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Homo Sapiens Circular RNA 0079993 (hsa_circ_0079993) of the POLR2J4 Gene Acts as an Oncogene in Colorectal Cancer Through the microRNA-203a-3p.1 and CREB1 Axis

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Background: Worldwide, dietary changes have resulted in an increased incidence of colorectal cancer (CRC). Circular RNAs (circRNAs) are involved in tumorigenesis of several human tumors, but their role in CRC remains unknown. This study aimed to investigate the expression and effects of *Homo sapiens* (hsa)_circ_0079993 of POLR2J4 and its impact on CRC.

Material/Methods: Paired CRC tissue and adjacent normal colorectal tissue samples (N=41), and HCT116 and SW620 human CRC cells were studied. The expression of circ_0079993 and its parental gene, POLR2J4, were examined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Two small-interfering RNAs (siRNAs) against circ_0079993 were used to silence circ_0079993 expression in HCT116 and SW620 CRC cells. Cell proliferation was evaluated using the cell counting kit-8 (CCK-8) assay, colony formation, and *in vivo* tumor growth assays. The target miRNAs of circ_0079993 was predicted using TargetScan, and the interaction between circ_0079993 and its target miRNAs were verified by the dual-luciferase reporter (DLR) assay.

Results: In CRC tissue POLR2J4 expression was reduced, and circ_0079993 expression was increased compared with normal tissue. Knockdown of circ_0079993 significantly inhibited the proliferation of CRC cells *in vitro*. Also, circ_0079993 was predicted to sponge multiple miRNAs, miR-203a-3p.1 was verified as a target of circ_0079993, and circ_0079993 indirectly regulated mRNA expression of the CREB1 gene by sponging miR-203a-3p.1 in CRC cells. The use of anti-miR-203a-3p.1 reversed the inhibitory effects of circ_0079993 knockdown on CRC cell proliferation.

Conclusions: The findings supported that hsa_circ_0079993 acts as an oncogene in CRC through the miRNA-203a-3p.1/CREB1 axis.

MeSH Keywords: **Calcium-Calmodulin-Dependent Protein Kinase Type 1 • Colonic Neoplasms • MicroRNAs**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/916064>

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Background

Worldwide, colorectal cancer (CRC) affects millions of people and has a high morbidity and mortality rate [1]. The American Cancer Society (ACS) estimated that approximately 101,420 new cases of CRC would be diagnosed by the year 2019 in the US, with an estimated mortality of approximately 51,020 people [2]. In China, CRC is also considered to be a leading type of cancer [3]. Standard treatment for early-stage CRC includes surgical resection, chemotherapy, and radiotherapy. However, due to the complexity of the pathogenesis of CRC, patient prognosis has remained unchanged in the past decades [4]. The five-year survival rate of patients with advanced-stage CRC with metastatic disease is as low as 10% [3]. The majority of patients with advanced-stage CRC die of metastases and tumor recurrence after surgery [1,5]. Therefore, there remains an urgent need to continue to explore potential new treatment approaches for advanced-stage CRC.

It has been shown that less than 2% of the human genome encodes protein, and approximately 98% of the human transcriptome has no protein-encoding capacity, termed non-coding RNAs (ncRNAs) [6]. Due to the lack of protein-encoding ability, ncRNAs were once considered to be 'noise' during genomic transcription [7]. However, with advances in molecular research, ncRNAs have been shown to participate in several physiological processes, and abnormal expression of ncRNAs has been identified in human disease, including cancer, cardiovascular disease, and neurodegenerative disorders [8–10].

MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs), characterized by approximately 22 nucleotides (nt) and more than 200 nt, respectively, are the two main linear ncRNAs [11]. Previous studies have shown that miRNAs and lncRNAs are involved in tumorigenesis in several human cancers, including CRC, breast cancer, and lung cancer, by modulating mRNA expression of oncogenes or tumor inhibitory genes [12–15]. Unlike miRNAs and lncRNAs, circular RNA (circRNA) is a novel ncRNA with circular structure and without the 5' cap and 3' poly-A tail [16]. Recently, circRNA was also shown to be associated with tumorigenesis in several human cancers, including lung cancer, liver cancer, and oral cancer [17]. However, the roles and underlying molecular mechanisms of circRNAs in CRC remain unknown.

Therefore, this study aimed to investigate the expression and effects of *Homo sapiens* (hsa)_circ_0079993 of POLR2J4 and its effects in CRC tissues and cell lines.

Material and Methods

Colorectal cancer (CRC) tissue samples and cell lines

A total of 41 paired samples of colorectal cancer (CRC) tissue and adjacent normal colorectal tissue were obtained from patients who were diagnosed with CRC at Renmin Hospital of Wuhan University from 2013 to 2018. Written informed consent was provided by all the patients who participated in the study. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. The normal colonic cell line, NCM460, and five human CRC cell lines HT29, SW480, DLD-1, HCT-116, and SW620 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin and incubated in an atmosphere containing 5% CO₂ and 95% air at 37°C.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs of CRC tissue samples and cell lines were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After determining the quality of the RNA with NanoDro2000c (Thermo Scientific, Waltham, MA, USA), 2 µg of extracted RNAs were used as the template to synthesis cDNA using the Bestar™ qRT-PCR kit (#2220) (DBI Bioscience, Ludwigshafen, Germany). RT-PCR was performed using Bestar™ qPCR MasterMix (#2043) (DBI Bioscience, Ludwigshafen, Germany) on an ABI 7500 system (ABI Biosystems, Foster City, CA, USA). The primer sequences used in this study are shown in Table 1. The expression of circular RNA 0079993 (circ_0079993), POLR2J4, and CREB1 were normalized to GAPDH, and gene expression was quantified using the 2^{-ΔΔCt} method.

RNA transfection

Two small-interfering RNAs (siRNAs) against circ_0079993 (si-Circ#1 and si-Circ#2), mimics of miR-203a-3p.1, miR-450b-3p, miR-502-5p, and miR-1224-3p, and anti-miR-203a-3p.1, and the corresponding negative control (NC) for si-Circ#1/si-Circ#2 (si-NC) and miR-203a-3p.1 (miR-NC), were designed and obtained from GenePharma (Shanghai, China). All cell transfections were performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Cell counting kit-8 (CCK-8) assay

Cell viability of treated CRC cells was evaluated using the CCK-8 assay (Dojindo Molecular Technologies, Inc., Rockville, MD, USA), according to the manufacturer's instructions. Briefly,

Table 1. The primer sequences in this study.

