGGAGGCATTGGCTGATGAT-3′ and GAPDH-reverse, 5′-GAAGGCTGGGGCTCATTT-3′; U6-forward, 5′-CTCGCTTCCGCAAGCA-CA-3′ and U6-reverse, 5′-AACGCTTCAGAAATTGCGT-3′.

RNase R treatment

The current experiment was employed to assess the stability of circ_0067835. Five μg total RNA isolated from CRC HCT116 and SW480 cells was incubated with RNase R (Geneseed, Guangzhou, China) or without RNase R (MOCK) at 37°C for 30 min. Afterward, RNA levels of circ_0067835 and IFT80 were analyzed via qRT-PCR assay.

Cell proliferation assay

For cell counting kit (CCK)-8 assay, 4 × 10^5 transfected HCT116 and SW480 cells were seeded into 96-well plates and maintain for the indicated time (0 h, 24 h, 48 h or 72 h), followed by incubation with CCK-8 reagent for additional 4 h. Cell viability was evaluated by measuring the absorbance of every well at 450 nm by the use of a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

For colony formation assay, 800 transfected CRC cells were placed into 6-well plates and cultured for 10 days. Generated cell colonies (more than 50 cells) were subjected for fixation with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), dyeing with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and counting using Image J software (NIH, Bethesda, MD, USA).
Transwell assay
For invasion detection, 5 × 10⁴ transfected CRC cells in serum-free medium were placed in upper Transwell chambers (BD Biosciences, San Jose, CA, USA) precoated with Matrigel (BD Biosciences, San Jose, CA, USA). Moreover, medium added with 10% FBS was added in lower chambers. Twenty four hours later, cells attached to the lower side of the polycarbonate membrane were fixed and dyed, then counted under a microscope (E200, Nikon, Tokyo, Japan) with 100 × amplification. The mean of the number of cells in five random fields was recorded.

For migration analysis, 1 × 10⁴ transfected HCT116 and SW480 cells were seeded in upper chambers without Matrigel. The other procedures were the same as invasion detection.

Flow cytometry
This assay was employed to test CRC cell apoptosis using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA). After transfection, cells were harvested and double-stained with Annexin V-FITC and propidium iodide (PI) following the user’s manual. Apoptosis rate of CRC cells were distinguished by a flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were quantified as follows: live cells (Annexin V negative/PI negative); cells in early apoptosis (annexin V positive/PI negative); cells in the advanced stages of apoptosis (annexin V positive/PI positive); and necrotic cells (annexin V negative/PI positive). The apoptosis rate was expressed as the sum of the percentage of early apoptosis cells and late apoptosis cells.

Western blot analysis
The present assay was implemented according to previous research [26]. The antibodies were commercially procured from Abcam (Shanghai, China), namely, anti-phosphorylated (p)-PI3K (ab182651), anti-PI3K (ab180967), anti-p-AKT (ab192623), anti-AKT (ab179463), anti-MAPK1 (ab32081), anti-GAPDH (ab181602) and secondary antibody (ab205718).

Target prediction and validation
Circ_0067835/miRNA interaction was predicted by Circinteractome (https://circinteractome.nia.nih.gov/), and miR-873-5p was identified as a potential target. Additionally, miR-873-5p/mRNA interaction was predicted by TargetScan (http://www.targetscan.org/vert_71/), and 3’UTR of MAPK1 was observed to have binding sites with miR-873-5p.

For dual-luciferase reporter assay, wild-type reporter vectors of circ_0067835 and MAPK1 (circ_0067835 WT and MAPK1-3’UTR WT) harboring binding position with miR-873-5p, as well as mutant-type ones (circ_0067835 MUT and MAPK1-3’UTR MUT) containing corresponding binding position were all furnished by RIBOBIO Co. Ltd. (Guangzhou, China). Above constructs were respectively transfected with miR-873-5p (using miR-NC as a control) into HCT116 and SW480 cells by the use of Lipofectamine 3000. After 48 h, luciferase density was measured using Dual-Luciferase Detection Kit (Solarbio, Beijing, China).

