

## Circular RNA METTL15/miR-374a-5p/ESCO2 axis induces colorectal cancer development

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This study investigated the biological role and mechanism of circMETTL15 in colorectal cancer (CRC). Cancer tissues and matched adjacent normal tissues were collected. CircMETTL15, miR-374a-5p, and ESCO2 levels were detected by RT-qPCR and Western Blot. LoVo cells were selected for loss- and gain-of-function assays and rescue assays. Cell proliferation was detected by CCK-8 and colony formation tests, cell apoptosis and cell cycle were detected by flow cytometry, cell migration and invasion were detected by Transwell assay, and protein expression of ki-67, E-cadherin, N-cadherin, and cleaved caspase-3 was detected by Western blot. Through bioinformatics analysis and verification assays, the targeting relationship between circMETTL15, miR-374a-5p, and ESCO2 was studied. The results suggest that circMETTL15 was a stable circRNA that was highly expressed in CRC tissues and cells and was associated with tumor size, higher TNM staging, and lymph node metastasis in CRC patients. Functionally, knocking down circMETTL15 inhibited the proliferation, migration, invasion, and EMT of LoVo cells, and induced apoptosis. Overexpression of circMETTL15 showed the opposite effect. The effects of knockdown or overexpression of circMETTL15 on the biological behavior of LoVo cells were reversed by knockdown of miR-374a-5p or knockdown of ESCO2, respectively. Mechanistically, circMETTL15 acts as a ceRNA for miR-374a-5p to regulate ESCO2 expression, thereby promoting the biological behavior of LoVo cells. In conclusion, the results of this study reveal the role of circMETTL15 in CRC and the underlying molecular mechanism, which provides potential data support for the development of future CRC drugs.

**Keywords:** circular RNA METTL15, colorectal cancer, MicroRNA-374a-5p, the establishment of sister chromatid cohesion N-acetyltransferase 2

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**Abbreviations:** BCA, Bicinchoninic acid; CCK-8, Cell counting kit-8; ceRNA, Competing endogenous RNA; circMETTL15, Methyltransferase-like 15; circRNA, Circular RNA; CRC, Colorectal cancer; DMEM, Dulbecco's modified essential medium; EMT, Epithelial-mesenchymal transition; ESCO2, Establishment of sister chromatid cohesion N-acetyltransferase 2; FITC, Fluorescein isothiocyanate; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IHC, Immunohistochemistry; MUT, Mutant type; PI, Propidium iodide; RIP, RNA immunoprecipitation assay; RIPA, Radio-Immunoprecipitation Assay; RT-qPCR, Real-time reverse transcriptase-polymerase chain reaction; TNM, Tumor node metastasis; WT, Wild type

### INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of cancer deaths worldwide (Ameli-Mojarad *et*

*al.*, 2021). So far, surgery, chemotherapy, and radiotherapy have been the main treatment strategies for CRC (Shao *et al.*, 2021). Most patients are diagnosed in an intermediate or late stage because of the hidden early symptoms (Simon, 2016). In addition, limited efficacy, postoperative recurrence, and long-term metastasis lead to poor prognosis for CRC patients (Díaz-Tasende, 2018). Accumulating studies have shown that changes at the genetic and epigenetic levels of oncogenes and tumor suppressor genes are involved in the development and progression of CRC and act as potential therapeutic targets for CRC (Tsai *et al.*, 2021; Slattery *et al.*, 2017). Therefore, identifying these regulatory genes will offer new insights into the development of molecularly targeted therapies for CRC.

Circular RNA (circRNA) has been widely confirmed to be involved in cancer development (Zhang *et al.*, 2021) and is associated with drug resistance, differentiation, metastasis, angiogenesis, proliferation, migration, and apoptosis (Ameli-Mojarad *et al.*, 2021). For example, circPCLE1 promotes CRC epithelial-mesenchymal transition (EMT), glycolysis, and tumor-associated macrophage polarization (Yi *et al.*, 2022) and N-methyladenosine modification of circALG1 promotes CRC migration, invasion, and metastasis (Lin *et al.*, 2022). It can be seen that the regulation of circRNA has a profound impact on the occurrence and progression of CRC. As a member of circRNA, methyltransferase-like 15 (circMETTL15) is involved in the proliferation, metastasis, immune escape, and apoptosis of lung cancer (Zhang *et al.*, 2022). Based on this, we speculate that circMETTL15 may be involved in the regulation of CRC.

In recent years, the competing endogenous RNA (ceRNA) hypothesis has been widely proposed (Salmena *et al.*, 2011). We predicted circMETTL15-related miRNAs (miR-374a-5p) and miRNA target genes (The establishment of sister chromatid cohesion N-acetyltransferase 2, ESCO2). As a short non-coding RNA, miR-374a-5p negatively regulates gene expression and regulates various biological processes (Lin *et al.*, 2020; Guo *et al.*, 2021). miR-374a-5p abnormal expression has been tested in CRC indicating a correlation with patients' survival rates (Slattery *et al.*, 2015; Slattery *et al.*, 2017). ESCO2 is an evolutionarily conserved cohesion acetyltransferase (Zhu *et al.*, 2021) that can suppress the development of CRC (Guo *et al.*, 2018).

This study aimed to investigate the effect of circMETTL15 on the biological function of CRC cells and to discover the molecular mechanism of circMETTL15 with miR-374a-5p and ESCO2 in CRC.

