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CircRNA UBAP2 facilitates the progression of colorectal cancer by regulating miR-199a/VEGFA pathway

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Abstract. – OBJECTIVE: The aim of this study is to explore the regulatory mechanism of circRNA UBAP2 (circUBAP2) in colorectal cancer (CRC).

PATIENTS AND METHODS: The expression levels of circUBAP2, miR-199a, and VEGFA in tissues and cell lines were detected by RT-qPCR. The cell proliferation was examined by CCK-8 and colony formation assays. The migration and invasion abilities were evaluated by wound healing and transwell assays, respectively. Bioinformatics analysis and Luciferase activity assay were applied to determine the interaction between genes.

RESULTS: The expression of circUBAP2 was upregulated in CRC tissues and cell lines, and depletion of circUBAP2 suppressed the cell proliferation, migration, and invasion of CRC. Furthermore, miR-199a inhibitor abrogated the suppressive effect of circUBAP2 knockdown on CRC progression. Vascular endothelial growth factor A (VEGFA) was identified as a downstream target gene of miR-199a, and overexpression of VEGFA rescued the tumor phenotypes attenuated by circUBAP2 knockdown or miR-199a overexpression.

CONCLUSIONS: Our findings demonstrated that circUBAP2 facilitated CRC progression by sponging miR-199a to upregulate VEGFA. These findings implied that circUBAP2 may be a potential therapeutic biomarker for CRC.

Key Words:
CRC, CircUBAP2, MiR-199a, VEGFA.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide, which has become a severe threat to public health due to its high morbidity and mortality[1,2]. Although significant progress has been made in the diagnosis and surgical treatment of CRC over the past few decades, the 5-year survival rate of CRC patients is still unsatisfactory[3,4]. Therefore, it is imperative to identify novel targets to develop effective therapeutic strategies for CRC.

Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) with a ring structure[5]; their dysregulation is associated with tumorigenesis in various types of cancer, including CRC[6,7]. CircFMN2 promoted the cell proliferation of CRC by upregulating hTERT *via* sponging miR-1182[8]. CircRNA UBAP2 (circUBAP2) was reported to promote ovarian cancer progression by downregulating miR-114[9]. However, the underlying molecular mechanisms of circUBAP2 in CRC tumorigenesis are still unknown.

MiRNAs are a family of small ncRNAs of ~22 nucleotides in length, which regulate gene expression by complementary binding or complex mechanisms[10]. They play a vital role in the occurrence and development of cancers, including CRC. Of note, Yang et al[11] demonstrated that miR-125 inhibited the proliferation and invasion of CRC cells *via* binding to transcriptional co-activator tafazzin (TAZ). In addition, miR-199a was reported to function as a tumor suppressor in the occurrence and progression of different types of cancer[12]. Li et al[13] showed that miR-199a inhibited non-small cell lung cancer (NSCLC) progression by downregulating MAP3K1. CircRNAs have been reported to function as competing endogenous RNAs (ceRNAs) to regulate the development and progression of several types of cancers[14-16] which led to the hypothesis that circUBAP2 may promote CRC progression *via* miR-199a.

The present study demonstrated that circUBAP2 promoted CRC progression by regulating

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the miR-199a/VEGFA axis. Our research confirmed the oncogenic role of circUBAP2 in CRC and might provide novel potential mechanisms and theoretical basis for the targeted treatment of CRC.

Patients and Methods

Clinical Specimens

22 pairs of CRC tissues and para-carcinoma tissues were obtained from patients in the Third Affiliated Hospital of Soochow University. All subjects provided written informed consent prior to their inclusion. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.

Stable Cell Lines Generation and Transfection

CRC cell lines (SW620, HCT116), human normal colon mucosal cell line (NCM460) and 293T cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM; Corning Life Sciences, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, MD, USA) in a humidified incubator containing 5% CO_2 at 37°C. For transfection experiments, short-hairpin RNAs (shRNAs) against circUBAP2 (shcircUBAP2) and negative control (shNC), miR-199a mimics, miR-199a inhibitor and their correspondent negative controls were obtained from Genechem (Shanghai, China). The transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

CCK-8 Assay

Cells seeded into 96-well plates (2×10^4 cells/well) were incubated with 100 µL Dulbecco's Modified Eagle Medium (DMEM; Corning Life Sciences, MA, USA) for 48 h at 37°C and 5% CO_2. Following the addition of 10 µL CCK-8 solution (Dojindo, Molecular Technologies, Kumamoto, Japan) into each well, then incubated for an additional 2 h at 37°C and the absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Wound Healing Assay

The cells were plated into 6-well plates and grown to a fully confluent monolayer. A 200 µL pipette tip was used to generate artificial scratches. Images of the migrated cells were acquired at 0 and 24 h using an optical microscope (Leica DMI4000B, Milton Keynes, Bucks, UK).

