Am J Transl Res 2021;13(9):10038-10055
www.ajtr.org /ISSN:1943-8141/AJTR0135471

Original Article

CircRNA_002178 as a ceRNA promotes the development of colorectal cancer by regulating miR-542-3p/CREB1

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Received May 20, 2021; Accepted July 7, 2021; Epub September 15, 2021; Published September 30, 2021

Abstract: Objective: Colorectal cancer (CRC) is a malignant tumor commonly found in the digestive tract. This study aimed to explore the effect of circRNA_002178 as a competing endogenous RNA in the development of CRC by regulating the miR-542-3p/cAMP response element binding protein 1 (CREB1) axis and its molecular mechanism. Methods: The relative expressions of circ_002178, miR-542-3p, and CREB1 in patients' cell lines and CRC tissues were measured using Western blot and qRT-PCR. The localization and expression of circ_002178 were determined using fluorescence in situ hybridization and nucleocytoplasmic separation tests. The targeting relationships among circ_002178, miR-542-3p, and CREB1 were validated using RNA immunoprecipitation and dual luciferase reporter assays. The cells' proliferation, invasion, and colony forming ability were tested using CCK8, Transwell, and Clone formation assays, respectively. The cellular glucose consumption, lactification, and adenosine triphosphate (ATP) production were measured using glucose uptake colorimetric assay kits, lactate colorimetric assay kits and ATP assay kits, respectively. Results: The circ_002178 and CREB1 expressions were up-regulated in the CRC cells and tissues, and the miR-542-3p expression was down-regulated (all $P<0.05$). The circ_002178 knockdown inhibited the proliferation, invasion, colony formation, and glycolysis of the CRC cells *in vitro*, but the overexpression of circ_002178 induced the opposite result (both $P<0.05$). Our molecular mechanism study revealed that circ_002178, as the molecular sponge of miR-542-3p, promotes CREB1 expression. The downregulation of miR-542-3p or the overexpression of CREB1 is able to partly weaken the inhibition of CRC cells through the circ_002178 knockdown. Conclusion: circ_002178 promotes the invasion, proliferation, colony formation, and glycolysis of CRC cells by regulating the miR-542-3p/CREB1 axis, thus driving the development of CRC.

Keywords: Colorectal cancer, circ_002178, miR-542-3p, cAMP response element binding protein 1, glycolysis

Introduction

Colorectal cancer (CRC), commonly found in the digestive tract, is malignant and ranks third among cancers worldwide [1, 2]. CRC has high recurrence and metastasis rates, but progress has been made in the treatment of CRC through systematic methods such as chemoradiotherapy and immunotherapy [3, 4]. Abnormal mutations in molecules and genes facilitate the development of CRC [5]. Therefore, the exploration of molecular mechanisms involved in CRC occurrence and the identification of its potential biomarkers can contribute to the improvement of patient prognosis to a great degree.

Metabolic reprogramming is an important marker for the development of a malignant tumor, by which cancer development and metastasis occur [6]. The rapid growth of cancer cells leads to an insufficient oxygen supply in the microenvironment, and then metabolic reprogramming occurs in cancer cells to transform oxidative glucose metabolism into glycolysis, by which sufficient energy and substances are provided for the growth of cancer cells [7, 8]. The inhibition of metabolic reprogramming in cancer cells leads to the advances in cancer therapy.

circ_RNA has been demonstrated in several studies to play a vital role in cancer progres-

sion. For example, the overexpression of circMTO1 can inhibit gastric cancer cell growth, increase the apoptotic rate, and decrease cell invasion and migration [9]. High expressions of circABCB10 cause a low survival rate in patients with non-small cell lung cancer (NSCLC), and the knockdown of circABCB10 restrains NSCLC cell migration, proliferation and invasion [10]. circ_0038646 functions as a tumor promoter in CRC, and the knockdown of circ_0038646 significantly reduces the proliferation and migration of human CRC cells [11]. circ_002178, located on chr14:20811436-20811534, and also known as circ_0000519, is transcribed from RPPH1. circ_002178 has been shown to promote oral squamous cell carcinoma cell proliferation and migration by the activation of Akt/mTOR pathway [12]. The knockdown of circRNA_002178 defers breast cancer progression [13]. However, the biological effect of circ_002178 in CRC remains unclear. Hence, the potential effect of circ_002178 in CRC was explored in this study.

miRNAs can be competitively sponged by circ_RNA. Moreover, the vital effects of miR-542-3p in cancer has been explored on many occasions. It is reported that LINC00963 promotes the metastasis of prostate cancer by sponging tumor suppressor miR-542-3p [14]. LncRNA SNHG8 promotes osteosarcoma cell proliferation by down-regulating miR-542-3p [15]. Yang et al. found that miR-542-3p up-regulation inhibits CRC cell growth and invasion through the PI3K/AKT/survivin signal pathway [16]. miR-542-3p has been demonstrated by the above studies to play roles as a tumor suppressor. cAMP response element binding protein 1 (CREB1) has been found to play a carcinogenic role in various cancers. Imperatorin has been reported to inhibit the phosphorylation of CREB1, nuclear translocation, and its interaction with the TGF2 promoter, and to inactivate ERK signaling to suppress esophageal cancer invasion [17]. The overexpression of CREB1 weakens miR-133a-3p-mediated cell activity reduction and apoptosis and increases the cell cycle arrest in retinoblastoma [18]. LncRNA BLACAT1 knockdown down-regulates CREB1 using miR-519d-3p to inhibit CRC cell proliferation, migration, and invasion [19].

Based on the above studies, we aimed to systematically investigate the specific biological effects of circ_002178 on CRC and to explore whether circ_002178 plays a role in regulating the miR-542-3p/CREB1 axis, in order to provide effective targets and new strategies for the treatment of CRC.

Materials and methods

Bioinformatics analysis

Differential expression data sets of circ_RNA in CRC samples were retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/). The downstream targets of circ_RNA and miRNA were predicted using the miRWalk (http://mirwalk.umm.uni-heidelberg.de/) and StarBase (http://starbase.sysu.edu.cn) databases. *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway enrichment analyses of miRNA and mRNA were performed using the DIANA TOOLS database (http://snf-515788.vm.okeanos.grnet.gr/) and the Database for Annotation, Visualization, and Integrated Discovery (https://david.ncifcrf.gov/). A protein-protein interaction analysis for mRNA was conducted using the STRING database (https://string-db.org/).

The patients and the tissue samples

In this study, cancer tissues and para-carcinoma tissues from 60 CRC patients were included, and all the patients were diagnosed histopathologically with CRC between March 2020 and August 2020 and treated in our hospital. None of the patients had undergone radiotherapy, chemotherapy, or any other anticancer treatments before the surgery. The patients with multiple clinical diseases, a history of other malignant tumors, or with recurrent CRC were excluded from this study. Only patients with an initial diagnosis of CRC were enrolled. The excised tissues were placed in liquid nitrogen and stored in a freezer at -80°C. All the patients signed a written informed consent, and this study was approved by the ethics committee of our hospital (Approval number: 2021 Ethics Review No. 007). The CRC patients' clinical characteristics are shown in **Table 1**.

Cell culture

Normal human colonic epithelial cell line NCM460 (ZKC1143) was provided by the

Table 1. Characteristics of the CRC patients

Characteristic	Total (n=60)
Mean age (years ± SD)	54.2±13.7
Gender	
Male	35 (58.3%)
Female	25 (41.7%)
Localization	
Rectum	15 (25.0%)
Colon	45 (75.0%)
Tumor size	
≤4 cm	28 (46.7%)
>4 cm	32 (53.3%)
T classification	
T1/T2	20 (33.3%)
T3/T4	40 (66.7%)
Lymph nodes metastasis	
N0	16 (26.7%)
N1/N2	44 (33.3%)
Histological differentiation	
High/moderate	34 (56.7%)
Low	26 (43.3%)

Note: CRC: colorectal cancer; SD: standard deviation.

Beijing Zoman Biotechnology Co., Ltd., China. Human CRC cell lines HCT116 (CL-0096), SW480 (CL-0223) and HT29 (CL-0118) were provided by the Procell Life Science & Technology Co., Ltd., Wuhan, China. All the cells were cultured in DMEM (D5030, Merck KGaA, Germany) supplemented with 10% fetal bovine serum (A3160901, Thermo Fisher, USA), 100 U/mL penicillin (ST488-1, Beyotime Biotechnology, Shanghai, China) and 100 mg/mL streptomycin (ST488-2, Beyotime Biotechnology, Shanghai, China), and incubated in a 5% CO_2 incubator at 37°C.