## MATERIALS AND METHODS

### Clinical samples

Between 2016 and 2019, 78 cancer tissues and matched adjacent normal tissues ( $\geq 3$  cm from cancer tissue) were surgically resected from CRC patients (excluding those receiving radiotherapy or chemotherapy) at Renji Hospital, Shanghai Jiaotong University School of Medicine. All tissues were kept at  $-80^{\circ}\text{C}$ . All clinical and pathological diagnoses were validated by two independent pathologists according to the AJCC and UICC guidelines. This study was approved by the Ethics Committee of Renji Hospital, Shanghai Jiaotong University School of Medicine. Each patient was informed about the content.

### Cell culture

LoVo, Caco-2, SW620, SW480, and NCM460 cells were maintained in DMEM, HT-29 and HCT-116 were in DMEM/F12 medium, while DLD-1 was in RPMI-1640 medium. All the media (Gibco, CA, USA) were added with 10% fetal bovine serum, 1% penicillin, and streptomycin, and all cell lines were offered by the Chinese Academy of Sciences Culture Collection (Shanghai, China) and grown in a 5%  $\text{CO}_2$  humidified incubator at  $37^{\circ}\text{C}$ .

### RNA analysis

With Trizol reagent (Takara, Japan), circRNA, mRNA, and miRNA were extracted from tissues and cells for reverse transcription after detection on Nanodrop 2000 (Thermo Fisher Scientific). In the reverse transcription system, PrimeScript RT master mix (Takara) was utilized for mRNA and circRNA, while for miRNA, Bulge-Loop<sup>TM</sup> miRNA Quantitative Real-Time Polymerase Reaction Starter Kit (RIBOBIO, China) was applied. cDNA amplification was implemented in the ABI Prism 7500 Sequence Detection System with TB Green Premix Ex Taq II (Takara). The PCR cycling parameters are as follows:  $95^{\circ}\text{C}$  for 30 s followed by 40 cycles at  $95^{\circ}\text{C}$  for 5 s and  $60^{\circ}\text{C}$  for 30 s. mRNA and circRNA were normalized to GAPDH, while miRNA was to U6. The calculation of relative expression was dependent on the  $2^{-\Delta\Delta\text{CT}}$  method. Primers are listed in Table 1.

**Table 1. Primer sequences**

Genes	Primers
circMETTL15	F: 5'-GCCAGCATCGTTGCAGATT-3'
	R: 5'-GCTGTTGGGTCTCTGTCCAA-3'
miR-374a-5p	F: 5'-CGCGCGTTATAATACAACCTGA-3'
	R: 5'-GCAGGGTCCGAGGTATTC-3'
GAPDH	F: 5'-CACCCACTCTCCACCTTTG-3'
	R: 5'-CCACCACCTGTTGCTGTAG-3'

Note: F, forward; R, reverse; circMETTL15, circular RNA METTL15; miR-374a-5p, microRNA-374a-5p; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

### Nucleic acid electrophoresis

Agarose gel electrophoresis (2%) was performed using 45 mmol/L Tris-boric acid and 1 mmol/L EDTA (TBE). DNA was separated by electrophoresis at 120 V

for 30 min, taking marker L (50-500 bp) as the DNA marker (Beyotime, China). UV irradiation was used to examine the bands.

### Actinomycin D and RNase R

RNase R (3 U/g, Epicenter) was used to treat RNA (10  $\mu\text{g}$ ) from LoVo cells. After incubation at  $37^{\circ}\text{C}$  for 30 min, circRNA and linear mRNA were detected using reverse transcription and RT-qPCR.

Actinomycin D (2  $\mu\text{g}/\text{mL}$ , Sigma) was adopted to treat LoVo cells for 4, 8, 12, and 24 h, followed by checking the stability of linear mRNA and circRNA by RT-qPCR.

### Cell transfection

siRNA or pcDNA 3.1 overexpression vectors targeting circMETTL15 and ESCO2, and miR-374a-5p mimic/inhibitor were constructed and provided by Genepharma (Shanghai, China). The oligonucleotides and plasmids were transiently transfected into LoVo cells using Lipofectamine 3000 (Invitrogen) and gene expression was analyzed by RT-qPCR and western blot after 48 h.

### CCK-8 assay

LoVo cells (2000 cells/well) were plated in 96-well plates and added with 10  $\mu\text{L}$  of CCK-8 reagent (Sangon Biotech, Shanghai, China) at the indicated time points (24, 48, and 72 h). Absorbance at 450 nm was measured using an iMark microplate reader (Bio-Rad) after 2 h.

### Colony formation test

LoVo cells were grown at 1000 cells/well in 6-well plates to allow colony formation at  $37^{\circ}\text{C}$ . The colonies were observed every 3 days, and after 2 weeks, the colonies fixed with paraformaldehyde were counted under a microscope (Olympus) after 0.1% crystal violet staining.

### Flow cytometry

Cells were fixed overnight at  $-20^{\circ}\text{C}$  in 75% alcohol, washed 3 times, and detected by a cell cycle analysis kit (Beyotime). Cells were digested by trypsin, washed in cold PBS, and analyzed by an Annexin V-FITC/PI kit (Vazyme, Nanjing, China) to assess cell apoptosis. Cell cycle distribution and apoptosis rate were determined on the BD FACSCanto II (BD Biosciences).

### Transwell assays

LoVo cells were suspended in a fresh medium (100  $\mu\text{L}$ ) and added to the upper Transwell chamber (BD Bioscience). Matrigel coating (BD Bioscience) was required for the invasion test but not the migration test. After 24-h induction, cells appearing in the lower chamber were fixed with paraformaldehyde and stained with 0.1% crystal violet for microscopic observations ( $\times 100$ , Olympus).