Gene	Primer sequences
GAPDH	Forward: 5'-TATGATGATATCAAGAGGGTAGT-3' Reverse: 5'-TGTATCCAAACTCATTGTCATAC-3'
Hsa_circ_0079993	Forward: 5'-AAGATCGAGACTGCCCTC-3' Reverse: 5'-AGTGGTTGCTGCCTCCTAT-3'
POLR2J4	Forward: 5'-AACATCAGGGGAGTTGGGAG-3' Reverse: 5'-CTTGCACTTGGGCTTAGCA-3'
CREB1	Forward: 5'-CTGCCTCTGGAGACGTACAA-3' Reverse: 5'-CAAGCACTGCCACTCTGTTT-3'

treated CRC cells were collected and seeded into 96-well plates at a density of 2×10^4 cells/well, and cultured at 37°C for 24 h. Then, 10 μ l of CCK-8 solution (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to each well and incubated at 37°C for a further 10 min. The absorbance values were detected at 450 nm.

Colony formation assay

Cell proliferation of treated CRC cells was assessed using the colony formation assay. Briefly, treated CRC cells were plated into six-well plates at a concentration of 2,000 cells/well. Cells were cultured at 37°C for 14 days, followed by cell fixation using 4% paraformaldehyde and stained with Giemsa solution. The number of colonies was counted using microscopy.

In vivo assay of tumor growth

In vivo tumor growth was assessed in 7-week-old male BALB/c mice. All animal studies were approved by the Institutional Animal Care and Use Committee of the Renmin Hospital of Wuhan University. Briefly, HCT116 cells that were stably transfected with small-interfering RNAs (siRNAs), si-NC or si-Circ#1+#2 were harvested and re-suspended with culture medium (1×10^5 cells/ml). Then, 200 μ l of HCT116 cell suspensions were subcutaneously injected into the left flank of the mice. Tumor volume was examined every 3 days until day 36 after cell inoculation. Tumor volumes were calculated from the measurements of length \times width² \times 0.5.

Dual-luciferase reporter assay

To verify the interaction between circ_0079993 and miR-203a-3p.1 in HCT116 and SW620 cells, the wild type (WT) and mutant (Mut) fragments of circ_0079993 containing putative miR-203a-3p.1 binding sites were amplified and inserted into the pGL3 vector (Promega, Madison, WI, USA) to form circ_0079993-WT and circ_0079993-Mut recombinant plasmids. Then, HCT116 and SW620 cells (1×10^4 cells/well) were plated into 96-well plates and maintained at 37°C overnight, followed by the

co-transfection of miR-203a-3p.1 mimics and circ_0079993-WT or circ_0079993-Mut. The Firefly and Renilla luciferase activities of treated HCT116 and SW620 cells were detected with the Dual-Luciferase Assay System (Promega, Madison, WI, USA), and Renilla luciferase activity was normalized to the Firefly luciferase activity. The interaction between circ_0079993 and microRNAs, miR-450b-3p, miR-502-5p, miR-587 or miR-1224-3p, as well as CREB1 and miR-203a-3p.1, were also verified to be the same as circ_0079993 and miR-203a-3p.1.

DAVID online Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

The miRNAs with putative target sites with circ_0079993 were predicted using TargetScan 7.1 (http://www.targetscan.org/vert_71/). Biological pathways of the circ_0079993 target miRNAs underwent DAVID gene-annotation enrichment analysis (<https://david.ncifcrf.gov>).

Statistical analysis

Data were presented as the mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed using GraphPad Prism version 7 (GraphPad Prism Software, La Jolla, CA, USA) to compare the difference between groups. A P-value <0.05 was considered to be statistically significant.

Results

Homo sapiens circular RNA 0079993 (hsa_circ_0079993) of the POLR2J4 was highly expressed in colorectal cancer (CRC) and was correlated with poor prognosis

Analysis of the genomic location and formation of circ_0079993 showed that circ_0079993 was the circularized product of the mRNA of POLR2J4, and its circular structure was verified by Sanger sequencing (Figure 1A). Analysis was undertaken of the expression of POLR2J4 in 275 samples of colorectal cancer (CRC) and 349 corresponding normal tissue samples based on The Cancer Genome Atlas (TCGA) data from the Gene Expression Profiling Interactive Analysis (GEPIA) website (<https://bio.tools/gepia>). The results showed that POLR2J4 expression was significantly lower in the CRC samples compared with the normal samples (Figure 1B). In the overall survival (OS) analysis, patients with CRC with high POLR2J4 expression had a worse prognosis than patients with low POLR2J4 expression (Figure 1C). These findings suggested that POLR2J4, the parental gene of circ_0079993, might play a role in the tumorigenesis of CRC.

To further investigate the role of circ_0079993 in CRC, its expression was studied by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 41 paired samples