Cell transfection

The plasmids with CREB1 overexpressions and circ_002178 overexpressions or knockdowns were all provided by VectorBuilder (Guangzhou) Inc., China. The miR-542-3p overexpression and suppressor vectors were provided by the Shanghai GenePharma Co., Ltd., China. The sequences are as follows: OE-CREB1: 5'-TGTACGCTAGCGCATCGACGCC-3'; OE-circ_002178: 5'-ACGATCTAGCGAGCCTATGCCTGGC-3'; si-circ_002178: 5'-CCTACGACGATCGCGATCATAACCCCGC-3'; miR-542-3p mimic: 5'-AUCGCCUUUUCUACCGGCGAGC-3'; miR-542-3p inhibitor: 5'-UUCAGCGAUCUGAGCGCUA-3'. The SW480 cells were transfected according to the Lipofectamine 3000 Transfection Reagent (L3000-0007, Thermo Fisher, USA) instructions for the subsequent tests 48 h later.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The RNAs were isolated from the tissues and cells according to the Trizol reagent (R0016, Beyotime Biotechnology, Shanghai, China) instructions. 5 μg of RNAs were used as the template, and the reverse transcription reaction was conducted using BeyoRT™ III First Strand cDNA Synthesis kits (D7178L, Beyotime Biotechnology, Shanghai, China) according to the instructions. The polymerase chain reaction (PCR) was performed using PowerUp SYBR™ Green Master Mix (A25742, Thermo Fisher, USA) on a 7500 fast real-time fluorescent quantitative PCR system (ABI, USA). The program was set to react at 50°C for 2 min, 95°C for 2 min, 95°C for 3 s, and 60°C for 30 s, for 40 cycles. GAPDH was used as the internal reference of circ_002178 and CREB1, and U6 was used for the internal reference of miR-542-3p. The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were provided by the Shanghai GenePharma Co., Ltd., China. The sequences are shown in **Table 2**.

Western blot

The total proteins in the cells were extracted with a RIPA buffer (MT0066, Beijing Biolab Technology Co., Ltd., China) and quantitated using BCA Protein Assay kits (23227, Thermo Fisher, USA). 50 μg of total proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis at a constant voltage of 120 V, and the separated proteins were transferred to polyvinylidene fluoride membranes (FFP24, Beyotime Biotechnology, Shanghai, China) at 60 V and 100 mA. The membranes were blocked with 5% skimmed milk powder for 1 h. The primary antibodies CREB1 (1:1000, ab32515, Abcam, USA) and GAPDH (1:2000, ab8245, Abcam, USA) were added to bind to the antigens on the membrane and incubated overnight at 4°C. The next day, the membranes were washed three times with the Tris buffered saline Tween, for 5 min each time. Horseradish peroxidase coupled goat anti-rabbit secondary antibody IgG (1:1000, ab181236, Abcam, USA) was added

Table 2. qRT-PCR primer sequences

Gene name	Sequence (5'-3')
circ_002178	Forward: TAAGCCTCGCGCGATCTACGACGACTCAGG
	Reverse: AGCCTAGAGACTCCGCCTAGGCGTCTGCCGC
miR-542-3p	Forward: GCGCGATATCGCGACGAGCGACC
	Reverse: TTAAGCGAGCTATCGCGCGCGAGCG
CREB1	Forward: TTAAGCGAGCTAGCGCCCGCGACGAGCCC
	Reverse: TAGGCTCAGGAGCGAGCGACGCCCGAA
GAPDH	Forward: AAGCCGATCTACGCGCGGAGAGCGCC
	Reverse: CCTAGCGACGGCGGCTTAGCGAACCCG
U6	Forward: TAGGAACCGCAGTCACGGCGGCGATCCGCG
	Reverse: CCGAGCAGCTATCGACGCGTCTGGAAAGC

Note: qRT-PCR: quantitative real-time polymerase chain reaction.

and incubated for 2 h. The membranes were washed three times with the Tris buffered saline Tween, for 5 min per time. The proteins were developed using BeyoECL Moon (P0018FS, Beyotime Biotechnology, Shanghai, China). The proteins were quantitated using Image J software with GAPDH as the control.

Stability test

The RNAs were isolated from the cells using Trizol reagent. 15 μg of RNAs were taken, mixed with RNase R enzyme (RNR07250, NovoBiotechnology Co., Ltd., Beijing, China) and incubated in a 5% CO_2 incubator at 37°C. Another 15 μg of RNAs, not mixed with RNase R enzyme, were taken as the negative control (NC). After four-hours of incubation, the relative circ_002178 expression was measured using qRT-PCR.

Fluorescence in situ hybridization (FISH)

The experiment was completed using circ_RNA FISH kits (Bes1002, Guangzhou BersinBio Co., Ltd., China). The cells grew on the coverslip and were placed in a 6-well plate and fixed in 4% paraformaldehyde for 20 min. The subsequent hybridization steps were completed according to the kit instructions. The coverslip was sealed after airing and photographed under a laser scanning confocal microscope. The stained images were observed with the cell nuclei in blue fluorescence and the circ_002178 probe in red fluorescence.

Nucleocytoplasmic separation test

The cell nuclei and cytoplasms were separated and purified using a NE-PER Nuclear Cytoplasmic Extraction Kit (78835, Thermo Fisher, USA) as per the instructions. The relative circ_002178 expression in the cytoplasms and nuclei were measured using qRT-PCR, with GAPDH and U6 as the controls for the cytoplasms and nuclei, respectively.

Dual luciferase reporter assay

The specific binding sites between circ_002178 and miR-542-3p, miR-542-3p and CREB1 were predicted through the StarBase database. The 3'UTR fragment containing miR-542-3p in circ_002178 and CREB1 was amplified and cloned into a pGL3 vector to construct the circ_002178-WT and CREB1-WT reporter plasmids. The wild-type 3'UTR fragment was mutated using a QuickMutation™ Site-Directed Mutagenesis Kit (D0206, Beyotime Biotechnology, Shanghai, China), amplified and cloned into a pGL3 vector to construct the circ_002178-MUT and CREB1-MUT reporter plasmids. The above reporter plasmids were co-transfected with miR-542-3p NC or an miR-542-3p mimic into the SW480 cells as per the manufacturer's instructions for the Lipofectamine 3000 Transfection Reagent (L3000007, Thermo Fisher, USA). Forty-eight hours later, the relative luciferase activity was determined using a Dual Luciferase Reporter Gene Assay Kit (RG027, Beyotime Biotechnology, Shanghai, China).

RNA immunoprecipitation assay (RIP)

The binding of circ_002178 to miR-542-3p and miR-542-3p to CREB1 was verified using an RNA Immunoprecipitation Kit (P0101, Geneseed Biotech Co., Ltd., Guangzhou, China) as per the instructions. The circ_002178, miR-542-3p, and CREB1 enrichment levels were measured using qRT-PCR.

CCK8

The cells' proliferation ability was assessed using CCK8. The cells were seeded in a 96-well plate at 2×10^3 cells/well and supplemented with medium, and 10 μL of CCK8 solution (C0037, Beyotime Biotechnology, Shanghai, China) was added into each well and incubated in an incubator for 1 h. The absorbance values at 450 nm were measured using a multi-

mode microplate reader (BMG LABTECH, Germany) at incubation for 0, 24, 48, and 72 h.

Transwell

The cell invasion was determined using a 24-well Transwell chamber. Matrigel (M8370, Beijing Solarbio Science & Technology Co., Ltd., China) was added into the upper chamber. The SW480 cells were cultured in a serum-free medium and seeded in the upper chamber precoated with Matrigel. The medium containing 10% fetal bovine serum was added into the lower chamber for induction. The chamber was incubated in a 5% CO_2 incubator at 37°C for 24 h. The chamber was removed, and the non-invading cells were wiped off with cotton swabs. The invaded cells under the membrane were fixed with paraformaldehyde and stained with 1% crystal violet (G1062, Beijing Solarbio Science & Technology Co., Ltd., China). Five visual fields were selected randomly under an inverted microscope (Olympus, Japan) for photographing and observing. The number of invaded cells was counted.

Clone formation assay

SW480 cells were seeded in a 6-well plate at 1×10^3 cells/well, and incubated at 5% CO_2 and 37°C for 15 d. The cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet to count the cells in the colony.

The glucose consumption, lactate level, and adenosine triphosphate level measurements

The cells were lysed, and the glucose and adenosine triphosphate (ATP) levels in the cell lysates were measured using a Glucose Uptake Colorimetric Assay Kit (MAK083, Merck KGaA, Germany) and an ATP Assay Kit (S0026, Beyotime Biotechnology, Shanghai, China). Some cell supernatant was taken, and the extracellular lactate production was measured using Lactate Colorimetric Assay kits (K667, AmyJet Scientific Co., Ltd., Wuhan, China). The operation was conducted according to the kit's instructions. The above quantification results were normalized using the concentrations of the lytic cell proteins.

Statistical analysis

In this study, the data were analyzed using SPSS23.0 statistical software. The data were expressed as the mean ± standard deviation. The comparisons between two independent groups or among groups were performed using t tests or one-way analyses of variance followed by Tukey's post hoc test. Receiver operating characteristic (ROC) curves were drawn using GraphPad Prism 9.0 software. The correlations were analyzed using the Pearson method. All the *in vitro* experiments were performed three times. $P<0.05$ indicated a significant difference.