### Protein analysis

Protein samples collected by RIPA buffer were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis after quantification using a BCA kit (Beyotime). Next, the samples were transferred to polyvinylidene fluoride membranes and blocked with 5% non-fat milk for 2 h. After reaction with the primary antibody overnight at  $4^{\circ}\text{C}$ , the membranes were incubated with the secondary antibody (1:5000, CST, MA, USA) for 2

h. The protein blots which were visualized by Omni-ECL chemiluminescent reagent (EpiZyme, China) were then analyzed by Image Lab. Primary antibodies: ESCO2 (ab86003, Abcam), E-cadherin (610181, BD Biosciences), N-cadherin (22018-1-AP, Proteintech), cleaved caspase-3 (ab2302, Abcam), Ki-67 (ab15580, Abcam).

### Dual-luciferase reporter assay

Genepharma constructed PGL4 luciferase reporters (Promega) containing circMETTL15 wild-type sequence or mutant sequence (with or without miR-374a-5p binding site), namely circMETTL15-WT and circMETTL15-MUT, respectively. Likewise, ESCO2 3'UTR-WT and ESCO2 3'UTR-MUT were also constructed. The luciferase reporter vector was co-transfected with miR-374a-5p mimic or mimic NC into LoVo cells using Lipofectamine 3000 (Invitrogen) and its luciferase activity was determined in the Dual Luciferase Assay System (Promega) after 48 h.

### RIP experiment

Under the guidance of the Magna RIP kit (Millipore), LoVo cells were lysed and mixed with Ago2 antibody-conjugated or IgG antibody-conjugated magnetic beads. RNA complexes on magnetic beads were eluted to isolate total RNA for calculation of RNA expression by RT-qPCR.

### Observation of tumor formation

Animal experiments were approved by the Animal Ethics Committee of Renji Hospital, Shanghai Jiaotong University School of Medicine (Approval number:

201511F760). LoVo cells ( $1 \times 10^6$ , 100  $\mu$ L) stably transfected with si-circMETTL15 or si-NC were injected subcutaneously into the axilla of 5-week-old male BALB/c nude mice ( $n=5$ /group). After 1 week, the tumor volume ( $\text{length} \times \text{width}^2/2$ ) was calculated and recorded once a week. Five weeks later, mice were euthanized to resect tumors for IHC analysis (Zhou *et al.*, 2021).

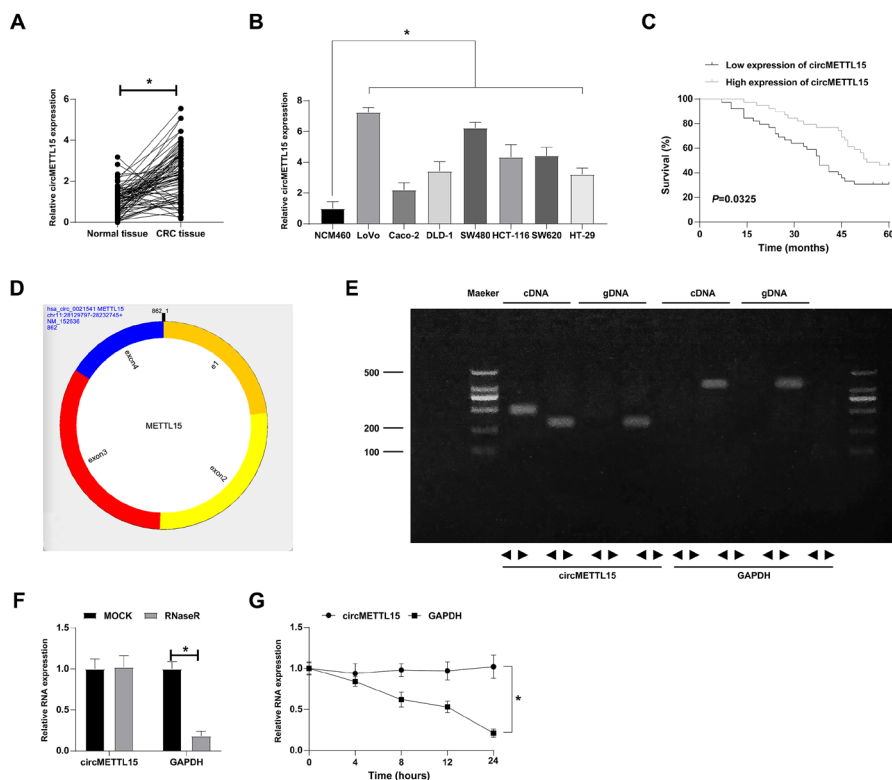
### Data analysis

At least 3 biological replicates were run for each experiment. All statistical analyses were performed using GraphPad Prism 9.0 software (La Jolla, CA, USA). Bilateral comparisons were run by Student's *t*-test, while multiple comparisons were by one-way ANOVA. The correlation between circMETTL15 expression and clinicopathological data was assessed by Chi-square test, while that between circMETTL15 expression and overall survival was by Log-rank (Mantel-Cox) test.  $P < 0.05$  was considered as significant difference.