Transwell Assay

Transwell assay was utilized to determine the cell invasion ability. Briefly, diluted Matrigel (Yeasen Biotech, Shanghai, China) was added onto the membrane of the upper chamber. The transfected cells were then seeded into the upper chambers of the transwell insert. Next, 600 µL Dulbecco's Modified Eagle Medium (DMEM; Corning Life Sciences, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, MD, USA) was added to the lower chamber. Following incubation for 48 h, cells in the lower chamber were fixed with 95% ethanol and stained with crystal violet. The invaded cells were observed under a microscope and counted in five random fields.

Luciferase Reporter Assays

The pmirGLO-circUBAP2-Wt/Mut and pmirGLO-VEGFA-Wt/Mut reporter plasmids were obtained from GenePharma (Shanghai, China). Subsequently, 293T cells were co-transfected with pmirGLO-circUBAP2-Wt/Mut or pmirGLO-VEGFA-Wt/Mut plasmids and miR-199a or NC mimics. The luciferase activity was evaluated by Luciferase Reporter Analysis system (Promega, Madison, WI, USA).

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was determined using a Nano Drop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa Biotechnology Ltd., Dalian, China). PCR amplification reaction was prepared using a SYBR® Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method, and U6 and GAPDH were employed as the internal controls. The primer sequences were listed in Table I.

Statistical Analysis

All data are presented as mean±standard deviation (SD) of three individual experiments. Statistical analyses were performed using SPSS 18.0 software (IBM Inc., Chicago, IL, USA) and comparative analyses were assessed using one-

Table I. The primer sequences for RT-qPCR

Gene	Sequence of primers (5′-3′)
circUBAP2 forward	AGCCTAGAGCCAACTCCTTTG
circUBAP2 reverse	TCAGGTTGAGATTTGAAGTCAAGA
miR-199a forward	GCGGCGGACAGTAGTCTGCAC
miR-199a reverse	ATCCAGTGCAGGGTCCGAGG
VEGFA forward	AGGAGGAGGGCAGAATCATCA
VEGFA reverse	CTCGATTGGATGGCAGTAGCT
U6 forward	CTCGCTTCGGCAGCACATATACTA
U6 reverse	ACGAATTTGCGTGTCATCCTTGCG
GAPDH forward	GATGATCTTGAGGCTGTTGTC
GAPDH reverse	CAGGGCTGCTTTTAACTCTG

way analysis of variance (ANOVA) and Student's *t*-test. $p<0.05$ was considered as statistically significant.

Results

CircUBAP2 Was Upregulated in CRC Tissues and Cell Lines and Facilitated CRC Progression

RT-qPCR results demonstrated that circUBAP2 expression was significantly upregulated in CRC tissues (or cell lines including SW620 and HCT116) compared with that in the normal tissues (or human normal colon mucosal cell line NCM460) (Figure 1A and B), suggesting that circUBAP2 might act as an oncogenic factor for CRC progression. To further investigate the biological function of circUBAP2 in CRC, circUBAP2 knockdown (shcircUBAP2) stable cell lines (SW620 and HCT116) were constructed. The knockdown efficiency of circUBAP2 was confirmed by RT-qPCR (Figure 1C). CCK-8 and colony formation assays suggested that proliferation ability was decreased in CRC cells transfected with shcircUBAP2 (Figure 1D and E). Furthermore, wound healing and transwell assays revealed that CRC cell migratory and invasive abilities were suppressed following silencing of circUBAP2 (Figure 1F and G). In summary, our results suggested that circUBAP2 knockdown inhibited CRC cell progression.