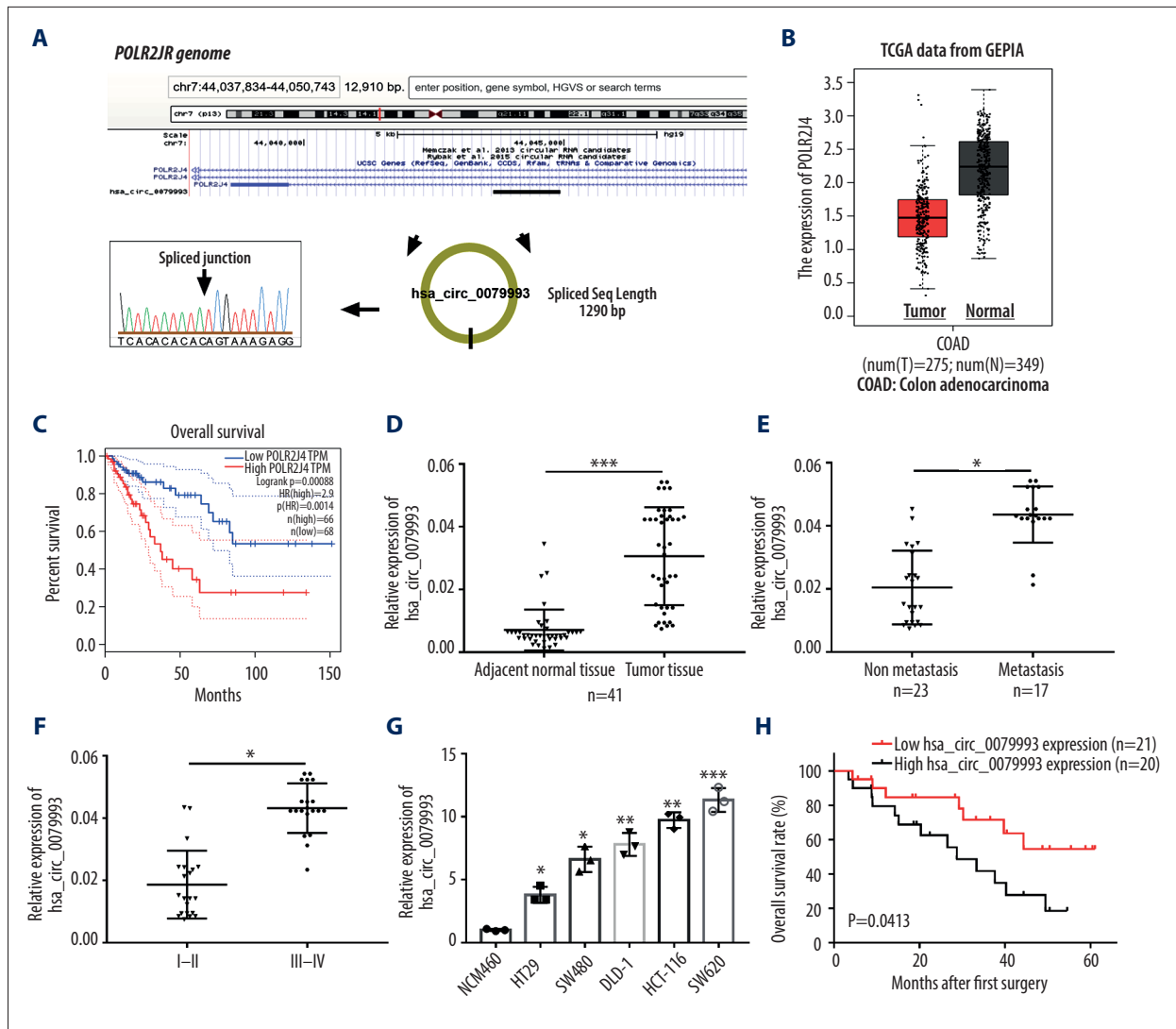


Figure 1. *Homo sapiens* circular RNA 0079993 (hsa_circ_0079993) was highly expressed in colorectal cancer (CRC) and was associated with poor prognosis. **(A)** Schematic diagram of the genomic location and formation of circ_0079993. Sanger sequencing was used to confirm its circular structure. **(B)** Expression of circ_0079993 was analyzed based on The Cancer Genome Atlas (TCGA) data from the Gene Expression Profiling Interactive Analysis (GEPIA) website. **(C)** Overall survival (OS) of patients with CRC with low or high POLR2J4 expression. **(D)** Expression of circ_0079993 was detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in 41 pairs of CRC tissues and adjacent normal tissues (***) $P < 0.001$. **(E)** Expression of circ_0079993 in metastatic CRC tissues (n=17) and non-metastasis tissues (n=23) (* $P < 0.05$). **(F)** Expression of circ_0079993 in CRC tissues with different clinical stages (* $P < 0.05$). **(G)** Expression of circ_0079993 was examined in five CRC cell lines (HT29, SW480, DLD-1, HCT-116, and SW620) and a normal colonic cell line (NCM460) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **(H)** Overall survival (OS) of patients with CRC with low or high expression of circ_0079993.

of CRC and adjacent normal colorectal tissues. The results showed that circ_0079993 expression was significantly up-regulated in CRC tissues compared with adjacent normal tissues (Figure 1D). Compared with the non-metastatic CRC tissues, circ_0079993 expression was significantly increased in metastatic tissues (Figure 1E). Also, circ_0079993 expression in advanced-stage CRC (stage III/IV) was greater than in early-stage CRC (stage I/II) (Figure 1F).

Evaluation of circ_0079993 expression in CRC cell lines showed that when compared with the normal colonic cell line, NCM460, circ_0079993 expression was significantly upregulated in the five CRC cell lines, HT29, SW480, DLD-1, HCT-116, and SW620. The HCT-116 and SW620 cells showed the highest circ_0079993 expression (Figure 1G). Also, patients with CRC with high circ_0079993 expression showed significantly reduced overall survival (OS) rate when compared with patients