## RESULTS

### circMETTL15 is abnormally highly expressed in CRC

To investigate whether circMETTL15 participates in CRC, its expression was analyzed in 78 cancer tissues and matched adjacent normal tissues by RT-qPCR. CircMETTL15 was abnormally highly expressed in the tumor samples compared with paired normal tissues (Fig. 1A). Also, circMETTL15 expression was analyzed in CRC cell lines, and elevated circMETTL15 was demonstrated in CRC cell lines than in NCM460 cell line



**Figure 1. circMETTL15 is abnormally highly expressed in colon cancer**

(A) RT-qPCR to detect circMETTL15 in CRC tissues and paired normal tissues. (B) RT-qPCR to detect circMETTL15 in human colorectal cancer cell lines and human colon epithelial mucosa cell line (NCM460). (C) Correlation analysis between circMETTL15 and survival rate of CRC patients. (D) Genetic information of circMETTL15. (E) Agarose gel electrophoresis confirmed the amplification of circMETTL15 by diverging primers in cDNA but not in gDNA. (F) RNase R experiment verified the ring structure of circMETTL15. (G) Actinomycin D experiment verified the ring structure of circMETTL15; data are expressed as mean  $\pm$  S.D. ( $N=3$ ).

**Table 2. Relationship between circMETTL15 and clinicopathological features of CRC patients**

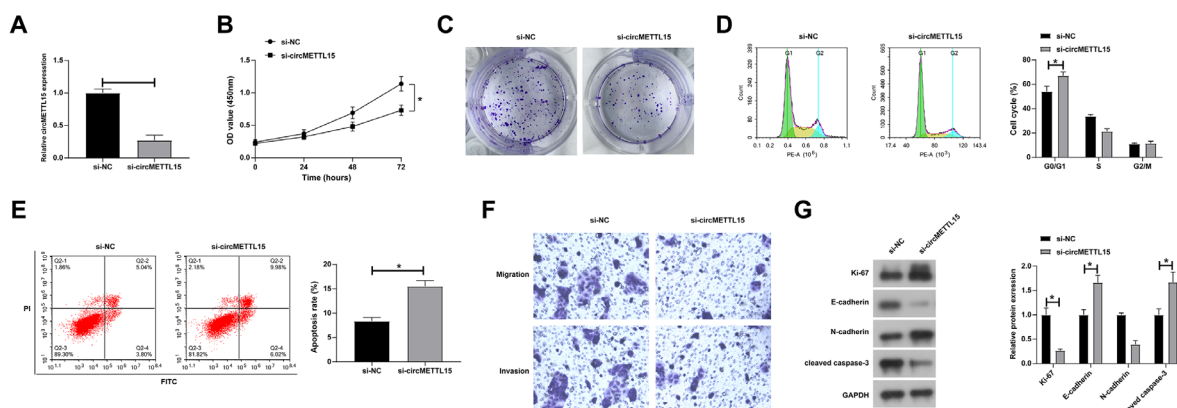
Characteristic	Cases n=78	The expression of circMETTL15		P
		Low (n=39)	High (n=39)	
<b>Gender</b>				
Male	48	23	25	0.6416
Female	30	16	14	
<b>Age (year)</b>				
≤ 60	14	5	9	0.2379
> 60	64	34	30	
<b>Tumor size</b>				
< 3 cm	52	33	19	0.0008
≥ 3 cm	26	6	20	
<b>TNM staging</b>				
I/II	19	14	5	0.0176
II/IV	59	25	34	
<b>Lymph node metastasis</b>				
Positive	34	12	22	0.0224
Negative	44	27	17	
<b>Distant metastasis</b>				
Positive	29	13	16	0.4821
Negative	49	26	23	

(Fig. 1B). Next, the relationship between circMETTL15 levels and clinicopathological characteristics of CRC patients was explored, and the data emphasized that the abundance of circMETTL15 was positively associated with tumor size, TNM stage, and lymph node metastasis

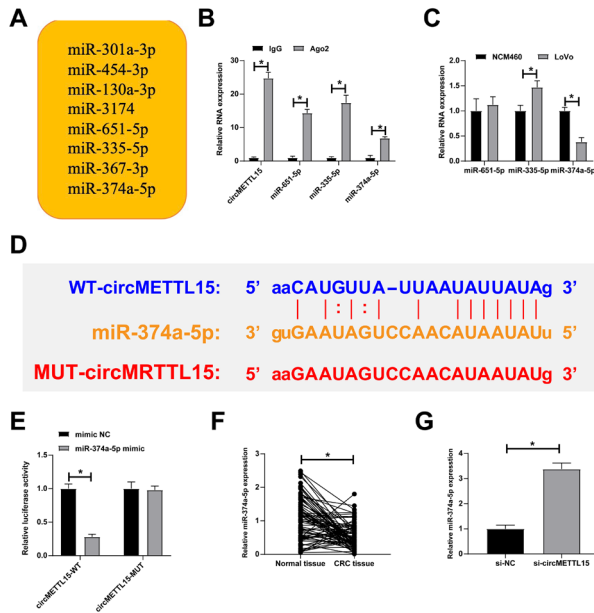
(Table 2). “Log-rank (Mantel-Cox) test” survival analysis showed that CRC patients with high circMETTL15 had poorer overall survival (Fig. 1C). Subsequently, the genetic information of circMETTL15 was determined from the bioinformatics website circbase. circMETTL15 was generated from exon 1 to exon 4 of the METTL15 gene with a full length of 862 bp (Fig. 1D). Agarose gel electrophoresis indicated that circMETTL15 was successfully detected by convergent primers for gDNA and cDNA, but it could only be generated from cDNA by different primers in LoVo cells (Fig. 1E). To further verify the stability of circMETTL15, RNase R and actinomycin D were used. CircMETTL15 was resistant to digestion by RNase R and had a longer half-life compared with linear mRNA (Fig. 1F, G).