Results

The potential tumor-promoting action of up-regulated circ_002178 in CRC

A data set named GSE126095 in the GEO database was analyzed, and the circ_RNAs of the Top 50 with a differential expression were selected for visualization. The results showed that the expression of circ_002178 was up-regulated in CRC (**Figure 1A**). Our analysis of the cancer tissues and normal para-carcinoma tissues of the 60 CRC patients revealed that the circ_002178 expression was higher in the CRC tissues than it was in the normal para-carcinoma tissues (**Figure 1B**, $P<0.05$). The ROC curve analysis results showed that circ_002178 is highly sensitive and reliable for CRC diagnosis (**Figure 1C**, $P<0.05$). The above results indicated an up-regulated circ_002178 expression in CRC, which might play a tumor-promoting action.

circ_002178 was highly expressed in CRC cells and participated in CRC cell proliferation and colony formation

The cell experiments revealed an up-regulated circ_002178 expression in the CRC cell lines HCT116, SW480 and HT29, relative to normal human colonic epithelial cell line NCM460 (**Figure 2A**, $P<0.05$). SW480 was selected as the subject, in view of its highest expression of the circ_002178 in SW480 cells. The function of circ_002178 on the SW480 cells was observed using transfecting plasmids with a knockdown or an overexpression of circ_002178 in the SW480 cells. The qRT-PCR showed that the knockdown of circ_002178 significantly inhibited the expression of circ_002178, and the overexpression of circ_002178 increased the expression of circ_002178 (**Figure 2B**, both $P<0.05$). The proliferation

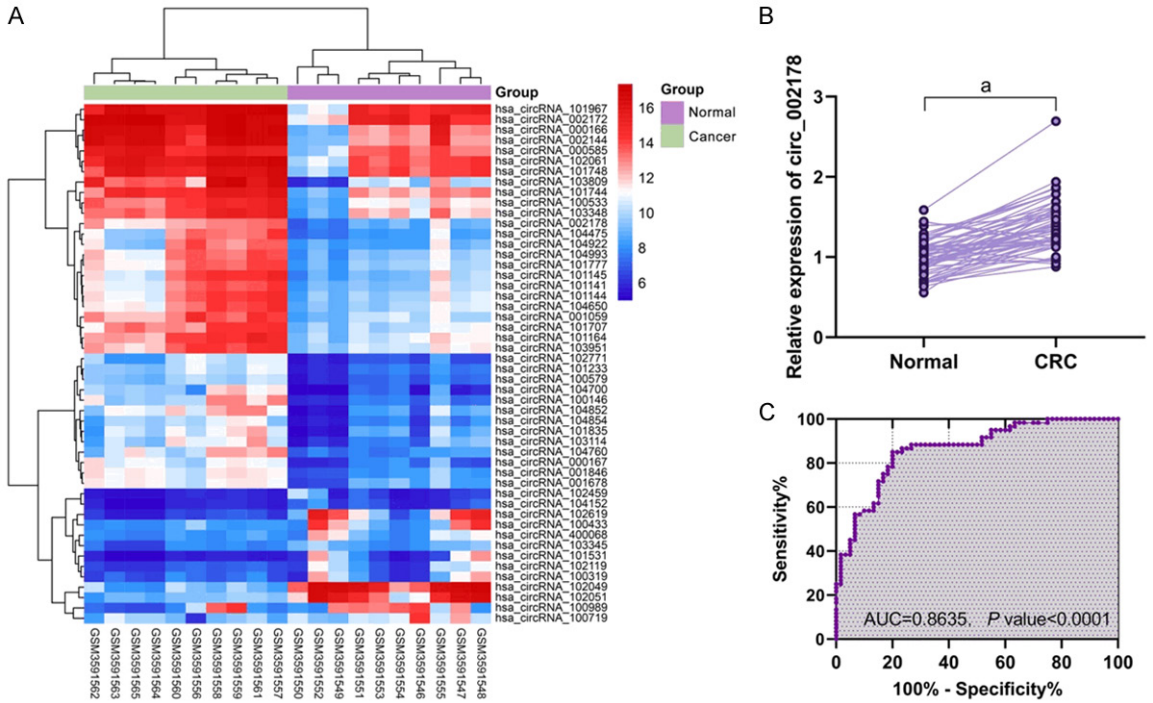


Figure 1. circ_002178 expression profile. A: The GSE126095 data set analysis results (Normal: N=10, Cancer: N=10); B: The circ_002178 expression in tissues (Normal: N=60, CRC: N=60); C: circ_002178 sensitivity analyzed by the ROC curve. Compared with the normal group, [a]P<0.05. CRC: colorectal cancer; ROC: receiver operating characteristics; AUC: area under the curve.

ability and number of clone formations of the SW480 cells were reduced after the knockdown of circ_002178, while the results were the opposite after the overexpression of circ_002178 (**Figure 2C** and **2D**, both P<0.05). The above experiments demonstrated that circ_002178 knockdown is able to inhibit the malignant development of cells by suppressing the cell proliferation and colony formation.

circ_002178 participated in the CRC cell invasion and glycolysis

The active invasion and the abnormal glycolysis of the cancer cells both participate in the development of cancer. In this study, the cell invasion and glycolysis were assessed using Transwell, lactate production, and glucose consumption. The experiments showed that the knockdown of circ_002178 significantly inhibited the number of invaded cells and reduced the glucose consumption and the lactate and ATP production, but the overexpression of circ_002178 had a facilitation effect on the above indicators (**Figure 3**, all P<0.05). The above experimental data indicated that circ_002178 knockdown can inhibit cells' malignant biological behavior by suppressing CRC cell invasion and glycolysis.

Competitive sponging of miR-542-3p by circ_002178 in the CRC cells

circ_RNA has been shown in a few studies to act as a competing endogenous RNA (ceRNA) to competitively absorb miRNA. In order to confirm whether circ_RNA functioned as a ceRNA, the subcellular localization of circ_002178 was predicted and the stability was tested. The FISH results showed that circ_002178 is co-expressed with the cytoplasms (**Figure 4A**). A nucleocytoplasmic separation test showed that circ_002178 is highly expressed in the SW480 cytoplasms (**Figure 4B**, P<0.05). The RNase R enzyme degrades linear RNA, but this has no significant effect on the expression of circ_002178 (**Figure 4C**, P>0.05). Based on the above results, we confirmed that circ_002178 can carry out the function by acting as a ceRNA.

The downstream miRNA of circ_RNA was predicted and confirmed using the StarBase data-