MiR-199a Inhibitor Partially Abrogated the Inhibitory Effect of CircUBAP2 Knockdown on CRC Phenotypes

Bioinformatics prediction results displayed that miR-199a has the binding ability for circUBAP2 (Figure 2A). Luciferase reporter assay was conducted to validate the interactions between circUBAP2 and miR-199a, and results revealed that miR-199a decreased the Luciferase activity of pmirGLO-circUBAP2-Wt vector, while no significant alteration in the Luciferase activity of pmirGLO-circUBAP2-Mut vector was observed (Figure 2B). Additionally, miR-199a mimics reduced circUBAP2 expression, suggesting miR-199a could directly target circUBAP2 and inhibit its expression (Figure 2C). To further determine the effect of miR-199a on shcircUBAP2-silenced CRC cells, miR-199a inhibitor was introduced into shcircUBAP2-expressing SW620 cells. The data showed that miR-199a expression was increased by circUBAP2 knockdown and the restoration of miR-199a expression occurred after the transfection of miR-199a inhibitor (Figure 2D). CCK-8 and colony formation assays exhibited that cell proliferation suppressed by knockdown of circUBAP2 was increased by inhibition of miR-199a in SW620 cells (Figure 2E and F). Besides, the repression of cell invasion and migration abilities caused by silencing of circUBAP2 was abolished by miR-199a inhibitor (Figure 2G and H). Taken together, miR-199a downregulation reversed the inhibitory effect of circUBAP2 knockdown on the proliferation and mobility of CRC cells.

MiR-199a Could Directly Target VEGFA and Inhibit its Expression

According to TargetScan (www.targetscan.org), VEGFA has a binding site for miR-199a in the 3'UTR region (Figure 3A). Luciferase reporter assay indicated that miR-199a mimics and miR-199a inhibitor markedly reduced and increased the luciferase activity compared with the correspondent negative controls respectively, while there was no effect on cells transfected with mutant 3'-UTR of VEGFA (Figure 3B). More-