### circMETTL15 inhibition blocks CRC cell proliferation, invasion, migration, and EMT and promotes apoptosis

Subsequently, the effects of circMETTL15 on the biological behavior of CRC cells were investigated by loss of function assay. si-circMETTL15 was transfected into LoVo cells to downregulate circMETTL15 (Fig. 2A). CCK-8 and colony formation assays were utilized to evaluate cell proliferation ability. It was observed that knockdown of circMETTL15 reduced LoVo cell proliferation rate (Fig. 2B) and colony formation capacity (Fig. 2C). Then, cell cycle was detected by flow cytometry. As shown in Fig. 2D, knockdown of circMETTL15 increased the proportion of cells in G0/G1 phase and decreased the proportion of cells in G2/M phase. To investigate the effect of circMETTL15 on apoptosis, Annexin V-FITC/PI kit was used to evaluate apoptosis rates. As shown in Fig. 2E, circMETTL15 knockdown resulted in an increased apoptosis rate. In addition, the invasion and migration ability of LoVo cells was examined by Transwell assay. The results showed that circMETTL15 knockdown reduced the number of cells that invaded and migrated (Fig. 2F). At last, the changes of apoptosis, proliferation, and EMT-related proteins were observed by Western blot. As shown in Fig. 2G, after circMETTL15 downregulation, N-cadherin and Ki-67 expression decreased but cleaved caspase-3 and E-cadherin expression increased in LoVo cells. These data suggest that circMETTL15 knockdown inhibits LoVo cell proliferation, invasion, migration, and EMT, pro-



**Figure 2. circMETTL15 inhibition blocks CRC cell proliferation, invasion, migration, and EMT and promotes apoptosis** circMETTL15 was downregulated by transfection of siRNA targeting circMETTL15 into LoVo cells. (A) RT-qPCR to detect circMETTL15. (B) CCK-8 to detect cell proliferation rate. (C) Colony formation assay to detect cell proliferation. (D) Flow cytometry to detect cell cycle. (E) Flow cytometry to detect apoptosis. (F) Transwell assays to detect cell invasion and migration. (G) Western blot to evaluate Ki-67, E-cadherin, N-cadherin, and cleaved caspase-3 expression; data are expressed as mean  $\pm$  S.D. (N=3).



**Figure 3. circMETTL15 binds to miR-374a-5p** (A) Schematic diagram showing the potential miRNA of circMETTL15 based on circinteractome and StarBase. (B) RIP assay confirmed that three miRNAs and circMETTL15 were enriched in LoVo cells. (C) RT-qPCR to detect miR-374a-5p in LoVo cells. (D) Potential binding sites of circMETTL15 and miR-374a-5p predicted based on starbase. (E) Dual luciferase reporter assay to verify the targeting relationship between circMETTL15 and miR-374a-5p. (F) RT-qPCR to detect miR-374a-5p in CRC tissues and paired normal tissues. (G) RT-qPCR to confirm that circMETTL15 knockdown inhibited miR-374a-5p expression; data are expressed as mean ± S.D. (N=3).

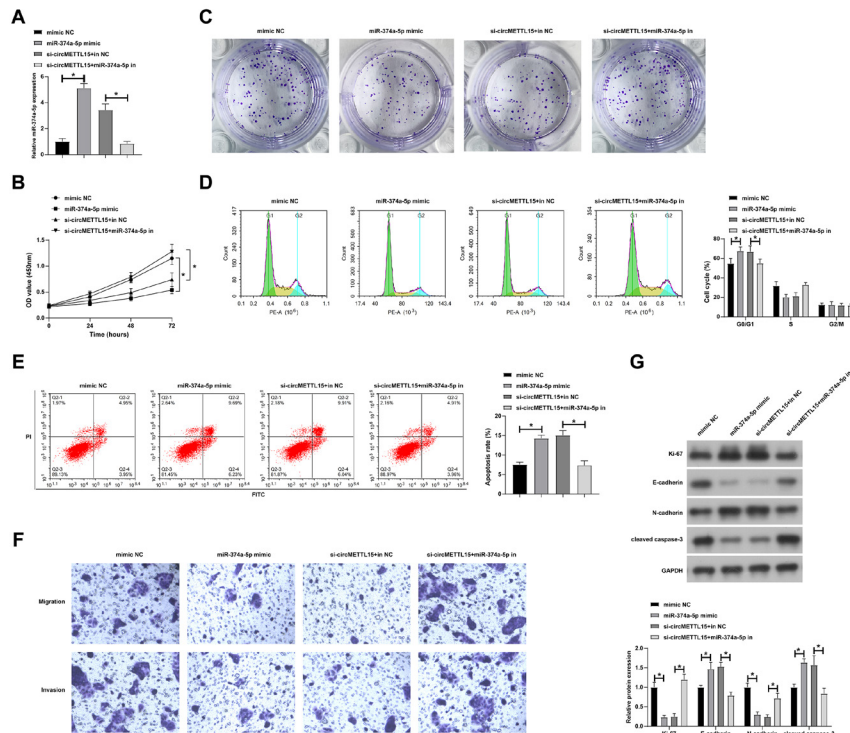
motes apoptosis, and increases the proportion of G0/G1 phase cells.