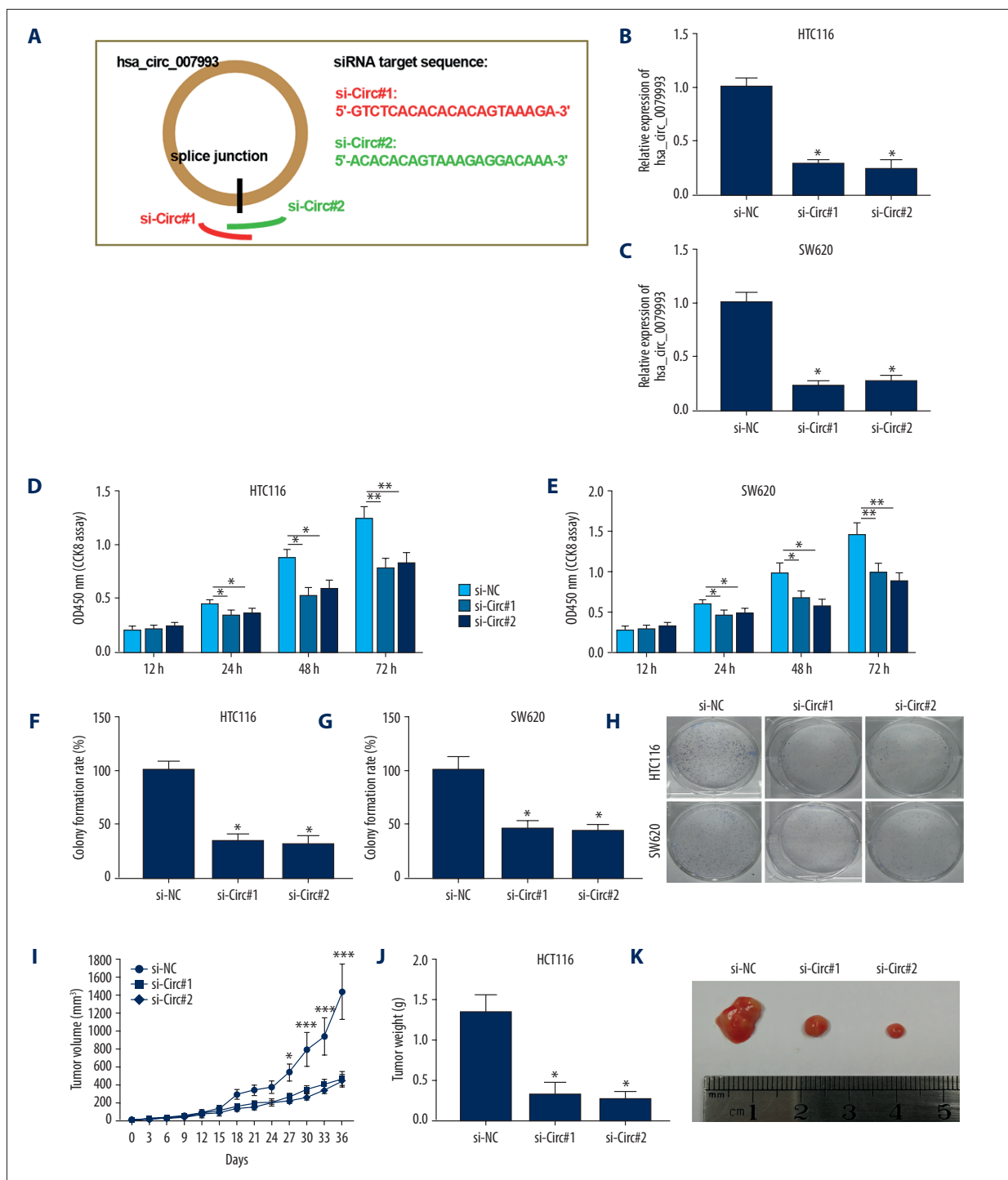


Figure 2. *Homo sapiens* circular RNA 0079993 (hsa_circ_0079993) knockdown inhibited colorectal cancer (CRC) cell growth *in vitro* and *in vivo*. (A) The sequence and target location of circ_0079993 small-interfering RNAs (siRNAs) (si-Circ#1, si-Circ#2). (B, C) Knockdown efficiency of circ_0079993 was examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in HCT116 and SW620 CRC cells (* P<0.05). (D, E) Effects of circ_0079993 knockdown on cell viability of HCT116 and SW620 cells was evaluated by the cell counting kit-8 (CCK-8) assay at 12, 24, 48, and 72 h after siRNAs transfection (* P<0.05, ** P<0.01). (F-H) Colony formation assay was performed to detect the effects of circ_0079993 knockdown on cell proliferation (* P<0.05). (I-K) *In vivo* tumor growth assay was performed to assess the effects of circ_0079993 knockdown on tumor growth (* P<0.05, *** P<0.001).

Table 2. The predicted microRNAs that interacted with hsa_circ_0079993 identified by the TargetScan algorithm.

miRBase ID	Scores (site 1)	Scores (site 2)	miRBase ID	Scores (site 1)	Scores (site 2)
hsa-miR-203	99	n/a	hsa-miR-625	85	n/a
hsa-miR-1224-3p	95	n/a	hsa-miR-1182	83	n/a
hsa-miR-769-3p	95	84	hsa-miR-615-3p	82	n/a
hsa-miR-502-5p	95	n/a	hsa-miR-663b	82	n/a
hsa-miR-431	94	n/a	hsa-miR-136	81	n/a
hsa-miR-450b-3p	94	83	hsa-miR-661	81	n/a
hsa-miR-149	93	66	hsa-miR-1257	80	n/a
hsa-miR-599	92	n/a	hsa-miR-1278	80	n/a
hsa-miR-874	92	n/a	hsa-miR-579	77	91
hsa-miR-1236	91	n/a	hsa-miR-1200	75	n/a
hsa-miR-411	91	n/a	hsa-miR-767-3p	75	n/a
hsa-miR-548k	91	n/a	hsa-miR-330-5p	74	n/a
hsa-miR-614	91	n/a	hsa-miR-646	73	n/a
hsa-miR-146b-3p	90	n/a	hsa-miR-324-5p	72	n/a
hsa-miR-526b	90	n/a	hsa-miR-513a-5p	72	n/a
hsa-miR-576-3p	88	n/a	hsa-miR-1238	70	n/a
hsa-miR-1184	87	n/a	hsa-miR-638	70	70
hsa-miR-498	87	n/a	hsa-miR-942	68	n/a
hsa-miR-520g	86	n/a	hsa-miR-326	66	n/a
hsa-miR-520h	86	n/a	hsa-miR-516a-5p	47	n/a
hsa-miR-758	86	n/a	hsa-miR-622	46	n/a
hsa-miR-924	86	91	hsa-miR-127-5p	41	n/a

with low circ_0079993 expression (Figure 1H). These results suggested that circ_0079993 might have a role in the pathogenesis of CRC.

Circ_0079993 knockdown inhibited CRC cell growth *in vitro* and *in vivo*

To assess the biological functions of circ_0079993 in CRC, we examined the effects of circ_0079993 knockdown on proliferation of CRC cells *in vitro* and *in vivo*. Two specific small-interfering RNAs (siRNAs) against circ_0079993 (si-Circ#1, si-Circ#2) were designed and synthesized to knockdown the expression of circ_0079993 in HCT116 and SW620 cells (Figure 2A). The knockdown efficiency of si-Circ#1 and si-Circ#2 were evaluated by qRT-PCR in HCT116 and SW620 cells (Figure 2B, 2C). The results from the CCK-8 assay showed significant inhibition of cell viability of HCT116 and SW620 cells transfected with si-Circ#1 and si-Circ#2 when compared with cells transfected with negative control siRNA (si-NC) (Figure 2D, 2E). In the colony formation assay, the number of colonies was significantly reduced in the HCT116 and SW620 cells transfected

with si-Circ#1 and si-Circ#2 when compared with the si-NC group (Figure 2F–2H). The effects of circ_0079993 knockdown on tumor growth were assessed using an *in vivo* tumor growth assay. HCT116 cells stably transfected with si-Circ#1, si-Circ#2, or si-NC were inoculated subcutaneously into the left flank of nude mice. All mice developed xenograft tumors at the injection site, and tumor volume and weight were measured. Compared with the si-NC group, the tumor volume and weight were significantly reduced in the si-Circ#1 and si-Circ#2 groups (Figure 2I–2K).