RNA immunoprecipitation (RIP) assay was conducted with a RIP Kit (Thermo Fisher Scientific, Waltham, MA, USA). In brief, HCT116 and SW480 cells were transfected with miR-NC or miR-873-5p for 48 h. Then, cell lysate of HCT116 and SW480 cells was incubated with magnetic beads combined with anti-Ago2 (ab186733) or anti-IgG antibody (ab133470) at 4°C overnight. Then, bound RNA was extracted and subjected to qRT-PCR analysis.

In vivo tumorigenesis study
Prior to the animal experiment, the ethical approval from the Ethics Committee of Weifang People’s Hospital was obtained. Twelve BALB/c mice (male) (5 weeks old; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were subjected to hypodermic injection into the right flanks with HCT116 cells stably expressing sh-NC or sh-circ_0067835, named sh-NC group and sh-circ_0067835 group (n = 6). Later, tumor size was recorded with a caliper once a week (Volume = length × width²/2). After four weeks, all animals were sacrificed for tumor collection. Then, tumors were weighed and subjected for circ_0067835, miR-873-5p and MAPK1 expression evaluation. Proliferation of tumors was assessed by immunohistochemistry with the antibody specific for Ki67 (ab16667, Abcam), Vectastain ABC Kit and 3,3’-diaminobenzidine (DAB) substrate (Vector Laboratories, Peterborough, UK) as reported [27].

Statistical analysis
All assays were performed with three biological replicates and four technical replicates for each biological replicate. SPSS software 21 (SPSS, Chicago, USA) and GraphPad Prism 7 (GraphPad Software, USA) were used for data processing and figures drawing. Data were expressed as mean ± standard deviation. Student’s t-test or analysis of variance was applied to analyze statistical difference. Kaplan-Meier analysis and log-rank test were employed to determine the survival rate of CRC patients. Correlation among the expression of circ_0067835, miR-873-5p and MAPK1 in 33 cases of CRC tissues was assessed via Pearson’s correlation analysis. p < 0.05 indicated a statistically significant difference.
**Results**

**Expression levels of circ_0067835 in CRC tissues and cells**

We firstly detected the expression of circ_0067835 in CRC tissues and cells. In contrast to the normal tissues, circ_0067835 expression was upregulated in CRC tissues (Fig. 1A). Moreover, circ_0067835 expression was higher in CRC cells (DLD1, SW480 and HCT116 cells) relative to FHC cells (Fig. 1B). Based on the median of circ_0067835 expression level, 33 CRC patients were divided into High circ_0067835 expression group and Low circ_0067835 expression group. We found that patients with high circ_0067835 expression had worse survival ($p = 0.0186$) (Fig. 1C) than the low ones. Additionally, circ_0067835 expression was closely correlated with the size, TNM stage, distant metastasis, and lymph node metastasis of these tumors (Table 1). As shown in Fig. 1D, circ_0067835 was generated by the back-splicing of exons 4-9 of IFT80 mRNA. Furthermore, circ_0067835, rather than the corresponding IFT80 linear mRNA, was resistant to RNase R (Fig. 1F).

**Circ_0067835 deficiency impeded the proliferative and metastatic abilities of CRC cells**

As illustrated in Fig. 2A-B, the expression of IFT80 mRNA (linear transcript of circ_0067835), rather than that of circ_0067835, was significantly declined in HCT116 and SW480 cells due to RNase R digestion, indicating the stability of circ_0067835 in CRC cells. Given the higher expression of circ_0067835 in CRC tissues and cells, we then studied the function of circ_0067835 in CRC development. siRNAs targeting (si-circ_0067835#1 and si-circ_0067835#2) were introduced into HCT116 and SW480 cells to reduce circ_0067835 expression, with si-NC as the negative control. Furthermore, si-circ_0067835#1 induced a lower circ_0067835 expression in CRC cells, so it was selected for the remaining assays (Fig. 2C-D). The data derived from the CCK-8 assay suggested that circ_0067835 deficiency reduced the cell viability of HCT116 and SW480 cells (Fig. 2E-F). Moreover, circ_0067835 knockdown also reduced the clonogenicity of CRC cells (Fig. 2G). Transwell assay revealed that the circ_0067835 depletion-induced the repressed migration and invasion of HCT116 and SW480 cells (Fig. 2H-I). Additionally, knockdown of circ_0067835 enhanced the apoptotic rate in CRC cells (Fig. 2J). HCT116 and SW480 cells with circ_0067835 depletion showed downregulated levels of p-PI3K/PI3K and p-AKT/AKT, suggesting that circ_0067835 knockdown inactivated PI3K/AKT pathway (Fig. 2K-L).