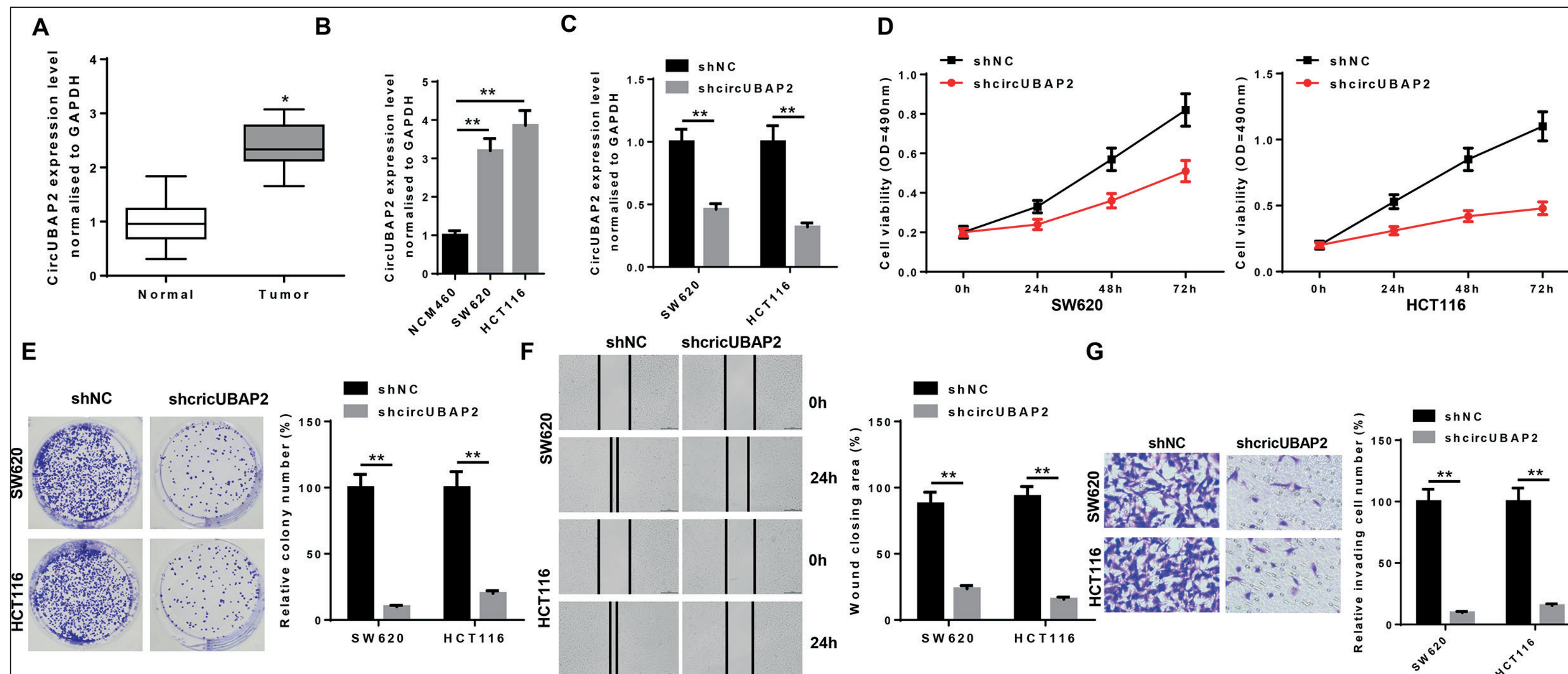

Figure 1. CircUBAP2 was upregulated in CRC tissues and cell lines, and promoted CRC progression. **A,** RT-qPCR analysis showed the relative expression of circUBAP2 expression in CRC tissues and para-carcinoma tissues, n=22. **B,** RT-qPCR analysis showed the relative expression of circUBAP2 expression in CRC cell lines (SW620 and HCT116) and human normal colon mucosal cell line (NCM460). **C,** RT-qPCR analysis showed the relative expression of circUBAP2 expression in SW620 and HCT116 transfected with shNC or shcircUBAP2. **D-E,** CCK-8 and colony formation assays showed the cell proliferation of SW620 and HCT116 transfected with shNC or shcircUBAP2 (magnification ×20). **F-G,** Wound healing and transwell assays showed the migration and invasion abilities of SW620 and HCT116 transfected with shNC or shcircUBAP2 (magnification ×40). The data were presented as mean ± SD (*p<0.05, **p<0.01).