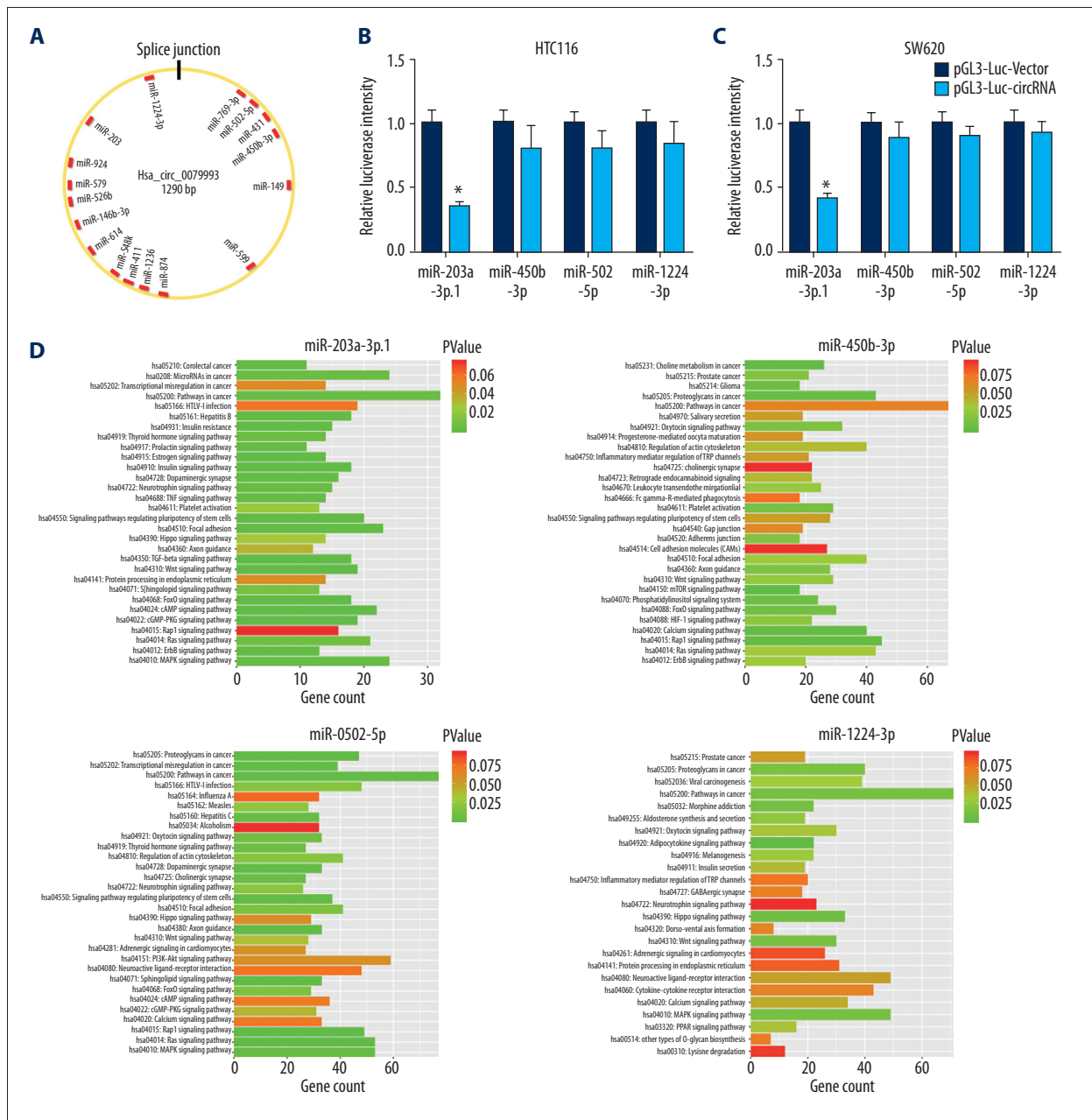
Circ_0079993 sponged to multiple miRNAs in CRC cells.

The target miRNAs of circ_0079993 were predicted using TargetScan version 7.2. The findings showed that circ_0079993 interacted with multiple miRNAs (Table 2, Figure 3A). We selected four miRNAs, miR-203a-3p, miR-450b-3p, miR-502-5p, and miR-1224-3p for further study. In the dual-luciferase reporter assay, of the four miRNAs, miR-203a-3p reduced the luciferase activity of HCT116 and SW620 cells driven by pGL3-Luc-circ_0079993 (Figure 3B, 3C). Analysis of the enrichment

pathways of miR-203a-3p.1, miR-450b-3p, miR-502-5p, and miR-1224-3p was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure 3D). We also constructed the circRNA-miRNA-mRNA network of circ_0079993 within its four target miRNAs and found that the mRNA participated in multiple signaling pathways, including the adipocytokine signaling pathway, the cGMP-PKG signaling pathway, the mTOR signaling pathway, and the Ras signaling pathway (Figure 3E).

Circ_0079993 regulated the expression of CREB1, a target gene of miR-203a-3p.1, by sponging miR-203a-3p.1.

In the circRNA-miRNA-mRNA network of circ_0079993/miR-203a-3p.1, CREB1 was identified as a target gene of miR-203a-3p.1. Therefore, we then investigate the association between CREB1 and circ_0079993/miR-203a-3p.1. Relative circ_0079993 and CREB1 expression were detected in the miR-203a-3p.1 mimic treated HCT116 and SW620 cells using qRT-PCR. Compared with the NC mimics group, circ_0079993 expression in HCT116 and SW620 cells was not affected by miR-203a-3p.1 (Figure 4A). However, CREB1 expression of HCT116 and SW620 cells was