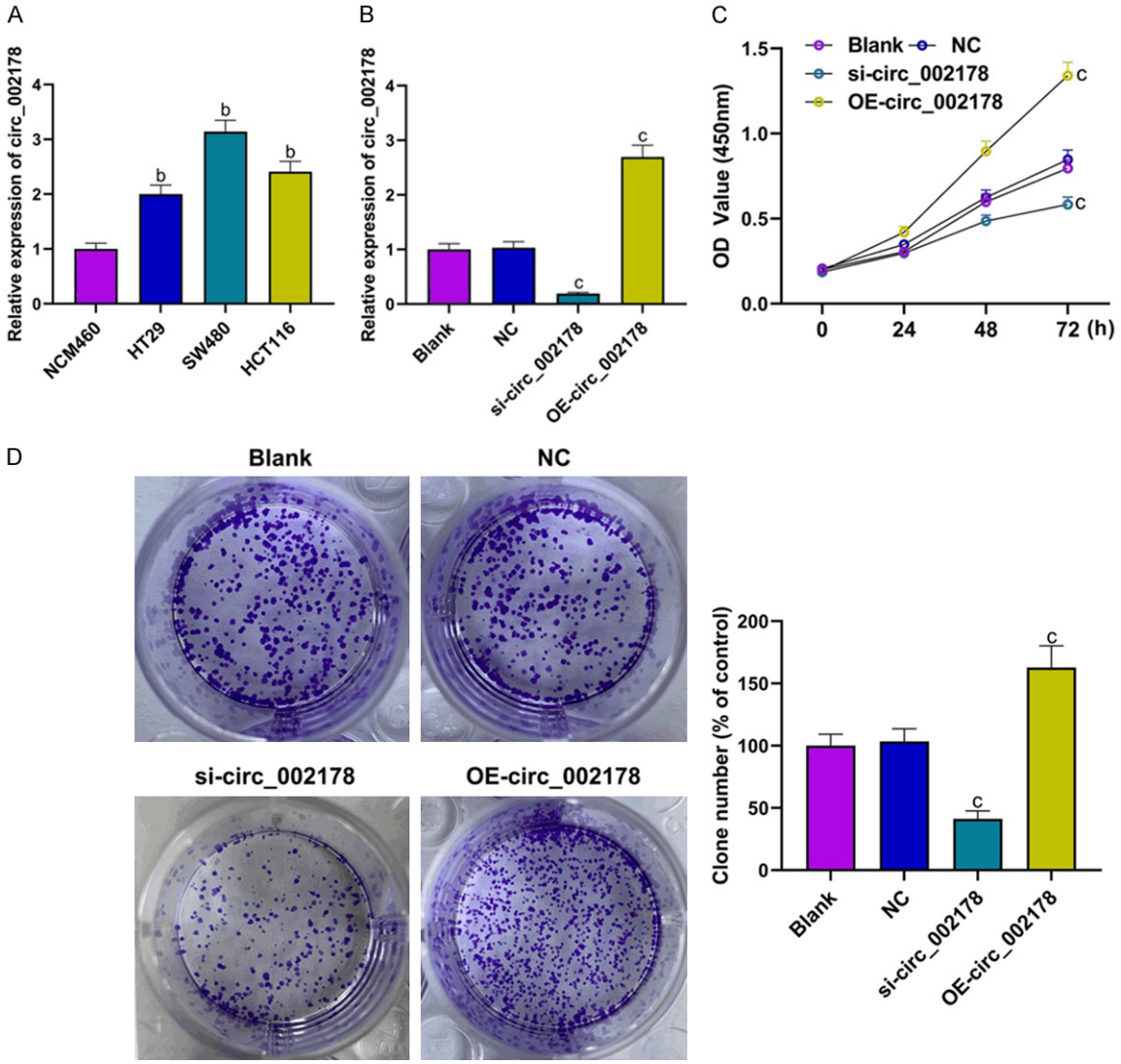


Figure 2. circ_002178 was highly expressed in the CRC cells and participated in the CRC cell proliferation and colony formation. A: The circ_002178 expressions in cells; B: The plasmid transfection efficiency measured using qRT-PCR; C: The cell proliferation ability; D: The cell colony forming ability. Compared with the NCM460 group, [b]P<0.05; compared with the NC group, [c]P<0.05. NC: negative control; OD: optical density; CRC: colorectal cancer; qRT-PCR: quantitative real-time polymerase chain reaction.

base. The results showed that hsa-miR-1296-5p, hsa-miR-328-3p and hsa-miR-542-3p all had specific binding sites with circ_002178 (**Figure 4D**). miR-542-3p was chosen as the focus of this study because it has an antitumor effect on CRC and is enriched in the cancer-related pathways (**Figure 4E**). Our qRT-PCR assays found down-regulated miR-542-3p expressions in the CRC tissues (**Figure 4F**, P<0.05) and the CRC cell lines (**Figure 4G**, all P<0.05). The miR-542-3p expression had a negative correlation with the circ_002178 expression (**Figure 4H**, both P<0.05). The knockdown of circ_002178 in the SW480 cells promoted the miR-542-3p expression, and the overexpression of circ_002178 inhibited the expression of miR-542-3p (**Figure 4I**, both P<0.05). The specific binding sequences of miR-542-3p and circ_002178 are shown in **Figure 4J**. A dual luciferase reporter assay was designed to confirm the targeting relationship between miR-542-3p and circ_002178. The results showed that the luciferase activity was significantly decreased in the circ_002178-WT+miR-542-3p mimic group (**Figure 4K**, P<0.05). An RIP assay discovered that miR-542-3p and circ_002178 were highly enriched in Ago2 compared with IgG (**Figure 4L**, P<0.05).

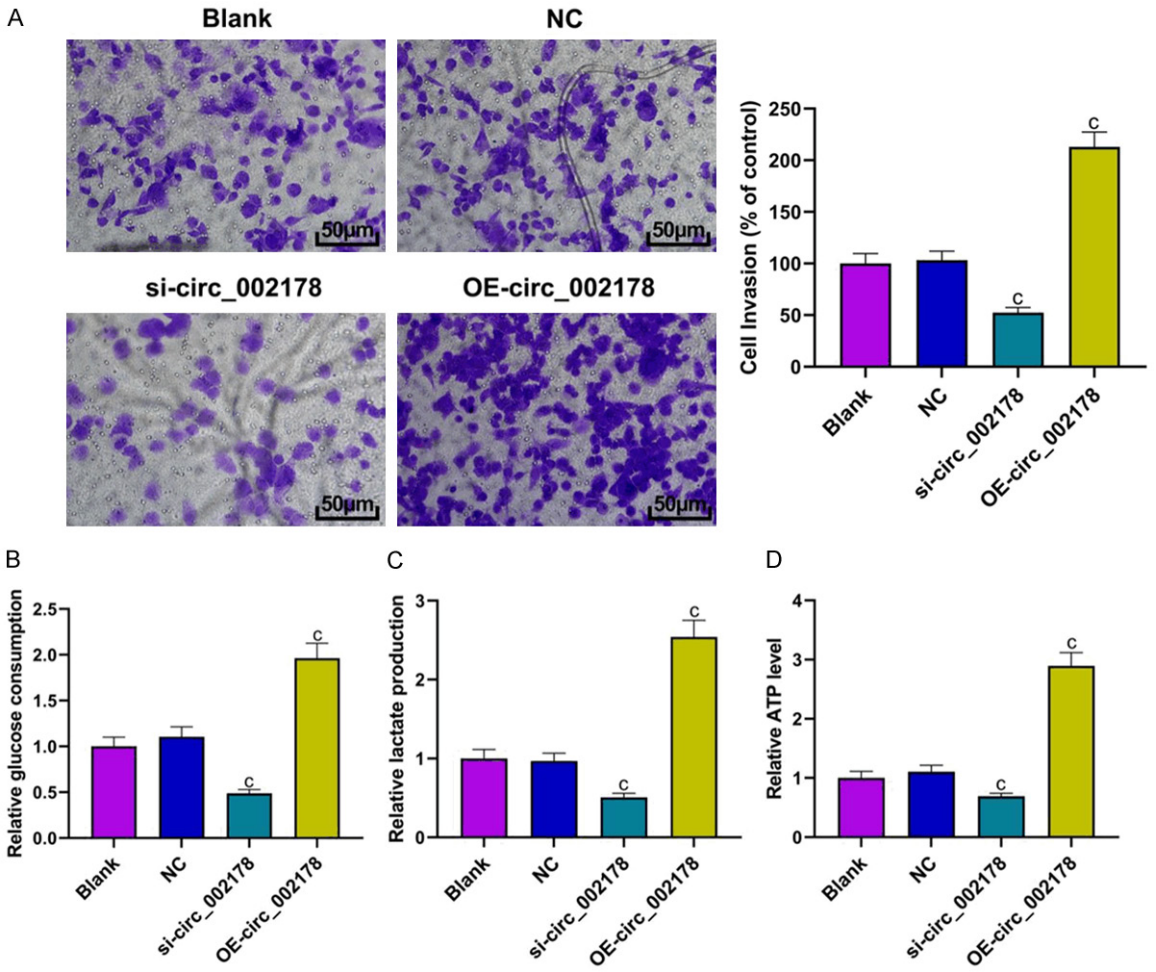


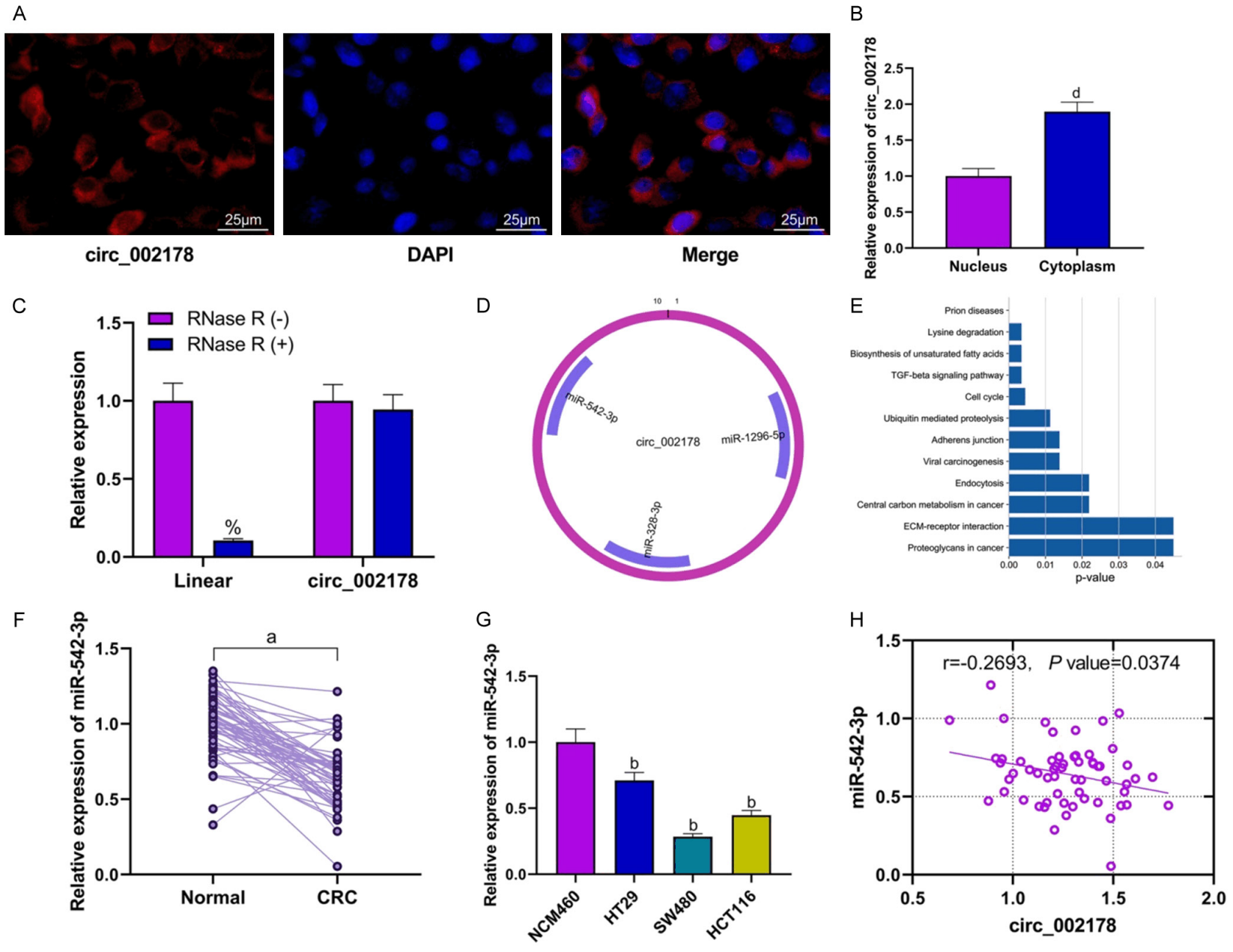
Figure 3. The participation of circ_002178 in the CRC cell invasion and glycolysis. A: The cell invasion ability (200×); B: The glucose consumption level; C: The lactate production; D: The ATP production. Compared with the NC group, $^{c}P<0.05$. NC: negative control; CRC: colorectal cancer; ATP: adenosine triphosphate.