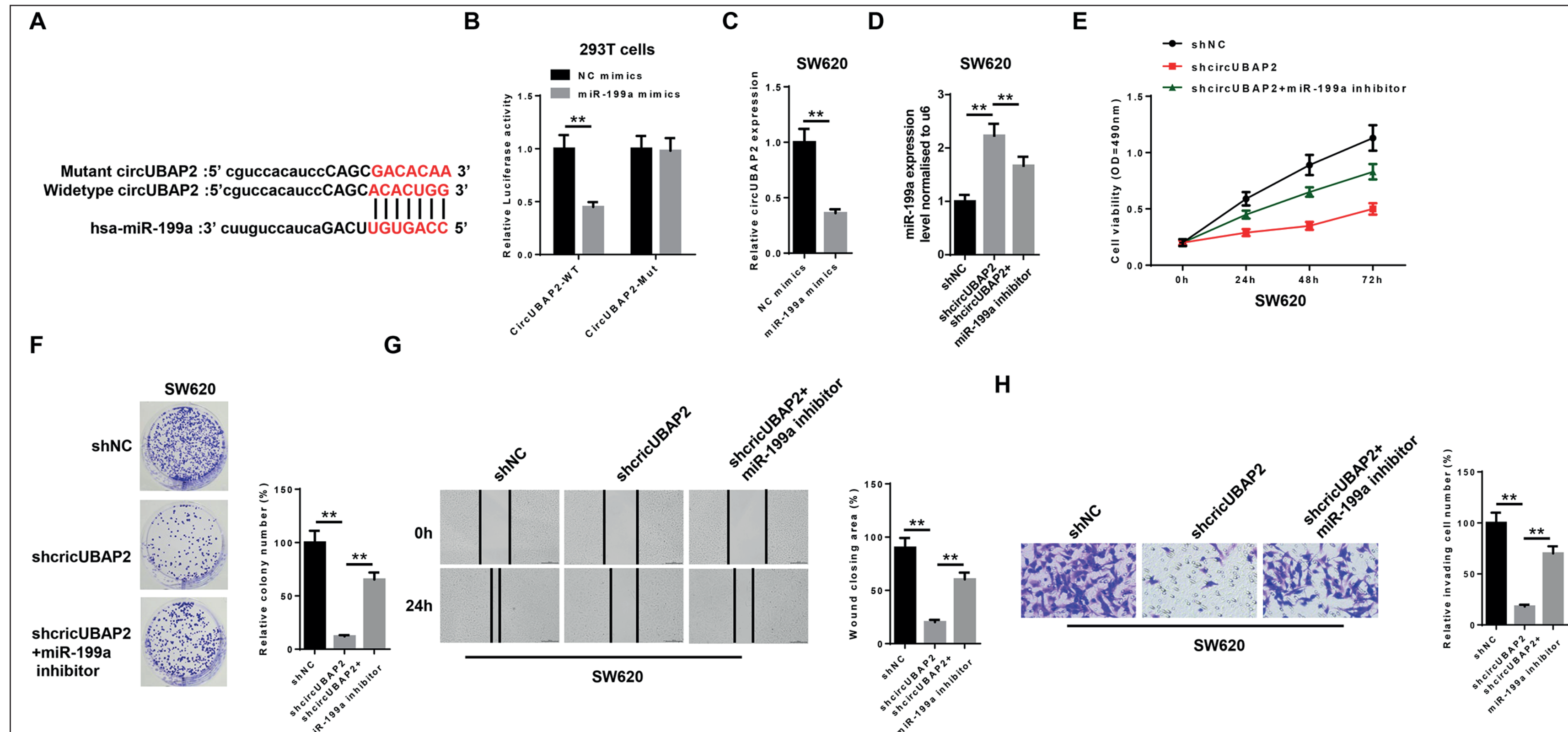

Figure 2. MiR-199a inhibitor rescues circUBAP2 knockdown-attenuated progression of CRC cells. **A,** Bioinformatic prediction of the putative binding site at 3'-UTR of circUBAP2 by miR-199a. **B,** Luciferase reporter assay showed luciferase activity in 293T cells transfected with NC mimics, miR-199a mimics. **C,** RT-qPCR analysis showed the relative expression of miR-199a in SW620 cells transfected with NC mimics, miR-199a mimics. **D,** RT-qPCR analysis showed the relative expression of miR-199a in SW620 cells transfected with shNC, shcircUBAP2, shcircUBAP2 plus miR-199a inhibitor. **E-F,** CCK-8 and colony formation assays showed the cell proliferation of SW620 cells transfected with shNC, shcircUBAP2, shcircUBAP2 plus miR-199a inhibitor (magnification ×20). **G-H,** Wound healing and transwell assays showed the cell migration and invasion abilities of SW620 cells transfected with shNC, shcircUBAP2, shcircUBAP2 plus miR-199a inhibitor (magnification ×40). The data were presented as mean ± SD (**p<0.01).

over, RT-qPCR analysis revealed that the VEGFA expression was obviously reduced in miR-199a mimics transfected cells (Figure 3C). Our results demonstrated that miR-199a directly interacted with VEGFA and significantly suppressed its expression level.

CircUBAP2 promoted CRC cell progression via the miR-199a/VEGFA axis

To investigate whether VEGFA mediated circUBAP2/miR-199a-regulated CRC phenotypes, VEGFA-overexpressed shcircUBAP2 and miR-199a mimics SW620 cells were established. The transfection efficiency was determined by RT-qPCR (Figure 4A). CCK-8 and colony formation assays showed suppression of circUBAP2 or overexpression of miR-199a reduced cell proliferation, which was reversed by the upregulation of VEGFA (Figure 4B and C). Moreover, overexpression of VEGFA counteracted the inhibitory effect of circUBAP2 knockdown or miR-199a overexpression on cell migration and invasion (Figure 4D and E). Collectively, these data revealed that circUBAP2/miR-199a/VEGFA axis promoted the development and progression of CRC.

Discussion

CircRNAs act as vital modulators in the tumorigenesis and development of diverse malignancies[17,18]. Wang et al[19] showed that the overexpression of circ-UBE2D2 was associated with poor prognosis and promoted breast cancer progression by regulating the expression of miR-1236 and miR-1287. Yao et al[20] demonstrated that circ_0058124 promoted papillary thyroid cancer progression *via* NOTCH3/GATAD2A signaling. However, only a limited number of studies have evaluated circRNA-mediated CRC progression. The present study revealed that circUBAP2 was upregulated in CRC tissues and cell lines. Furthermore, the depletion of circUBAP2 suppressed the cell proliferation, migration, and invasion of CRC, suggesting that circUBAP2 could serve as a carcinogenic factor in CRC progression.