**Circ_0067835 acted as a sponge of miR-873-5p**

Expression percentage of circ_0067835 cytoplasmic and
Fig. 2  Circ_0067835 deficiency impeded the proliferative and metastatic abilities of CRC cells. (A-B) qRT-PCR assay for the relative expression of circ_0067835 and IFT80 mRNA in RNA isolated from HCT116 and SW480 cells treated with RNase R or not (MOCK). (C-D) qRT-PCR assay for relative expression of circ_0067835 in HCT116 and SW480 cells transfected with si-NC, si-circ_0067835#1 or si-circ_0067835#2. (E-L) HCT116 and SW480 cells were transfected with si-NC or si-circ_0067835#1. (E-F) CCK-8 assay for the cell viability of transfected cells. (G) Colony formation assay for the clonogenicity of transfected cells. (H-I) Transwell assay for the migration and invasion of transfected cells. (J) Flow cytometry for the apoptotic rate in transfected cells. (K-L) Western blot assay for the levels of p-PI3K/PI3K and p-AKT/AKT proteins in transfected cells. *p < 0.05, **p < 0.01.
nuclear RNA from HCT116 and SW480 cells was analyzed by qRT-PCR assay after the subcellular fractionation experiment. Results revealed that most of circ_0067835 transcripts were observed in the cytoplasm, making it potential to sponge miRNAs [28] (Fig. 3A-B). Circular RNA Interactome was searched to predict the target miRNAs of circ_0067835, and a putative miR-873-5p binding region was discovered within circ_0067835 (Fig. 3C). Transfection with miR-873-5p mimic effectively increased miR-873-5p expression in HCT116 and SW480 cells compared with the miR-NC control (Fig. 3D). Dual-luciferase reporter assay indicated that CRC cells co-transfected with circ_0067835 WT and miR-
miR-873-5p could target MAPK1

Using the TargetScan miRNA prediction program, MAPK1-3’-UTR was discovered to endow with a target sequence for miR-873-5p (Fig. 5A). Dual-luciferase reporter assay proved that miR-873-5p gain of function triggered an about 60% reduction of luciferase activity in HCT116 and SW480 cells co-transfected with MAPK1-3’-UTR WT, while MAPK1-3’-UTR MUT abolished the suppression of miR-873-5p (Fig. 5B-C). RIP assay manifested that miR-873-5p could specifically bind with MAPK1-3’ UTR (Fig. 5D-E). As shown in Fig. 5F, MAPK1 mRNA expression was apparently overexpressed in CRC tissues compared with normal tissues. Western blot assay validated the upregulation of MAPK1 protein in CRC cells (DLD1, SW480 and HCT116 cells) in contrast to FHC cells (Fig. 5G). Additionally, an inverse correlation between the expression of miR-873-5p and MAPK1 mRNA was found in CRC tissues (Fig. 5H). The data of western blot and qRT-PCR confirmed the miR-873-5p-mediated negative regulation on MAPK1 expression in HCT116 and SW480 cells (Fig. 5I).

miR-873-5p functioned as a CRC suppressor by targeting MAPK1

Knowing that miR-873-5p could target MAPK1, we then investigated the co-effects of miR-873-5p and MAPK1 on the cellular behaviors of CRC cells. The transfection of pcDNA-MAPK1 efficiently elevated MAPK1 protein level in HCT116 and SW480 cells in comparison to pcDNA-con (Fig. 6A). Overexpression of MAPK1 relieved the miR-873-5p-5p-induced the decreased expression of MAPK1 in HCT116 and SW480 cells (Fig. 6B). Furthermore, miR-873-5p gain of function repressed the cell viability (Fig. 6C-D), clonogenicity (Fig. 6E), migration and invasion (Fig. 6F-G), as well as the activation of PI3K/AKT pathway (Fig. 6L-J), while facilitated apoptosis (Fig. 6H) of CRC cells, which were attenuated by introduction of MAPK1.