**circMETTL15 binds to miR-374a-5p**

Given that circRNAs primarily act as miRNA sponges in the cytoplasm, online databases (<https://starbase.sysu.edu.cn> and <https://circinteractome.nia.nih.gov>) predicted the possible miRNAs that bind to circMETTL15, and screened 8 kinds of overlapping of miRNAs (Fig. 3A). RIP experiments confirmed that three of the eight miRNAs were enriched with circMETTL15 (Fig. 3B), but only miR-374a-5p was downregulated in LoVo cells (Fig. 3C). Therefore, miR-374a-5p might be a downstream miRNA of circMETTL15. With their predicted potential binding sites (Fig. 3D), circMETTL15-WT and circMETTL15-MUT reporters were constructed, and their luciferase activity was observed by dual luciferase reporter assay. The results were consistent with the prediction, that is, circMETTL15 targets miR-374a-5p (Fig. 3E). RT-qPCR analyzed miR-374a-5p expression in patients' tissues and found its downregulation in CRC tissues (Fig. 3F). CircMETTL15 knockdown effectively increased miR-374a-5p expression in LoVo cells (Fig. 3G).

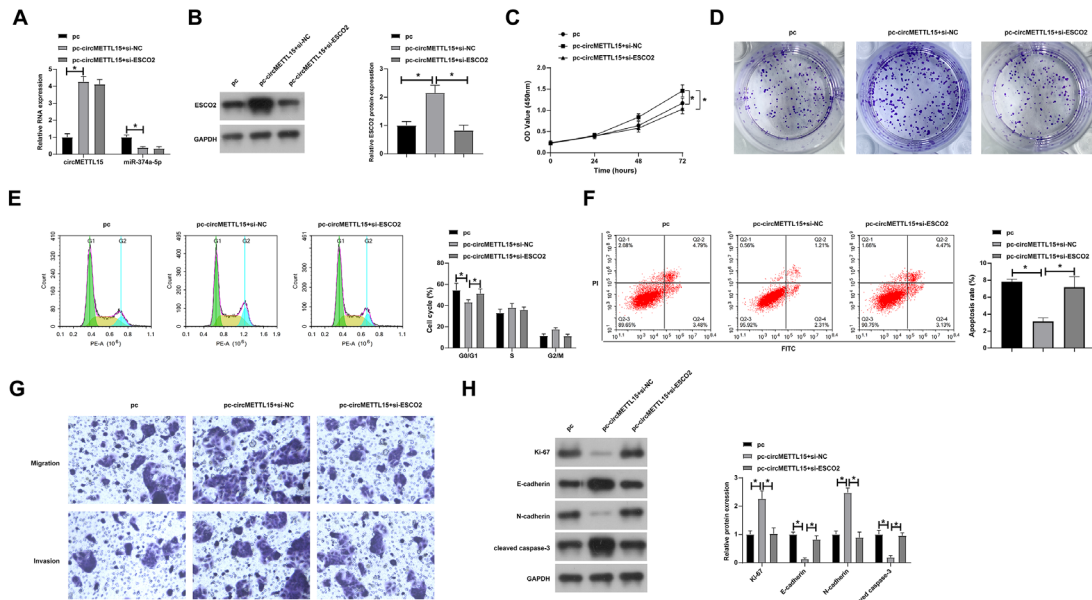
**The effects of circMETTL15 on CRC cell proliferation, invasion, migration, apoptosis, cell cycle, and EMT are reversed by the silencing of miR-374a-5p**

To explore whether miR-374a-5p is involved in the process of circMETTL15 regulating the biological behavior of LoVo cells, a functional rescue experiment was performed. Firstly, the expression changes of miR-374a-5p were evaluated by RT-qPCR. As shown in Fig. 4A, miR-374a-5p mimic and si-circMETTL15 both promoted miR-374a-5p expression, but miR-374a-5p inhibitor re-



**Figure 4. The effects of circMETTL15 on CRC cell proliferation, invasion, migration, apoptosis, cell cycle, and EMT are reversed by the silencing of miR-374a-5p** miR-374a-5p mimic/inhibitor and si-circMETTL15 were transfected into LoVo cells. (A) RT-qPCR to detect miR-374a-5p. (B) CCK-8 to detect cell proliferation rate. (C) Colony formation assay to detect cell proliferation. (D) Flow cytometry to detect cell cycle. (E) Flow cytometry to detect apoptosis. (F) Transwell assays to detect cell invasion and migration. (G) Western blot to evaluate Ki-67, E-cadherin, N-cadherin, and cleaved caspase-3 expression; data are expressed as mean ± S.D. (N=3).





**Figure 7.** circMETTL15 affects CRC cell proliferation, cell cycle, apoptosis, invasion, migration, and EMT processes by regulating miR-374a-5p/ESCO2 axis

pcDNA 3.1-circMETTL15 and si-ESCO2 were transfected into LoVo cells. (A) RT-qPCR to detect circMETTL15 and miR-374a-5p. (B) Western blot to detect ESCO2. (C) CCK-8 to detect cell proliferation rate. (D) Colony formation assay to detect cell proliferation. (E) Flow cytometry to detect cell cycle. (F) Flow cytometry to detect apoptosis. (G) Transwell assays to detect cell invasion and migration. (H) Western blot to evaluate Ki-67, E-cadherin, N-cadherin, and cleaved caspase-3 expression; data are expressed as mean  $\pm$  S.D. (N=3).