Previous studies[6,21] have demonstrated that circRNAs interact with miRNAs by acting as ceRNAs to regulate the proliferation and migration of tumor cells. Zhang et al[22] reported that circ_0000285 acted as a ceRNA to facilitate the progression of osteosarcoma *via* sponging miR-599. In addition, Yang et al[23] revealed that circITGA7 suppressed the proliferation of CRC by sponging miR-3187 to upregulate additional sex combs-like 1 (ASXL1) expression. In the current study, miR-199a was predicted to be a direct downstream target gene of circUBAP2 and the upregulation of miR-199a markedly suppressed circUBAP2 expression. Moreover, we found that miR-199a depletion abolished the inhibitory effect of circUBAP2 knockdown on cell phenotypes of CRC.

VEGFA belongs to the VEGF family, which plays a crucial role in cell proliferation, invasion, and angiogenesis in several malignancies[24,25]. Several miRNAs inhibit tumor growth, metastasis, and angiogenesis[26-28] by directly or indirectly targeting VEGFA. For example, Feng et al[26] discovered that miR-26b suppressed tumor growth of hepatocellular carcinoma by targeting

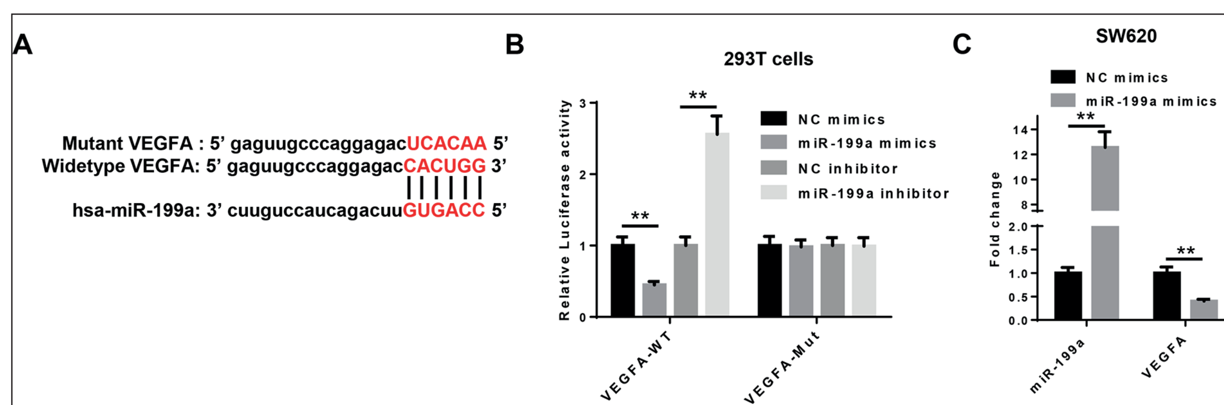

Figure 3. MiR-199a directly targets VEGFA. **A,** Bioinformatic prediction of the putative binding site at 3'-UTR of VEGFA by miR-199a. **B,** Luciferase reporter assay showed luciferase activity in 293T cells transfected with NC mimics, miR-199a mimics, miR-199a inhibitor and NC inhibitor. **C,** RT-qPCR analysis showed the relative expression of miR-199a and VEGFA in SW620 cells transfected with NC mimics, miR-199a mimics. The data were presented as mean ± SD (***p*<0.01).