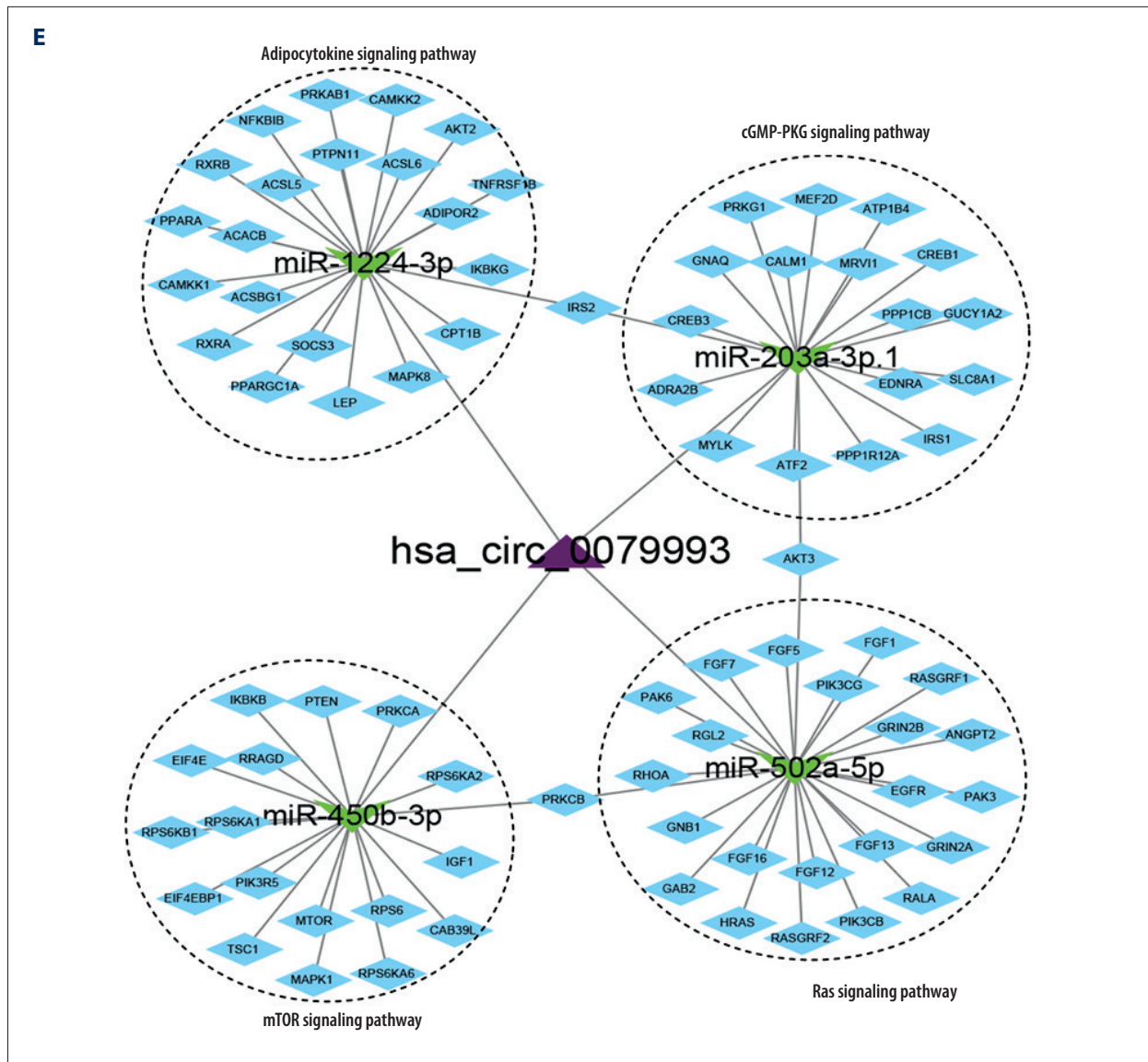


Figure 3. *Homo sapiens* circular RNA 0079993 (*hsa_circ_0079993*) sponged to multiple microRNAs (miRNAs) in colorectal cancer (CRC) cells. (A) Target miRNAs of *circ_0079993* predicted by TargetScan version 7.2. (B, C) Interaction between *circ_0079993* and miR-203a-3p.1, miR-450b-3p, miR-502-5p, or miR-1224-3p was verified by dual-luciferase reporter assay in HCT116 and SW620 cells (* $P < 0.05$). (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of *circ_0079993* targets four miRNAs (miR-203a-3p.1, miR-450b-3p, miR-502-5p, and miR-1224-3p). (E) The four key functional modules of the circRNA-miRNA-mRNA network, the adipocytokine signaling pathway, the cGMP-PKG signaling pathway, the mTOR signaling pathway, and the Ras signaling pathway.

significantly downregulated after transfection with miR-203a-3p.1 (Figure 4B). In the dual-luciferase reporter assay for miR-203a-3p.1 and *circ_0079993*, we found that the luciferase activity of HCT116 and SW620 cells was reduced by co-transfecting the cells with miR-203a-3p.1 and *circ_0079993*-WT (Figure 4C, 4D).

In the dual-luciferase reporter assay for miR-203a-3p.1 and CREB1, the luciferase activity of HCT116 and SW620 cells could only be reduced by co-transfecting the cells with miR-203a-3p.1

and CREB1-WT (Figure 4E, 4F). Also, miR-203a-3p.1 reduced the luciferase activity of HCT116 and SW620 cells transfected with pGL3-Luc-WT-CREB1-3'UTR. However, this effect could be blocked by the presence of *circ_0079993* (Figure 4G). The expression of CREB1 was significantly decreased in miR-203a-3p.1 treated HCT116 and SW620 cells, and the downregulation induced by miR-203a-3p.1 of CREB1 was blocked by *circ_0079993* (Figure 4H). These findings showed that *circ_0079993* could regulate the expression of CREB1 by sponging miR-203a-3p.1.

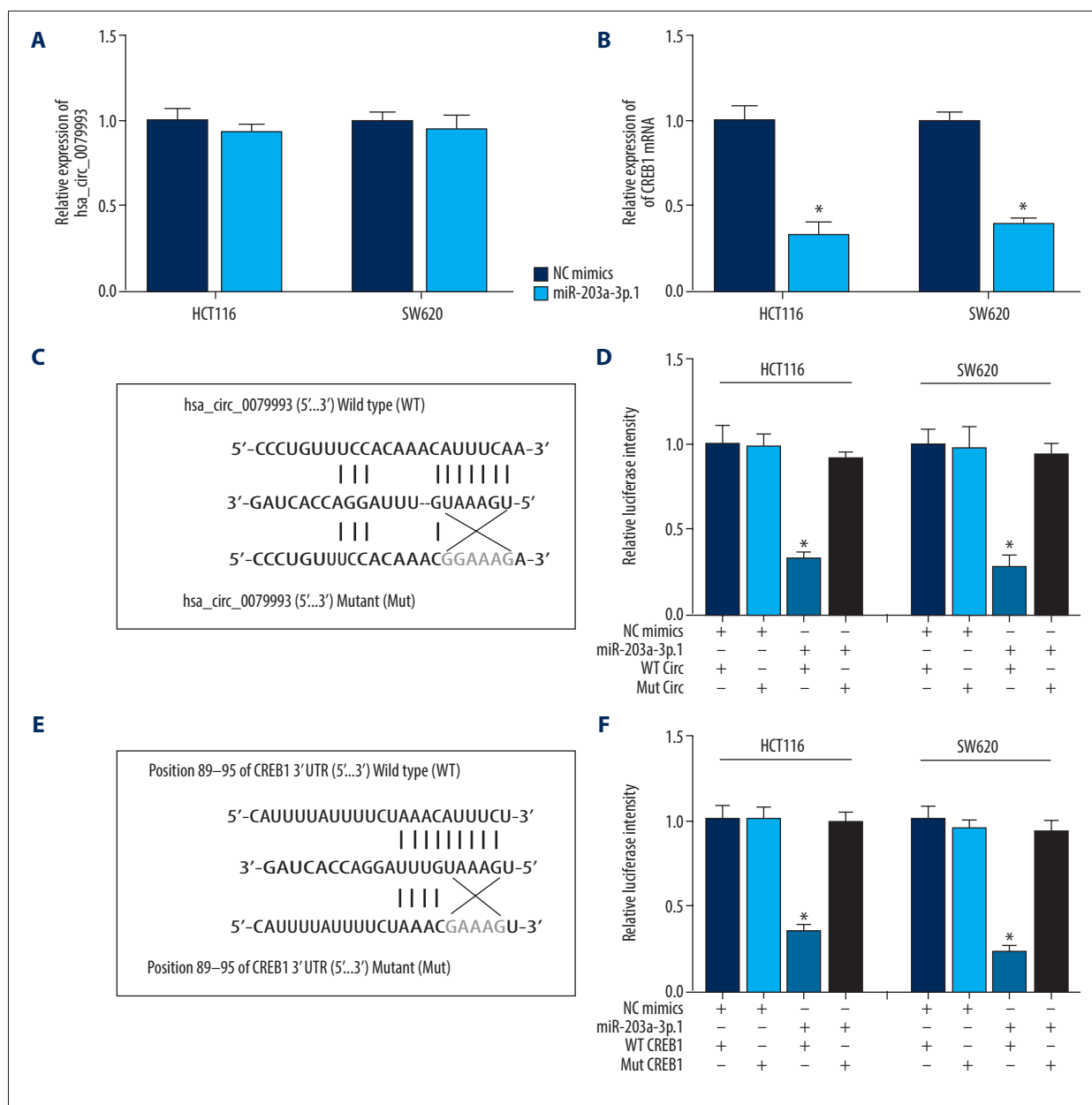
Anti-miR-203a-3p.1 reversed the inhibitory effects of circ_0079993 knockdown on the proliferation of CRC cells.

