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ORIGINAL RESEARCH

RETRACTED ARTICLE: Hsa_circRNA_000166 Promotes Cell Proliferation, Migration and Invasion by Regulating miR-330-5p/ELK1 in Colon Cancer

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Introduction: Circular RNAs (circRNAs), a novel class of non-coding RNAs, which are widely expressed in human cells, have essential roles in the development and progression of cancers. The aim of this study is to figure out the role of circ_000166 in colon cancer (CC) development and the signaling pathway involved.

Materials and Methods: HT29 and HCT116 cells were transfected with siRNA of circRNA, miRNA mimics and inhibitors. Cell proliferation, migration and invasion were examined using CCK-8 assay and transwell assay, respectively. Luciferase reporter assay was used to validate the targets of circRNA and miRNA. CC cells were implanted into nude mice subcutaneously to detect tumor growth.

Results: hsa_circRNA_000166 was significantly upregulated in the human CC tissue and in the CC cell lines. Knockdown of hsa_circRNA_000166 reduced cell viability, colony formation, migration and invasion in vitro and decreased tumor size and weight in vivo. Luciferase reporter assay revealed that miR-330-5p was the target of circRNA_000166. miR-330-5p could bind to 3′ untranslated region (3′UTR) of ELK1 to downregulate both mRNA and protein expression of ELK1. Dual inhibition of circRNA_000166 and miR-330-5p inhibited the suppression of cell proliferation, migration and invasion induced by si-circRNA_000166.

Conclusion: The data of this study demonstrated that the hsa_circRNA_000166 could upregulate the expression of gene *ELK1* by sponging miR-330-5p, which may contribute to a better understanding of the regulatory circRNA/miRNA/mRNA network and CC pathogenesis.

Keywords: colon cancer, circRNA_000166, miRNA-330-5p, ELK1

Introduction

Colon cancer (CC) is the third most common cancer (1.8 million new cases reported in 2018) in the world and the fourth one to cause death.[1,2] Global burden of CC is estimated to be augmented by 60%, which means more than 2.2 million new cases and 1.1 million deaths in 2030.[3] CC is considered as a "lifestyle" disease because the mortality and morbidity are associated with diet, obesity and carcinogenesis.[4–6] Many signaling pathways are involved in the development of CC such as PTEN-Akt, NF-κB, AMPK-COX-2, as well as ELK1.[7–9] ELK1 is one of the transcription factors belonging to ETS family,[10] which regulates cell proliferation, angiogenesis, differentiation and apoptosis.[11] Upregulation of ELK1 has been found to promote cervical cancer,[12] thyroid cancer progression[13] and urothelial tumorigenesis.[14] Therefore, ELK1 expression and activation plays a crucial role in tumorigenesis.

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microRNAs (miRNAs), a class of noncoding RNAs with ~22 nucleotides, can induce translational suppression through binding to the 3′-untranslated region (3′UTR) of their target mRNAs.[15] Dysregulation of miRNAs is linked to carcinogenesis.[16] More and more studies are focusing on interactions between circular RNAs (circRNAs) and miRNAs since biological effects of circRNAs are mainly mediated by miRNAs.[17,18] circRNAs are a novel class of non-coding RNAs with covalently closed continuous loops, which make circRNAs more stable than linear microRNAs.[19–22] Utilizing the high-throughput sequencing technology, more and more circRNAs have been found to involve in pathological process such as myocardial infarction, apoptosis, depression, as well as carcinomas.[23–26] circRNAs can act as a real sponge of miRNAs to regulate gene expression[26–28] and the circRNA-miRNA-mRNA network might play a key role in cancer related and non-cancer pathways.[29,30]

Recent studies have proved a global increase of circRNA expression in both CC cell lines and tumor tissues.[1,31] However, little is known about the role of circRNAs in the development of CC. This study aims to explore the role of circ_000166 on CC progression and the signaling pathway involved.

Materials and Methods

Cell Culture

Normal human colon mucosal epithelial cell line NCM460 and six colon cancer cell lines (HT29, HCT116, HCT8, LoVo, SW420 and SW620 cells) were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Eagles MEM (Sigma-Aldrich, USA) containing 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin/streptomycin (Invitrogen, USA) at 37 ºC with 95% air and 5% CO2.