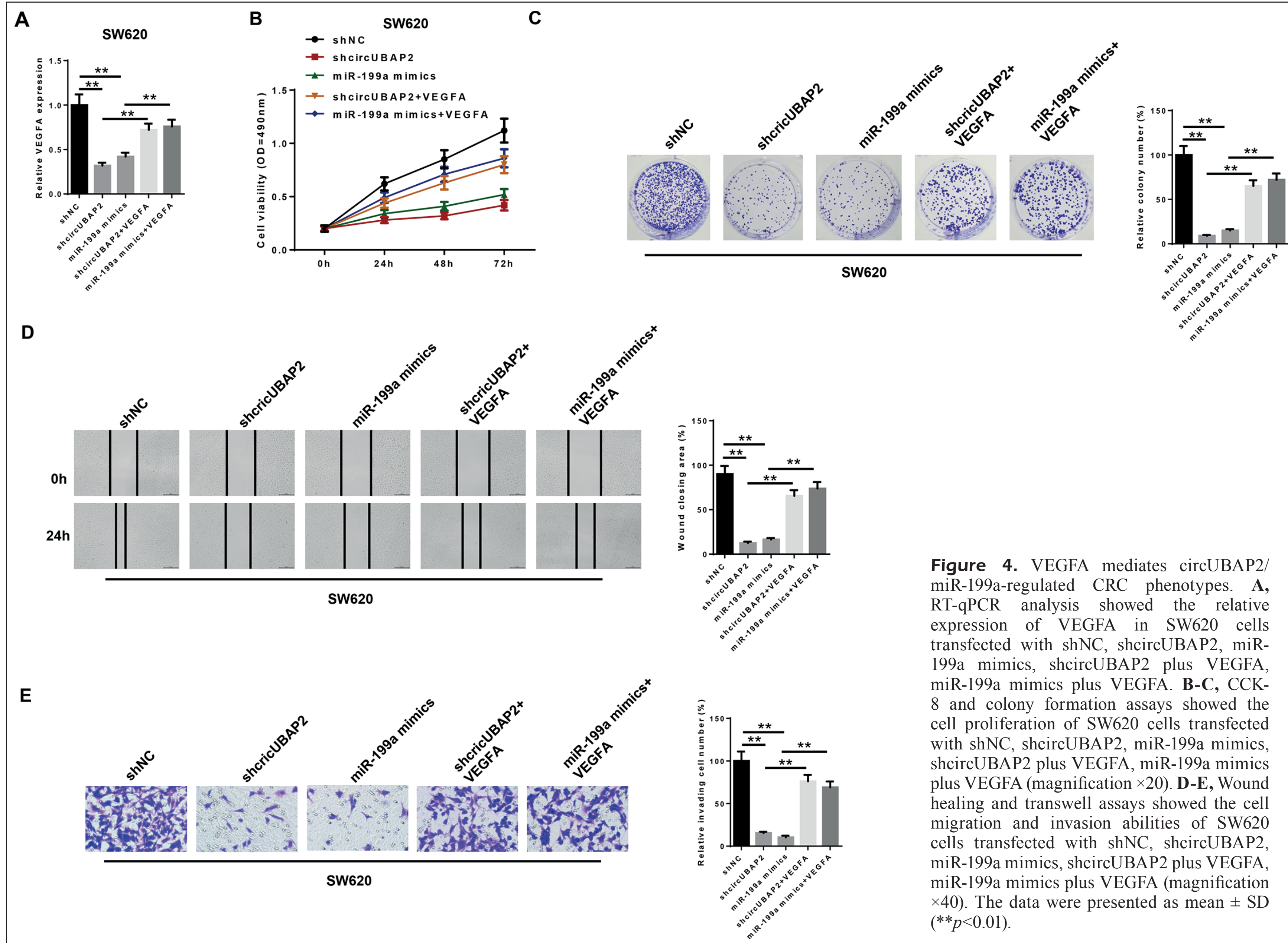

Figure 4. VEGFA mediates circUBAP2/miR-199a-regulated CRC phenotypes. **A,** RT-qPCR analysis showed the relative expression of VEGFA in SW620 cells transfected with shNC, shcircUBAP2, miR-199a mimics, shcircUBAP2 plus VEGFA, miR-199a mimics plus VEGFA. **B-C,** CCK-8 and colony formation assays showed the cell proliferation of SW620 cells transfected with shNC, shcircUBAP2, miR-199a mimics, shcircUBAP2 plus VEGFA, miR-199a mimics plus VEGFA (magnification ×20). **D-E,** Wound healing and transwell assays showed the cell migration and invasion abilities of SW620 cells transfected with shNC, shcircUBAP2, miR-199a mimics, shcircUBAP2 plus VEGFA, miR-199a mimics plus VEGFA (magnification ×40). The data were presented as mean ± SD (**p<0.01).

VEGFA via NF-κB/MMP-9 signaling. Wang et al[29] reported that miR-377 inhibited gastric cancer metastasis by downregulating VEGFA. In our study, VEGFA was predicted as a downstream target of miR-199a by bioinformatics analysis, which was further validated by Luciferase reporter assay. The results prompted us to make subsequent experiments to verify the directly regulatory axis between circUBAP2/miR-199a and VEGFA in CRC cells. Rescue experiments showed the overexpression of VEGFA enhanced the CRC progression attenuated by circUBAP2 knockdown or miR-199a overexpression.

Conclusions

Our results revealed that circUBAP2 upregulated VEGFA expression through sponging miR-199a and accelerated CRC progression. This study offers an improved understanding of the pathogenesis of CRC and may provide potential therapeutic strategies for CRC treatment. However, *in vivo* experiments are required to further validate these findings in follow-up studies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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