The above experimental data suggested that circ_002178 can act as a molecular sponge to directly regulate miR-542-3p in CRC cell lines.

Regulation of CREB1 by circ_002178/miR-542-3p in CRC cells

In order to further explore the downstream regulatory mechanism of circ_002178/miR-542-3p, the downstream target genes of miR-542-3p were investigated through the StarBase and miRWalk online prediction websites. The intersection of two websites was determined, and a total of 677 potential target genes were obtained (**Figure 5A**). A KEGG pathway enrichment analysis revealed that hsa05215, a prostate cancer pathway (**Figure 5B**), is a cancer-related pathway. Therefore, the genes enriched in this pathway were further analyzed. The protein-protein interaction analysis for the above enriched genes and CRC occurrence-related genes showed a strong association between CREB1 and the other genes (**Figure 5C**). A dual luciferase reporter assay found that the luciferase activity was decreased in the miR-542-3p mimic+CREB1-WT group versus the miR-NC+CREB1-WT group (**Figure 5E**, $P<0.05$). An RIP assay indicated that miR-542-3p and CREB1 were highly enriched in the Ago2 group versus the IgG group (**Figure 5F**, $P<0.05$). The targeting relationship between miR-542-3p and CREB1 was confirmed.

qRT-PCR found significantly up-regulated CREB1 expressions in the cancer tissues of the CRC patients (**Figure 6A**, $P<0.05$), and in the CRC cell lines (**Figure 6B**, $P<0.05$). The regulatory effect of circ_002178/miR-542-3p on CREB1 in the CRC cells was further determined. The experiment showed that the knock-


A
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Prion diseases
Lysine degradation
Biosynthesis of unsaturated fatty acids
TGF-beta signaling pathway
Cell cycle
Ubiquitin mediated proteolysis
Adherens junction
Viral carcinogenesis
Endocytosis
Central carbon metabolism in cancer
ECM-receptor interaction
Proteoglycans in cancer
p-value
F
Relative expression of miR-542-3p
a
Normal
CRC
G
Relative expression of miR-542-3p
b
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HT29
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miR-542-3p
circ_002178

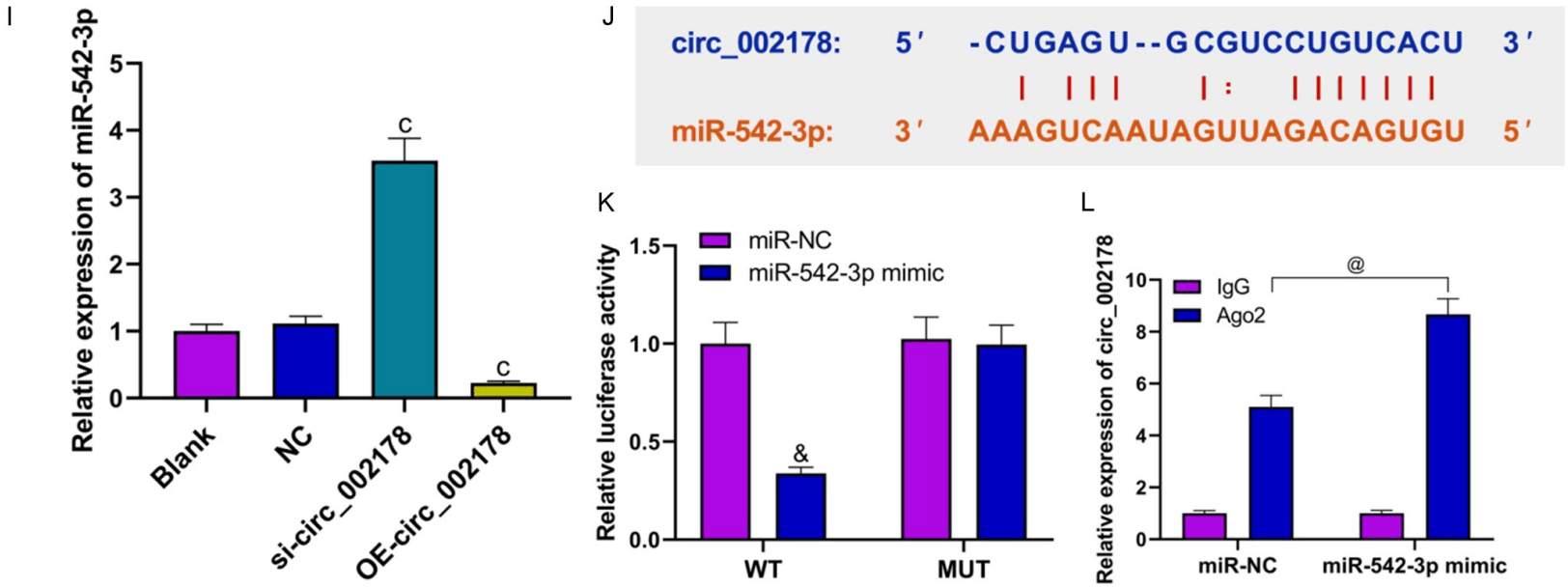


Figure 4. circ_002178 functions as a molecular sponge for miR-542-3p. A: The FISH results (400×); B: The expression of circ_002178 in the cytoplasms and nuclei; C: A stability test for circ_002178; D: circ_002178 and its target miRNAs; E: KEGG analysis results of the target miRNA for circ_002178; F, G: The expression of miR-542-3p in the tissues and cells (in tissues, Normal: N=60, CRC: N=60); H: A correlation analysis of circ_002178 and miR-542-3p (N=60); I: The effect of circ_002178 on the miR-542-3p expression; J: The specific binding sites of circ_002178 and miR-542-3p; K, L: The targeting relationship between circ_002178 and miR-542-3p validated using dual luciferase reporter and RIP assays. Compared with the Nucleus group, $^{d}P<0.05$; compared with the RNase R (-) group, $^{\%}P<0.05$; compared with the Normal group, $^{a}P<0.05$; compared with the NCM460 group, $^{b}P<0.05$; compared with the NC group, $^{c}P<0.05$; compared with the miR-NC+WT group, $^{\&}P<0.05$; compared with the IgG group, $^{@}P<0.05$. NC: negative control; CRC: colorectal cancer; FISH: fluorescence in situ hybridization; KEGG: *Kyoto Encyclopedia of Genes and Genomes*; RIP: RNA immunoprecipitation assay.