G0/G1 phase cells, and circMETTL15 affected the proliferation, apoptosis, cell cycle, invasion, migration, and EMT processes of CRC cells by regulating miR-374a-5p.

#### miR-374a-5p serves as a regulator of ESCO2 expression

To explore downstream targets regulated by miR-374a-5p, starbase, TargetScan and miRDB predicted seven overlapping candidate downstream genes (Fig. 5A). Three were identified to be enriched with miR-374a-5p by RIP screening (Fig. 5B), among which ESCO2 was found to be upregulated in CRC by TCGA database analysis (Fig. 5C). ESCO2 upregulation was also demonstrated in CRC patients and cell lines (Fig. 5D, E). Sub-

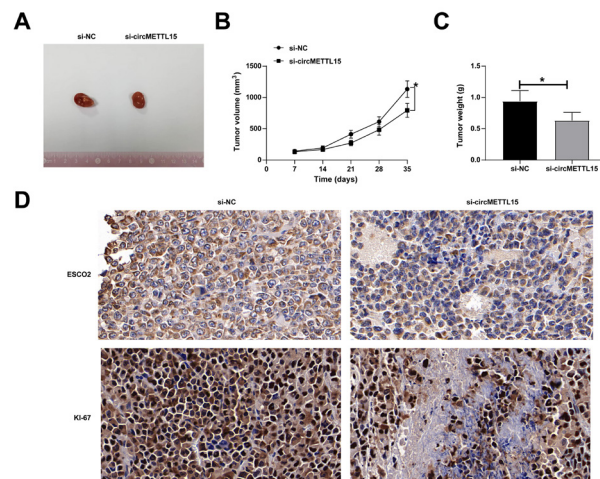
sequently, the targeting relationship between ESCO2 and miR-374a-5p was confirmed by dual luciferase reporter experiments (Fig. 5F, G). Furthermore, ESCO2 expression reduced accordingly when miR-374a-5p was overexpressed (Fig. 5H).

#### Knockdown of ESCO2 inhibits CRC cell proliferation, invasion, migration, and EMT and promotes apoptosis

Subsequently, the effects of ESCO2 on the biological behavior of LoVo cells were examined. Western blot assay showed that transfection of si-ESCO2 decreased ESCO2 expression in LoVo cells (Fig. 6A). CCK-8 and colony formation experiments showed that knockdown of ESCO2 inhibited cell proliferation rate and colony-forming ability (Fig. 6B, C). Flow cytometry showed that knockdown of ESCO2 resulted in an increased proportion of cells arrested in the G0/G1 phase (Fig. 6D) and resulted in increased apoptosis (Fig. 6E). Transwell assay showed that the invasion and migration abilities of cells were reduced after ESCO2 knockdown (Fig. 6F). Western blot analysis showed that ESCO2 inhibition decreased N-cadherin and Ki-67 expression and increased cleaved caspase-3 and E-cadherin expression (Fig. 6G). The above data indicated that knockdown of ESCO2 effectively inhibited CRC proliferation, invasion, migration, and EMT and promoted apoptosis.

#### circMETTL15 affects CRC cell proliferation, cell cycle, apoptosis, invasion, migration, and EMT processes by regulating miR-374a-5p/ESCO2 axis

To explore whether the miR-374a-5p/ESCO2 axis is involved in the process of circMETTL15 regulating the biological behavior of CRC cells, LoVo cells were subjected to a co-transfection design with pcDNA 3.1 overexpression vector targeting circMETTL15 and si-ESCO2. pcDNA 3.1 overexpression vector targeting circMETTL15 promoted circMETTL15 and ESCO2 expression



**Figure 8.** Knockdown of circMETTL15 inhibits CRC tumorigenesis *in vivo*

(A) Representative images of tumors. (B–C) Tumor volume and weight. (D) IHC staining to measure ESCO2 and Ki-67 expression in tumors; data are expressed as mean  $\pm$  S.D. (n=5).

and suppressed miR-374a-5p expression, while si-ESCO2 made ESCO2 expression partially recovered (Fig. 7A, B). CCK-8 and colony formation assays showed that overexpression of circMETTL15 promoted cell proliferation rate and cell cloning ability, but knockdown of ESCO2 reversed this phenomenon (Fig. 7C, D). Flow cytometry showed that overexpression of circMETTL15 reduced the proportion of cells arrested in G0/G1 phase, while knockdown of ESCO2 increased the proportion of G0/G1 phase cells (FIG. 7E). Annexin V-FITC/PI apoptosis kit tests showed that the overexpression of circMETTL15 reduced apoptosis rate, while knockdown of ESCO2 reversed this effect (Fig. 7F). Transwell assay showed that overexpression of circMETTL15 enhanced cell invasion and migration, but knockdown of ESCO2 reduced the number of invasion and migration cells (Fig. 7G). In addition, overexpression of circMETTL15 promoted expression of N-cadherin and Ki-67 and inhibited expression of cleaved caspase-3 and E-cadherin, but knockdown of ESCO2 prevented changes in these proteins (Fig. 7H). These data suggest that circMETTL15 promotes LoVo cell proliferation, invasion, migration, and EMT and reduces apoptosis by regulating the miR-374a-5p/ESCO2 axis.

#### Knockdown of circMETTL15 inhibits CRC tumorigenesis *in vivo*

At last, LoVo cells stably knocking down circMETTL15 were implanted into nude mice to explore the effect of circMETTL15 on the growth of CRC tumors *in vivo*. CircMETTL15 depletion suppressed tumor volume and weight (Fig. 8A–C). IHC staining showed that knockdown of circMETTL15 decreased ESCO2 expression and Ki-67 expression in tumors (Fig. 8D).