Circ_0067835 positively regulated MAPK1 expression by absorbing miR-873-5p

We then evaluated the impact of circ_0067835 on MAPK1 expression in CRC cells. As exhibited in Fig. 7A-B,
**Fig. 5** MiR-873-5p could target MAPK1. (A) Binding position between miR-873-5p and MAPK1-3' UTR predicted by TargetScan. (B-C) Dual-luciferase reporter assay for the luciferase activity of HCT116 and SW480 cells co-transfected with MAPK1-3' UTR WT or MAPK1-3' UTR MUT and miR-NC or miR-873-5p. (D-E) RIP assay and qRT-PCR assay for the binding potency between miR-873-5p and MAPK1-3' UTR. (F) qRT-PCR assay for the relative expression of MAPK1 expression in normal tissues and CRC tissues (n = 33). (G) Western blot assay for the protein level of MAPK1 in FHC, DLD1, SW480 and HCT116 cells. (H) Pearson correlation analysis for the correlation between the expression of miR-873-5p and MAPK1 in CRC tissues. (I) Western blot assay and qRT-PCR assay for the relative expression of MAPK1 in HCT116 and SW480 cells transfected with miR-NC, miR-873-5p, anti-miR-NC or anti-miR-873-5p. *p < 0.05, **p < 0.01.

**Fig. 6** MiR-873-5p functioned as a CRC suppressor by targeting MAPK1. (A) Western blot assay for the protein level of MAPK1 in HCT116 and SW480 cells transfected with pcDNA-con or pcDNA-MAPK1. HCT116 and SW480 cells were transfected with miR-NC, miR-873-5p, miR-873-5p+pcDNA-con or miR-873-5p+pcDNA-MAPK1. (B) Western blot assay and qRT-PCR assay for the relative expression of MAPK1 in transfected cells. (C-D) CCK-8 assay for the cell viability of transfected cells. (E) Colony formation assay for the clonogenicity of transfected cells. (F-G) Transwell assay for the migration and invasion of transfected cells. (H) Flow cytometry for the apoptotic rate in transfected cells. (I-J) Western blot assay for the levels of p-PI3K/PI3K and p-AKT/AKT proteins in transfected cells. *p < 0.05, **p < 0.01.
circ_0067835 depletion decreased the protein level of MAPK1 in HCT116 and SW480 cells, while miR-873-5p inhibitor significantly rescued it.

**Circ_0067835 knockdown repressed CRC tumor growth in vivo**

Subsequently, the role of circ_0067835 in vivo was determined by xenograft model assay. Compared with the volume of tumors in the sh-NC group, the size of those in the sh-circ_0067835 group was smaller (Fig. 8A-B). Additionally, tumors in the sh-circ_0067835 group exhibited lighter weight than those in the sh-NC group (Fig. 8C). Circ_0067835 and MAPK1 were downregulated in tumors in the sh-circ_0067835 group, while miR-873-5p was upregulated (Fig. 8D-E). The data of immunohistochemistry assay also confirmed the suppression of circ_0067835 silencing on tumor growth by detecting proliferating maker Ki67 (Fig. 8F).

**Discussion**

CircRNAs have become a research focus due to their involvement in many biological processes connected with malignant properties of CRC cells [29]. Dysregulated circRNAs has been implicated in human malignancies, which usually have the potential to be biomarkers for diagnosis, prognosis and/or significant roles in cancer development [30]. Herein, we identified the upregulation of circ_0067835 in CRC tissues and cells. Also, we demonstrated its functional actions and mechanisms in regulating CRC progression (Fig. 9).

Circ_0067835 could modulate the progression of temporal lobe epilepsy via absorbing miR-155 and upregulating FOXO3a expression [31]. Additionally, the circ_0067835/miR-155/FOXO3a was involved in liver fibrosis development, implying that circ_0067835 was a promising molecular target of liver fibrosis [32]. Feng et al. initially described the abnormal upregulation of circ_0067835 in tumor tissues of CRC patients and cells [12]. Additionally, interference of circ_0067835 hindered the proliferation, invasion, migration and tumorigenesis of CRC cells, suggesting it was a tumor promoter [12]. Likewise, we also observed that circ_0067835 expression was increased in CRC tissues and cells, and circ_0067835 was sustainable to RNase R digestion. Functionally, circ_0067835 depletion brought inhibitory influence on CRC cell growth, migration and invasion, as well as the activation of the PI3K/AKT pathway. Moreover, circ_0067835 sponged miR-324-5p to increase HMGA1 expression, thereby inducing development [33]. Collectively, circ_0067835 might serve as a pro-tumor stimulus.