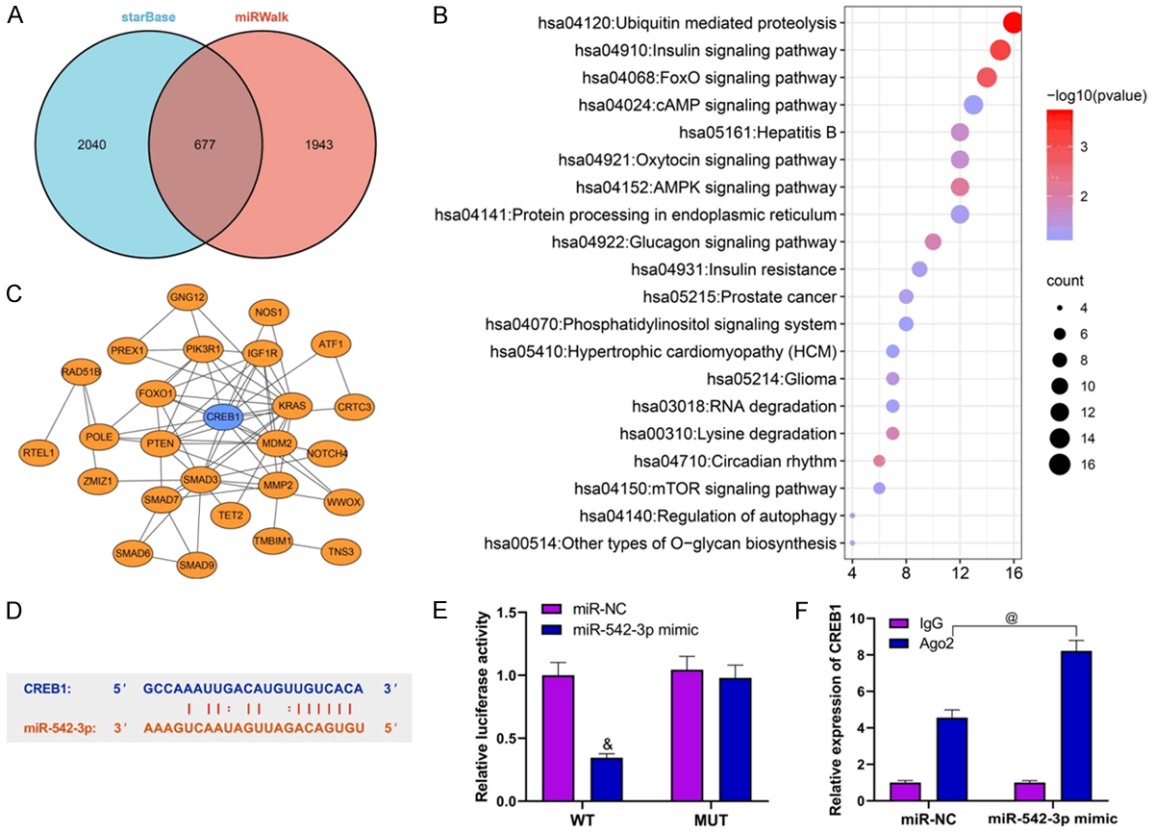


Figure 5. The targeting relationship between miR-542-3p and CREB1. A: The intersection of the StarBase and miRWalk online websites in predicting the target genes; B: The KEGG pathway enrichment analysis; C: The protein-protein interaction analysis; D: The specific binding sites of miR-542-3p and CREB1; E, F: The targeting relationship between miR-542-3p and CREB1 validated using a dual luciferase reporter assay and an RIP assay. Compared with the miR-NC+WT group, $^{\&}P<0.05$; compared with the IgG group, $^{@}P<0.05$. NC: negative control; KEGG: Kyoto Encyclopedia of Genes and Genomes; RIP: RNA immunoprecipitation assay.

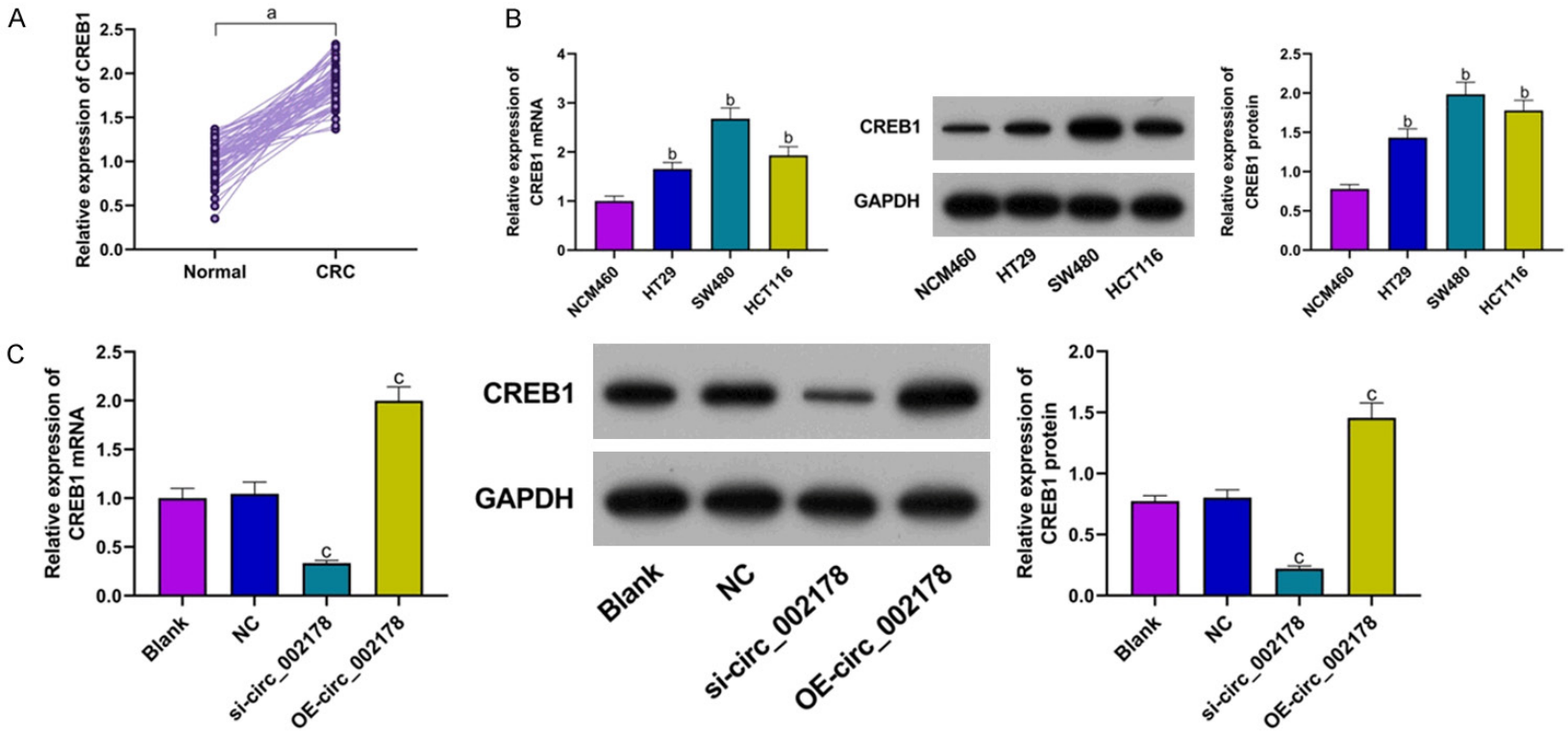
down of circ_002178 or the overexpression of miR-542-3p in the SW480 cells significantly inhibited the CREB1 expression, while the overexpression of circ_002178 or the miR-542-3p inhibitor significantly promoted the CREB1 expression (**Figure 6C** and **6D**, both P<0.05). The CREB1 expression had a positive correlation with the circ_002178 expression and a negative correlation with the miR-542-3p expression in the CRC tissues (**Figure 6E** and **6F**, both P<0.05). The above experimental data further suggest that the circ_002178/miR-542-3p axis can regulate CREB1 in CRC cells.

The circ_002178 knockdown inhibited CRC cell proliferation and colony formation through the miR-542-3p/CREB1 axis

The above experiments confirmed the regulatory relationship of circ_002178/miR-542-3p/CREB1. The miR-542-3p inhibitor and plasmids overexpressing CREB1 were transfected into the cells to observe their effects on the biological function of circ_002178. The CCK8 and clone formation assays showed that knockdown of circ_002178 remarkably inhibited the proliferation of and the number of clone formations in the SW480 cells, and the transfection of the miR-542-3 inhibitor or the plasmids overexpressing CREB1 in the SW480 cells partly offset the inhibition of the cell proliferation and the colony formation by the circ_002178 knockdown (**Figure 7**, P<0.05). The above experimental data indicated that the circ_002178 knockdown inhibited the cell proliferation and clone formation by regulating the miR-542-3p/CREB1 axis.