#### DISCUSSION

Late detection is the main reason for annual mortality in CRC (Ameli-Mojarad *et al.*, 2021), therefore, the search for an optimal biomarker for disease diagnosis and prognosis is necessary for identifying and treating CRC cases. Molecular biology and genomics have emphasized the significance of non-coding RNAs in tumor development (Chu *et al.*, 2021). Therefore, we attempted to investigate the effects of circMETTL15 involved in CRC, as well as related molecular mechanisms. Further experiments demonstrated that silence of circMETTL15 by suppressing miR-374a-5p and promoting ESCO2 expression could effectively inhibit the malignant behavior of CRC.

circRNAs are more suitable as new biomarkers for CRC and have potential clinical value in the early diagnosis and prognostic assessments of CRC (C. Lin *et al.*, 2022; Radanova *et al.*, 2021). For example, circ\_001659 has a good predictive value and probability ratio for the diagnosis of CRC and confers pro-tumor influence on CRC development (He *et al.*, 2021) and circMYLK is associated with poor prognosis and some unfavorable clinical features of CRC patients (Huang & Dai, 2021). Similarly, our findings suggested that a higher abundance of circMETTL15 was correlated with tumor size, TNM stage, lymph node metastasis, and poor overall survival. The results indicated that circMETTL15 may be a prognostic or diagnostic biomarker in CRC, which needs to be verified by detecting circMETTL15 expression in CRC serum in subsequent studies. In a recent study, circMETTL15 is highly expressed in lung cancer and regulates malignant progression and immune escape

(Zhang *et al.*, 2022). The results of this study further support the role of circMETTL15 as a cancer-promoting factor, which promotes ESCO2 expression by binding miR-374a-5p, thus promoting CRC development. The study speculates that circMETTL15 also has the function of regulating immune checkpoint in CRC, and it may be feasible to employ circMETTL15 as a new target for immunotherapy, but this needs to be explored in subsequent studies.

miRNAs have been widely recognized as key regulators in cancers (Yamamoto & Mori, 2016). According to the ceRNA network, circRNAs can bind to miRNAs, thereby reducing the biological functions of miRNAs (Jorgensen & Ro, 2022). The study predicted and then verified that miR-374a-5p was a downstream miRNA of circMETTL15 in CRC. miR-374a-5p is considered a tumor oncogenic factor in ovarian cancer (Hao, Huang, & Han, 2020), triple-negative breast cancer (Son *et al.*, 2019), gastric cancer (Ji *et al.*, 2019), whereas a tumor suppressor in non-small cell lung cancer (Guo *et al.*, 2021) and esophageal squamous cell carcinoma (Chen *et al.*, 2019). Notably, it is recorded that miR-374a-5p is downregulated in CRC and is related to patients' survival rate (Slattery *et al.*, 2017; Slattery *et al.*, 2015). The study continuously explored miR-374a-5p in CRC and, as expected, miR-374a-5p could suppress the malignant phenotype of CRC cells while miR-374a-5p downregulation rescued the effect of circMETTL15 silencing on the biological behavior of CRC cells.

For this study, the downstream target (ESCO2) of miR-374a-5p was screened, which was upregulated in CRC. ESCO2, a member of the Eco1 family, contributes to sister chromatid cohesion during cell cycle progression (Wang & Liu, 2020). It is well known that different states of cohesin are associated with different phases of the cell cycle (Alomer *et al.*, 2017) and ESCO2 is involved in cohesion-mediated DNA repair (Rahman, Jones, & Jallepalli, 2015). Because of this, ESCO2 has received increasing attention in cancer development. It is noted that ESCO2 promotes lung adenocarcinoma (Zhu *et al.*, 2021) and gastric cancer (Chen *et al.*, 2018). Notably, ESCO2 is recently reported as an epigenetic regulator to suppress tumor metastasis in CRC (Guo *et al.*, 2018). Based on this, a functional rescue experiment was performed to reveal that ESCO2 knockdown rescued the promotion of circMETTL15 overexpression on the malignant progression of CRC cells.

However, how ESCO2 interacts with other epigenetic regulators or pathways to influence the development and progression of CRC has not been thoroughly investigated. In the follow-up experiments, we should further carry out multi-center trials and animal experiments to clarify the therapeutic effect and mechanism of circMETTL15 on CRC. In addition, multicenter trials and animal experiments are needed to elucidate the role of circMETTL15 in CRC.

#### CONCLUSION

In conclusion, this study suggests that circMETTL15 sponges miR-374a-5p to regulate ESCO2 and promote CRC malignant behavior. These data provide new molecular targets for the development of CRC-targeted drugs and future combination therapies. However, the results of this study have so far only been verified in LoVo cells and animal experiments. Therefore, it is not suitable for clinical trials. More basic experiments (such as validation in more CRC cell lines) are needed in the



future to further explore the influence of the circMETTL15/miR-374a-5p/ESCO2 axis on CRC biological behavior.

## Declarations

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**Declaration of Conflicting Interests.** Authors declared no conflict of interest.

**Ethical approval.** All procedures performed in this study involving human participants were in accordance with the ethical standards of the Renji Hospital, Shanghai Jiaotong University School of Medicine research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All subjects were approved by Renji Hospital, Shanghai Jiaotong University School of Medicine.

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