RNA Extract and Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNA was extracted using Trizol reagent (TaKaRa, China) according to the manufacturer's instructions. 500 ng of total RNA was reverse transcribed to cDNA with the PrimeScript RT Master Mix (TaKaRa, China). The relative RNA expression was examined using the SYBR Premix Ex Taq II Kit (TaKaRa, China) on the StepOnePlus system (Applied Biosystems, USA). The primer sequences (Sigma-Aldrich, USA) used in this study are shown in Table 1. The data were calculated by means of the $2^{-\Delta\Delta Ct}$ method.

Table 1 Primer Sequences for qRT-PCR

Gene	Primer Sequences
GAPDH	*Forward: CCACATCGCTCAGACACCAT* *Reverse: CCAGGCGCCCAATACG*
circRNA_000166	*Forward: CCATATTGAATCACAGTGCGT* *Reverse: ACAGCGCAGTAAGGTGCTCG*
U6	Forward: CGCTTCGGCAGCACATATAC Reverse: TTCACGAATTTGCGTGTCAT
miR-330-5p	Forward: TCTCTGGGCCTGTGTCTTAGGC
ELK1	Forward: CCTTGCC[illegible]CTATGAC Reverse: CCTTG[illegible]GGTACTAC[illegible]TGAC

RNase R Treatment

Total RNA (10 μg) was incubated with or without 3 U·μg^{-1} of RNase R (Epicentre Biotechnologies, USA). After incubation at 37°C for 15 min, the RNA was subsequently purified by RNeasy MinElute Cleaning Kit (Qiagen, Germany) and then subjected to qRT-PCR.

Cell Viability and Colony Assay

Cell was seeded into sterile 96-well plates. After transfection, cell proliferation was measured at 0, 24, 48, 72 and 96 hours (h) using the Cell Counting Kit-8 (CCK-8) assay (Dojido, Japan) according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution was added to each well. The solution was then measured spectrophotometrically at 450 nm after 2-hour incubation at 37°C.

For colony formation assay, a total of 2000 stably transfected cells were seeded into 6-well plates and cultured for 2 weeks under standard conditions. Then, the colonies were washed with PBS, fixed with methanol and then stained with crystal violet. The number of clone spots was counted in 10 random view fields using a microscope (Olympus, Japan).

Cell Invasion and Migration Assays

Transwell assay was used to examine cell migration and invasion. For cell invasion assay, 1 × 10^6 of HT29 and HCT116 cells were seeded into the upper chamber of a 24-well insert (8-μm pore size; Corning Inc., USA) precoated with Matrigel. The upper chamber was filled with serum-free medium while the lower chamber was filled with FBS-contained medium. The cells in the upper chamber were removed and the invading cells were fixed with methanol and stained with crystal violet after incubation for 48 h. Cells

from five random fields were counted under a 200× microscope. For cell migration assay, the upper chambers were not coated with Matrigel, and the following protocols were the same as what was conducted for cell invasion assay.

Plasmid and Luciferase Reporter Assay

This protocol followed the published paper.[32] Briefly, the full-length of *ELK1* 3′-UTR containing (wt) and scrambled (mut) miR-330-5p binding sequence was inserted downstream of the firefly luciferase gene in psiCHECK2 to generate the psiCHECK2-*ELK* 3′UTR-wt or cirRNA_000166 wt plasmid and psiCHECK2-*ELK* 3′UTR-mut plasmid or cirRNA_000166 mut, respectively. The wt and mut plasmids subsequently were co-transfected into CC cells with negative control, miR-330-5p mimics, si-circRNA_000166 along with control Renilla luciferase expression plasmid (phRL-TK) using Lipofectamine 2000 (Invitrogen, USA). After 24 h, luciferase and renilla signals were assayed using the Dual-luciferase reporter Assay System (Promega, USA) according to the manufacturer's instructions.

Western Blotting

Protein was extracted using RIPA cell lysis buffer (Beyotime, China). 10 μg of protein was electrophoresed on a 10% polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Hybond; USA). Membranes were blocked for 1 h with 5% milk and then probed with the indicated primary antibodies and the appropriate secondary antibodies (Cell Signaling Technology, USA). Finally, blots were detected using a chemiluminescence reagent kit (Merck KGaA, Germany).