The knockdown of circ_002178 inhibited the CRC cell invasion and glycolysis through the miR-542-3p/CREB1 axis

The effect of the circ_002178/miR-542-3p/CREB1 axis on cell invasion and glycolysis was


A
Relative expression of CREB1
a
Normal
CRC
B
Relative expression of CREB1 mRNA
b
NCM460
HT29
SW480
HCT116
CREB1
GAPDH
Relative expression of CREB1 protein
C
Relative expression of CREB1 mRNA
c
Blank
NC
si-circ_002178
OE-circ_002178
CREB1
GAPDH
Relative expression of CREB1 protein

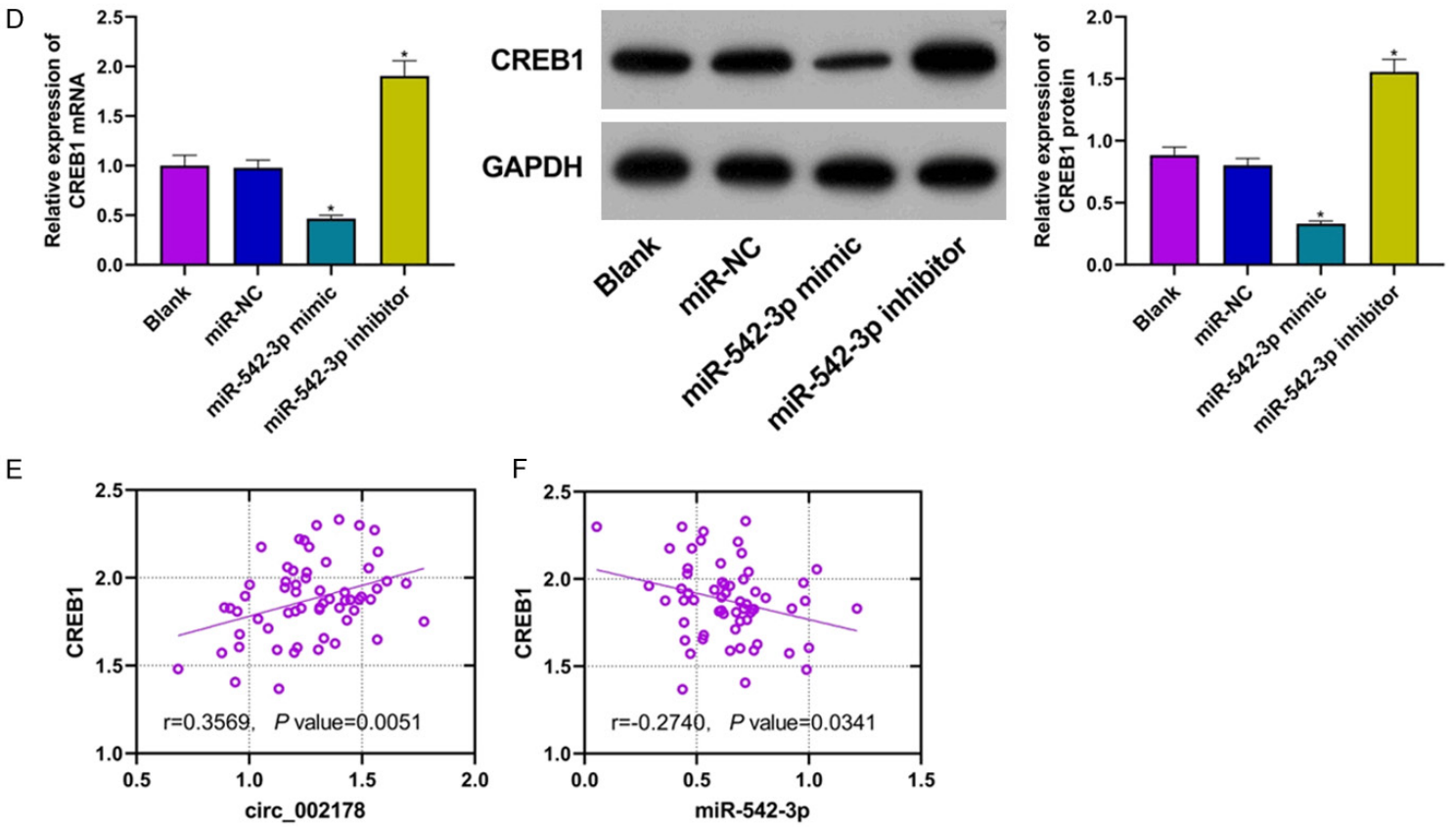


Figure 6. The regulatory effect of circ_002178/miR-542-3p on CREB1. A, B: The expression of CREB1 in the tissues and cells (in tissues, Normal: N=60, CRC: N=60); C, D: Effect of circ_002178/miR-542-3p on CREB1 expression; E, F: Correlation analysis between circ_002178/miR-542-3p and CREB1 (N=60). Compared with the Normal group, [a]P<0.05; compared with the NCM460 group, [b]P<0.05; compared with the NC group, [c]P<0.05; compared with the miR-NC group, *P<0.05. NC: negative control; CRC: colorectal cancer.

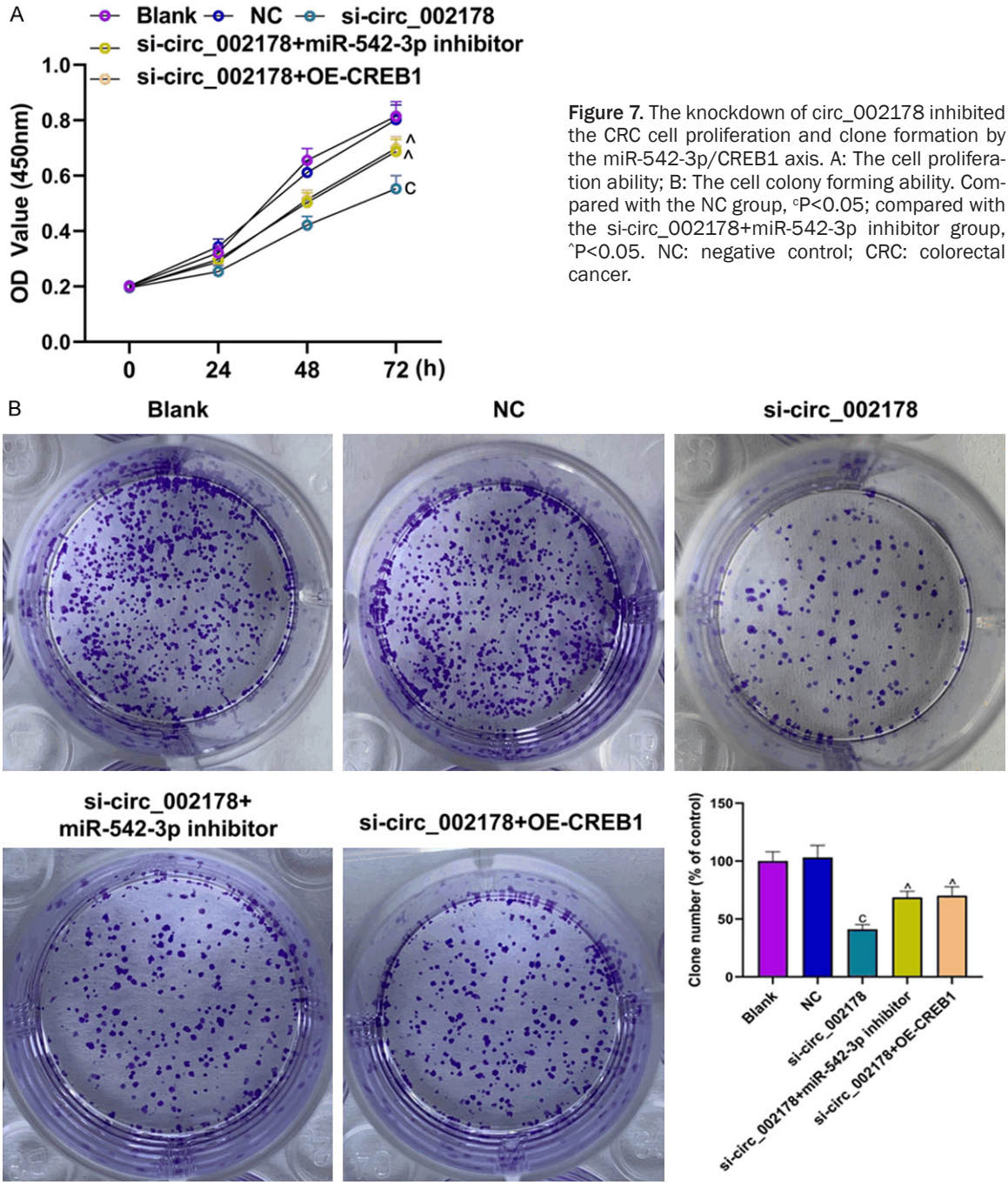


Figure 7. The knockdown of circ_002178 inhibited the CRC cell proliferation and clone formation by the miR-542-3p/CREB1 axis. A: The cell proliferation ability; B: The cell colony forming ability. Compared with the NC group, $^{c}P<0.05$; compared with the si-circ_002178+miR-542-3p inhibitor group, $^{\wedge}P<0.05$. NC: negative control; CRC: colorectal cancer.

measured using Transwell and corresponding kits. The results demonstrated that the knockdown of circ_002178 significantly inhibited the cell invasion and reduced the glucose consumption and lactate and ATP production, and the transfection of the miR-542-3 inhibitor or the plasmids overexpressing CREB1 in the cells partly offset the inhibitory effect of circ_002178 knockdown on the cell invasion, glucose consumption, and ATP and lactate production (**Figure 8**, $P<0.05$). The above experimental data indicate that the circ_002178 knockdown inhibits cell invasion and glycolysis by regulating the miR-542-3p/CREB1 axis.