Circ_0079993 was shown to act as a miRNA sponge for miR-203a-3p.1 in CRC cells. To determine whether miR-203a-3p.1 was involved in the biological effects of circ_0079993 in CRC, cell proliferation was assessed by the CCK-8 and colony formation assays when circ_0079993 was silenced in HCT116 and SW620 cells followed by treatment with anti-miR-203a-3p.1. The results from the CCK-8 assay showed that the inhibitory effects of si-Circ#1+#2 on cell viability of HCT116 and SW620 cells were reversed by anti-miR-203a-3p.1 (Figure 5A, 5B). Also, the findings from the colony formation assay showed

that the inhibitory effects of si-Circ#1+#2 on colony formation of HCT116 and SW620 CRC cells were also abolished by miR-203a-3p.1 (Figure 5C, 5D). These findings indicated that circ_0079993 derived from POLR2J4 might be involved in the progression of CRC by releasing CREB1 from miR-203a-3p.1 and sponging miR-203a-3p.1 (Figure 5E).

Discussion

Due to its widespread expression in human tissues and organs and complex biological functions, circular RNAs (circRNAs) have recently become an area of increasing research interest.



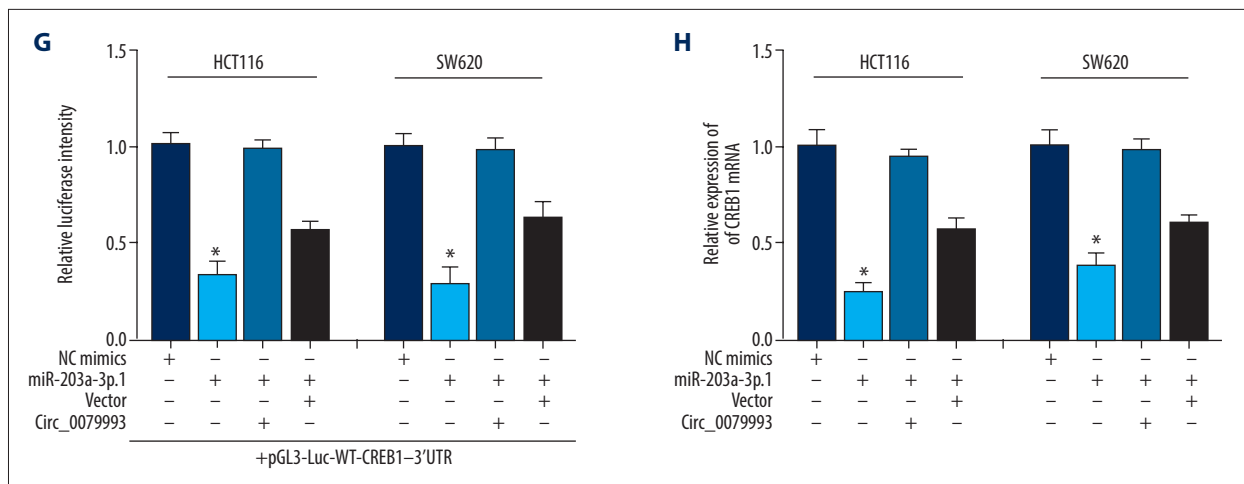


Figure 4. *Homo sapiens* circular RNA 0079993 (*hsa_circ_0079993*) regulated the expression of CREB1, a target gene of miR-203a-3p.1, by sponging miR-203a-3p.1. (A) Relative circ_0079993 expression and (B) relative mRNA expression of CREB1 in HCT116 and SW620 cells transfected with miR-203a-3p.1 and its mimics were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (* $P < 0.05$). (C, D) Interaction between circ_0079993 and miR-203a-3p.1 in HCT116 and SW620 cells was verified by the dual-luciferase reporter assay (* $P < 0.05$). (E, F) Interaction between miR-203a-3p.1 and CREB1 in HCT116 and SW620 cells was verified by dual-luciferase reporter assay (* $P < 0.05$). (G) Effects of miR-203a-3p.1 and circ_0079993 on the relative luciferase intensity of HCT116 and SW620 cells transfected with pGL3-Luc-WT-CREB1-3'UTR were detected by dual-luciferase reporter assay (* $P < 0.05$). (H) Relative mRNA expression of CREB1 in miR-203a-3p.1 or circ_0079993 treated HCT116 and SW620 cells were measured by qRT-PCR (* $P < 0.05$).

Previous studies have shown that circRNA has several biological activities, including tumor progression [18,19]. CircRNAs are expressed in specific tissues, which makes them promising diagnostic and therapeutic biomarkers in human cancers [20]. However, the roles of circRNAs in colorectal cancer (CRC) have been poorly understood.

The findings from the present study showed that the expression of *Homo sapiens* circular RNA 0079993 (*hsa_circ_0079993*), a novel circRNA derived from the mRNA of the POLR2J4 gene, was significantly increased in the CRC tissues and cell lines, and was associated with a worse prognosis. *In vitro* and *in vivo* studies performed in this study showed that circ_0079993 knockdown significantly inhibited CRC cell proliferation. Also, circ_0079993 was shown to act as a sponge for multiple miRNAs, and miR-203a-3p.1 was shown to interact with circ_0079993 and CREB1 in CRC cells directly. Importantly, circ_0079993 indirectly regulated the expression of CREB1 in CRC cells by sponging miR-203a-3p.1, which also blocked the inhibitory effects of circ_0079993 knockdown on CRC cell proliferation. To the best of our knowledge, the present study was the first to report the expression, function and underlying mechanisms of circ_0079993 in human CRC.