Tumor Xenografts in Nude Mice

Male BALB/c nude mice (6–8 weeks) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) and kept under the environment of 23 ± 2°C, 55 ± 15% humidity, 12 h light/12 h dark cycle. Negative control cells or treated cells with the indicated lentivirus vector with a concentration of 1×10^7/mL diluted in PBS. 0.1 mL of this solution was injected subcutaneously on the back flank of each mouse at day 0. Tumor size was measured with a caliper every 7 days until 35 days. The tumor weight was weighed every 7 days until 35 days.

All experiment procedures were approved and carried out following the ethical standards under a protocol approved by the Committee on Animal Welfare of Suqian First Hospital, and were executed conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (No. 85–23, 1996).[33]

Bioinformatics Analysis

CircInteractome (https://circinteractome.nia.nih.gov/) was used to predict miRNA-330-5p binding sites to the hsa_circRNA_000166 and TargetScan (http://www.targetscan.org/) was used to predict the potential miR-330-5p binding sites to 3′UTR of *ELK1* to study the possible crossing network among circRNA, miRNA and target mRNA.

Statistical Analysis

Results have been presented as mean ± SEM. All statistical analysis was performed via the Pearson chi-squared test, two-tailed Student's *t*-test, or analysis of variance (ANOVA) GraphPad Prism 7.0. (GraphPad Software, USA). $p < 0.05$ was considered statistically significant.

Results

circRNA_000166 Expression Was Upregulated in Colon Cancer Cell Lines and Tissues

To investigate the dysregulated circRNAs in CC tissue, we analyzed 10 pairs of human CC tissue and their adjacent normal tissue from GSE126094 database and figured out the top 15 upregulated and downregulated circRNAs (Figure 1A). hsa_circRNA_000166 was chosen for further study. qRT-PCR results of 30 pairs of human CC tissue and their adjacent normal tissue showed that circRNA_000166 expression was elevated in CC tissue (Figure 1B). The qRT-PCR data from six CC cell lines (HT29, HCT116, HCT8, LoVo, SW420 and SW620 cells) also demonstrated that circRNA_000166 expression was higher than that in NCM 460 cells (Figure 1C).

RNase R treatment was used to confirm the circular characteristics of circRNA_000166. The results manifested that the circRNA_000166 expression did not change while the linear control gene GADPH expression was significantly reduced with the treatment of RNase R in both HT29 and HCT116 cells (Figure 1D). Further experiments demonstrated that circRNA_000166 was mainly localized in cytoplasm (Figure 1E)