Discussion

circ_002178 has been found previously to down-regulate the miR-1258 levels and to reduce the inhibition of KDM7A by miR-1258,

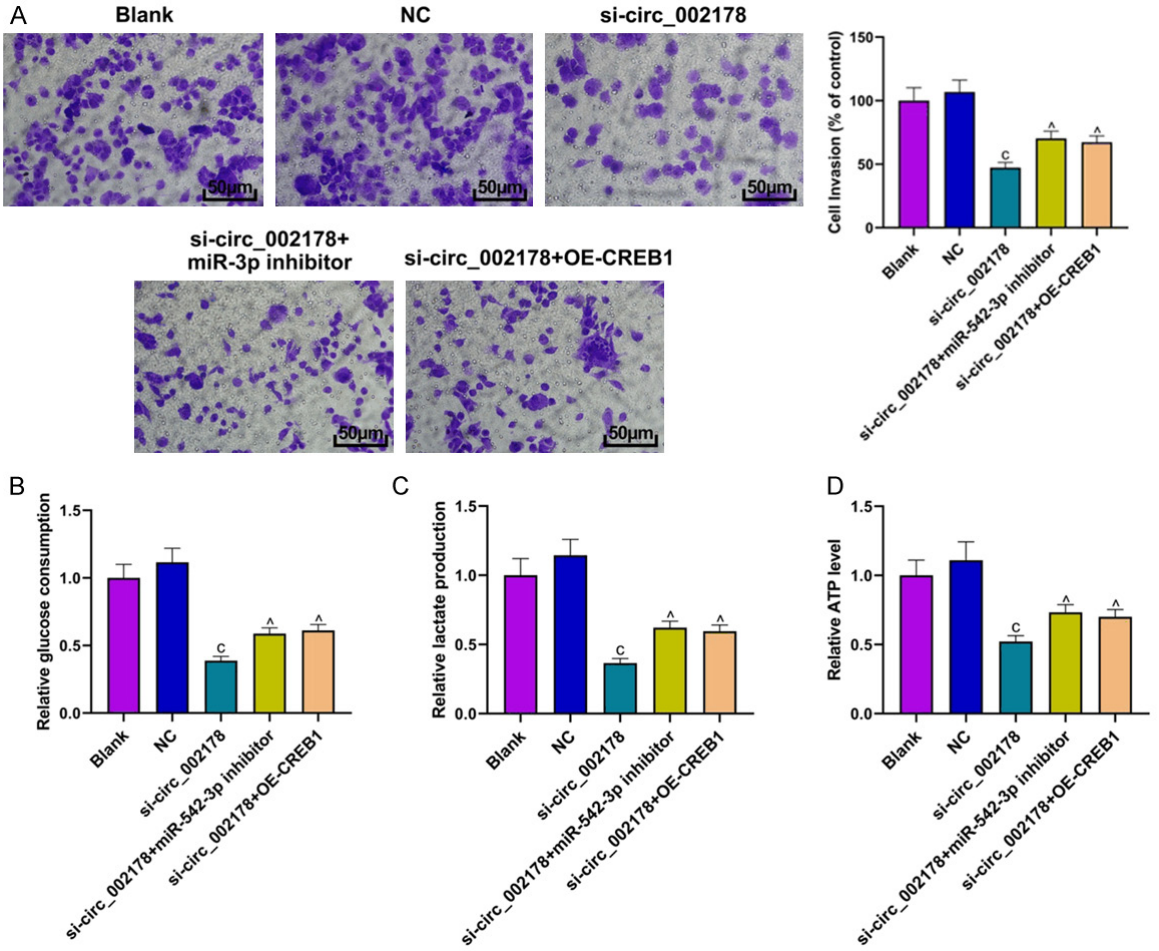


Figure 8. The knockdown of circ_002178 inhibited the CRC cell invasion and glycolysis by the miR-542-3p/CREB1 axis. A: The cell invasion ability (200×); B: The glucose consumption; C: The lactate production; D: The ATP production. Compared with the NC group, $^{c}P<0.05$; compared with the si-circ_002178+miR-542-3p inhibitor group, $^{\wedge}P<0.05$. NC: negative control; CRC: colorectal cancer; ATP: adenosine triphosphate.

thus promoting breast cancer cell growth and migration [20]. circ_002178 is able to act as a ceRNA to promote the expression of PDL1/PD1 in lung adenocarcinoma and to induce immune escape [21]. In this study, we found that circ_002178 can promote the progression of CRC.

It is widely known that cancer cells grow unrelentingly and are characterized by high invasion and metastasis rates. Glycolysis can provide substances for the pre-metastatic niche of cancer cells. Glycolytic metabolic reprogramming, also known as the Warburg effect, is a primary process in the development of all cancer cells [22]. In this process, glucose is consumed by the cancer cells and processed into lactate, which is then released into the cellular microenvironment for to promote cell invasion and metastasis. Lactate production and glucose consumption are important indicators for the assessment of glycolytic ability [23]. In this study, the transfection of circ_002178-knockdown plasmids in the CRC cells showed that knockdown of circ_002178 significantly inhibited the glucose consumption, lactate and ATP production, and glycolytic process, thus inhibiting cell proliferation, invasion, and colony forming ability. Therefore, the results indicated that circ_002178 promotes the metabolic reprogramming of CRC and pushes forward the development of CRC.

Many recent studies have revealed that circ_RNA can act as a ceRNA to relieve the inhibi-

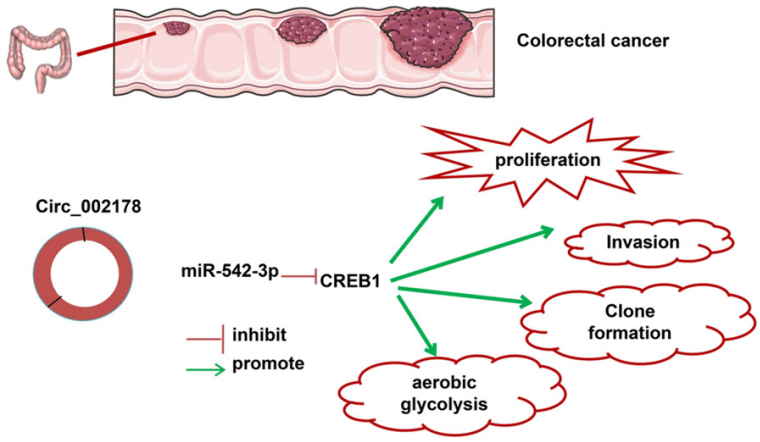


Figure 9. circRNA_002178, as a ceRNA, affects the proliferation, invasion, cloning, and aerobic glycolysis of colorectal cancer cells by regulating the miR-542-3p/CREB1 axis. ceRNA: competing endogenous RNA.

tory effect of miRNA on the target mRNA and form a biological regulatory network of circ_RNA-miRNA-mRNA [24]. In this study, our bioinformatics and experimental analyses revealed that circ_002178 regulates miR-542-3p in a targeting way. miR-542-3p has been confirmed in several studies to be a tumor suppressor. For example, circ_PGAM1 promotes the malignant progression of epithelial ovarian cancer by inhibiting the function of miR-542-3p [25]. miR-542-3p expression is significantly reduced in osteosarcoma tissues and cell lines, and the restoration of miR-542-3p expression can inhibit cell proliferation and induce cell apoptosis [26]. In addition, miR-542-3p can inhibit CRC cell proliferation, migration and invasion by targeting OTUB1 [27]. miR-542-3p inhibits CRC cell growth and invasion by targeting the regulation of cortin [28]. In this study, the miR-542-3p expression was lowered in CRC tissues and cell lines, which is consistent with previous studies and had a negative correlation with circ_002178 expression. The down-regulation of miR-542-3p partly offset the inhibitory effect of circ_002178 knockdown on CRC cell growth, suggesting that circ_002178 promotes tumor cell growth by down-regulating miR-542-3p.

Based on the above studies, the downstream target genes of miR-542-3p were screened. The bioinformatics websites and assay demonstrated that miR-542-3p can directly target the regulation of CREB1. CREB1 has been proved to be highly expressed in CRC and a potential tumor promoter [29]. CREB1 has been shown to be involved in the glycolysis of CRC [30]. In this study, CREB1 expression was increased in both the CRC tissues and cell lines, which is consistent with previous studies. The CREB1 expression has a positive correlation with circ_002178 expression in CRC, and circ_002178 can positively regulate CREB1 through miR-542-3p. The inhibitory effect of the circ_002178 knockdown on the CRC cells was partly offset by the overexpression of CREB1, suggesting that circ_002178 has a biological effect by up-regulating CREB1.

In this study, the function of circ_002178 was not further confirmed using in vivo experiments. Only miR-542-3p/CREB1 was selected as the downstream target of circ_002178 for investigation. Whether circ_002178 plays a biological effect through other mechanisms remains to be further explored. In addition, whether circ_002178 can affect the autophagy, pyroptosis, and ferroptosis of the CRC cells is also a direction for our subsequent studies.

In conclusion, circ_002178 up-regulates CREB1 expression by competitively sponging miR-542-3p to boost CRC cell glycolysis, proliferation, invasion, and clone formation. The mechanism diagram of this study is shown in **Figure 9**.

Disclosure of conflict of interest

None.

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