The RNA polymerase II subunit J (POLR2J) family includes four members, POLR2J1, POLR2J2, POLR2J3, and POLR2J4. The POLR2J4 gene (31040 bp) is located at the 7p13 locus on the short arm of chromosome 7 and is one of the genes encoding variants of the hRPB11 subunit of *Homo sapiens* circular RNA

0079993 (*hsa_circ_0079993*) [21]. To the best of our knowledge, no previous studies have shown an association between POLR2J4 and human cancer. Circ_0079993 from mRNA of POLR2J4 is a novel circRNA with unclear biological functions, and there have been no previously published studies.

According to the theory of competing endogenous RNA (ceRNA), circRNAs participate in the regulation of tumorigenesis of human cancers by acting as miRNAs sponges to affect corresponding mRNA expression [22,23]. Sponging the specific miRNAs in the circRNA-miRNA-mRNA axis is considered to be an intrinsic function of circRNA [24]. Therefore, we performed a bioinformatics analysis to construct the circRNA-miRNA-mRNA networks of circ_0079993. Circ_0079993 has been shown to interact with miR-203a-3p.1, miR-450b-3p, miR-502-5p, and miR-1224-3p, and miR-203a-3p.1 is associated with the progression of human tumors, including CRC [25], which was consistent with our findings.

The cAMP response element binding protein 1 (CREB1) gene encodes a critical transcription agent that regulates multiple stress signals and growth factors [26,27]. CREB1 has a key role in the initiation and progression of human tumors by acting as an oncogene [28]. CREB1 regulates and is regulated by the expression of miRNAs, forming a feedback loop [29], indicating a bidirectional interaction between CREB1 and miRNAs in human tumors. In this study, we demonstrated that miR-203a-3p.1 could negatively regulate CREB1 in CRC cells, and circ_0079993 could block these effects.

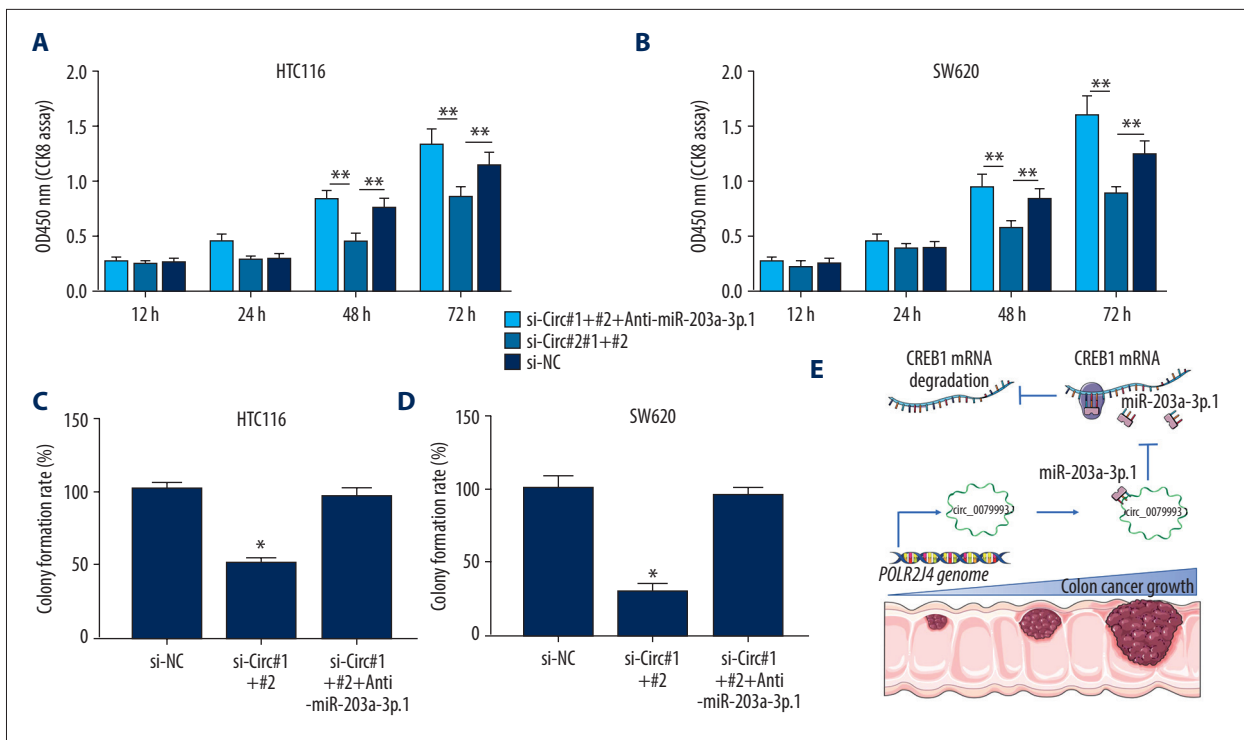


Figure 5. Anti-miR-203a-3p.1 reversed the inhibitory effects of circ_0079993 knockdown on the proliferation of colorectal cancer (CRC) cells. (A, B) The cell counting kit-8 (CCK-8) assay was performed to evaluate the cell viability of HCT116 and SW620 cells transfected with small-interfering RNAs (siRNAs), si-NC, si-Circ#1+#2, and si-Circ#1+#2+anti-miR-203a-3p.1 (** P<0.01). (C, D) Colony formation assay was used to detect the effects of anti-miR-203a-3p.1 on cell proliferation in circ_0079993 silenced HCT116 and SW620 cells (* P<0.05). (E) Diagram of the molecular mechanisms underlying the circ_0079993/miR-203a-3p.1/CREB1 axis in CRC.

Conclusions

The findings from this study showed that the *Homo sapiens* circular RNA 0079993 (hsa_circ_0079993), microRNA-203a-3p.1 (miR-203a-3p.1), and cAMP response element-binding protein (CREB1) axis had a role in the progression of colorectal cancer (CRC) cells *in vitro* and *in vivo*. The findings add to the understanding of the pathogenesis of CRC, but also may stimulate further studies to identify possible novel therapeutic targets for CRC. There may be further functional mechanisms for circ_0079993 in CRC, including protein encoding, which should be investigated further. It is likely that the circ_RNA regulation networks in the pathogenesis of CRC are highly

complex and that further studies are needed to characterize these molecules.

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Conflict of interest

None.

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