It was evidenced that circRNAs participated in cancer initiation and progression by serving as miRNA sponges [34]. In this paper, bioinformatic analysis relying on Circular RNA Interactome, dual-luciferase reporter assay and RIP assay verified that circ_0067835 targeted miR-873-5p. Former research revealed that miR-873-5p played tumor-suppressing role in the development of papillary thyroid cancer [35], gastric cancer [36], and colon cancer [37], as well as in CRC [22]. Li et al. suggested that miR-873-5p was lowly expressed in CRC tissues and cells, and overexpression of miR-873-5p impeded CRC cell proliferation and metastasis [38, 39]. Here, our data also suggested the downregulation of miR-873-5p in CRC tissues and cells. Functional assays showed that miR-873-5p induced suppressed effects on the proliferation and metastasis of CRC cells. Additionally, miR-873-5p depletion largely relieved circ_0067835 knockdown-induced CRC cell growth, mobility, and PI3K/AKT pathway inhibition.

MAPK1 was identified as a target gene of miR-873-5p by the TargetScan forecast, dual-luciferase reporter and RIP assays. MAPK signaling cascade is a crucial signaling module, involved in the enablement of varieties of cellular processes, including proliferation and differentiation [40]. MAPK1 is a member of MAPKs, which acts as an oncogene in multiple cancers, such as gastric cancer, ovarian cancer, multiple myeloma [41], andCRC [42]. In CRC, MAPK1 expression was upregulated and was targeted by miRNAs to take part in CRC progression [24, 43]. In accordance with previous reports, we found the increased expression
in CRC tissues and cells. Furthermore, the introduction of MAPK1 undermined miR-873-5p induced CRC development suppression.

Although the present research disclosed the regulatory mechanism of circ_0067835 in CRC progression, there are some limitations in the present study. First, although we found the expression tendency of circ_0067835 in CRC tissues and adjacent normal tissues, the sample size is relatively small. A larger patient population for further investigation and an in-depth exploration the possibility of using circ_0067835 as a diagnostic marker for CRC is needed. Second, circ_0067835 participates in the regulation of CRC progression, however, it remains unknown whether

![Fig. 8 Circ_0067835 knockdown repressed CRC tumor growth in vivo. HCT116 cells stably expressing sh-NC or sh-circ_0067835 were subcutaneously injected into BALB/c mice (n = 6). (A) Picture of generated tumors. (B) Volume of generated tumors measured once a week. (C) Weight of tumors measured at 4 weeks after injection. (D) qRT-PCR assay for the relative expression of circ_0067835 and miR-873-5p in generated tumors. (E) Western blot assay for the protein level of MAPK1 in generated tumors. (F) Immunohistochemistry assay showing the expression of Ki67 in tumors. *p < 0.05, **p < 0.01.](image1)

![Fig. 9 Schematic model of the circ_0067835/miR-873-5p/MAPK1/PI3K/AKT pathway in CRC progression. In CRC, circ_0067835 was upregulated and miR-873-5p was downregulated, resulting in increased expression of MAPK1. Increased level of MAPK1 activated the PI3K/AKT pathway, promoted cell proliferation, migration, and invasion, as well as inhibited cell apoptosis, thereby contributing to CRC progression.](image2)
circ_0067835 mediated the metastatic process of CRC. Therefore, the mechanism underlying circ_0067835 regulates CRC metastasis is worth in-depth study.

In conclusion, the upregulation of circ_0067835 and MAPK1, as well as the downregulation of miR-873-5p were identified in CRC. Moreover, circ_0067835 acted as an oncogenic agent in CRC via positively affect the growth and mobility of CRC cells, which was partly attributed to the miR-873-5p-MAPK1-P3K/AKT pathway. Our findings uncovered a novel molecular mechanism during CRC development and afforded a promising target for CRC treatment.

Ethics approval and consent to participate
Written informed consents were obtained from all participants and this study was permitted by the Ethics Committee of Weifang People’s Hospital.

Consent for publication
Not applicable

Availability of data and material
Not applicable

Competing interests
The authors declare that they have no conflict of interest.

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Authors’ contributions
Shouchao Li designed and performed the research; Shouchao Li, Feifei Sun analyzed the data; Shouchao Li wrote the manuscript. All authors read and approved the final manuscript.

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