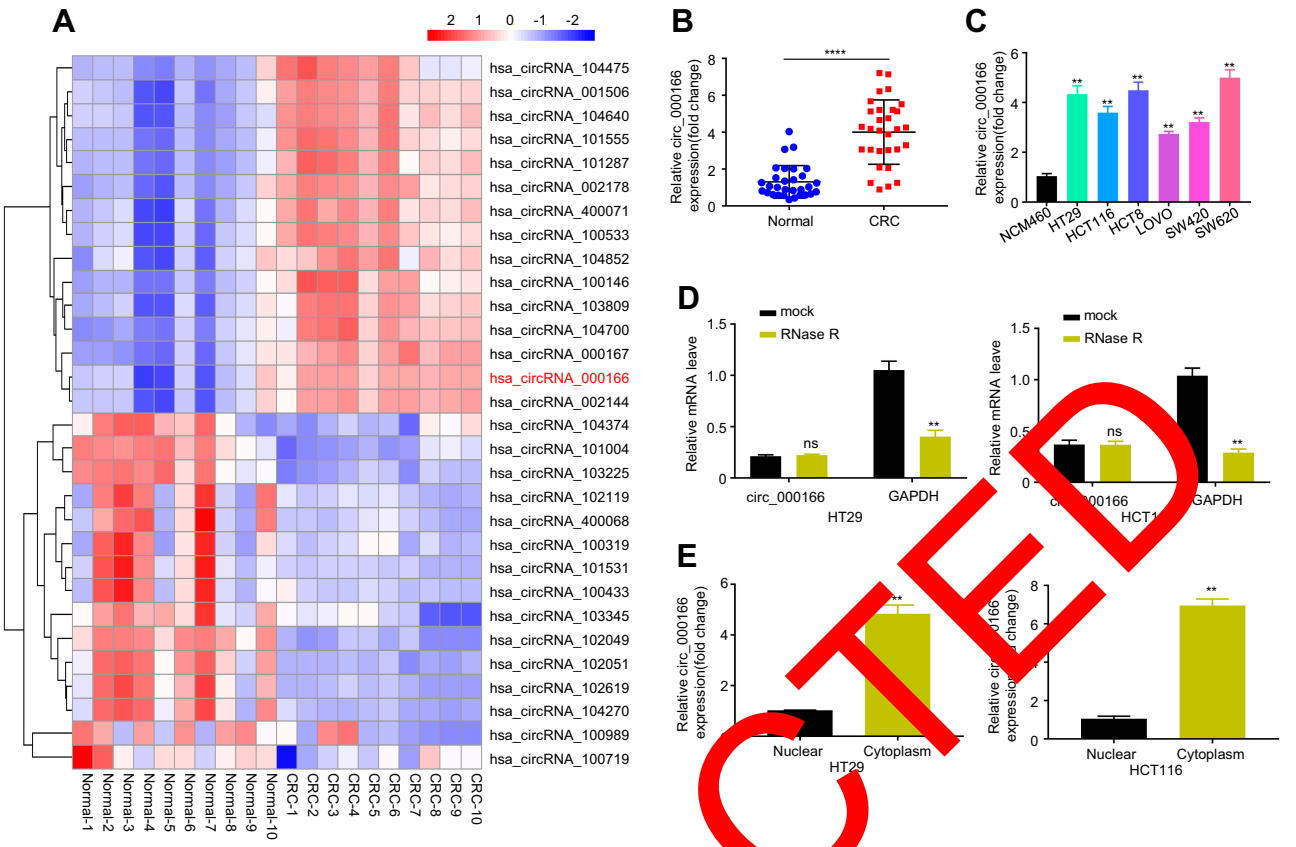

Figure 1 circRNA_000166 expression was increased in colon cancer. (**A**) Hierarchical clustering analysis of the top 15 upregulated and downregulated circRNAs in CC; (**B**) Relative circRNA_000166 expression in CC tissue and their adjacent normal tissue using qRT-PCR assay; (**C**) Relative circRNA_000166 expression in different CC cell lines (HT29, HCT116, HCT8, LOVO, SW420 and SW620 cells); (**D**) Relative circRNA_000166 expression and GAPDH in HT29 and HCT116 cells treated with RNase R; (**E**) Localization of circRNA_000166 in HT 29 and HCT116 cells. $^{**}p$ < $^{****}p$ < 0.0001; ns: no significance.
Abbreviation: CC, colon cancer.

circRNA_000166 Knockdown Inhibited Colon Cancer Proliferation, Migration and Invasion

To figure out the effects of circRNA_000166 in CC, Scramble RNA (si-NC) and circRNA_000166 siRNA (si-circRNA_000166) was transfected into HT29 and HCT116 cells. The data demonstrated that circRNA_000166 expression was significantly reduced (Figure 2A), indicating that the function of circRNA_000166 was inhibited. CCK-8 assay revealed that downregulation of circRNA_000166 decreased the proliferative ability of HT29 and HCT116 cells (Figure 2B). Inhibition of circRNA_000166 reduced the number of clone spots in HT29 and HCT116 cells compared with the control group (Figure 2C). 1×10^6 of HT29 and HCT116 cells were transfected with si-NC and si-circRNA_000166, and then implanted into nude mice subcutaneously. Subcutaneous tumor size and tumor weight were smaller in the si-circRNA_000166 group compared with si-NC group (Figure 2D). Transwell assay without and with Matrigel was used to examine HT29 and HCT116 cells migration and invasion, respectively. Both migrated cells and invaded cells were decreased in si-circRNA_000166 group (Figure 2E and F).

circRNA_000166 Sponged miR-330-5p

Many studies have revealed that circRNAs can act as a sponge of miRNAs to regulate gene expression.[26–28] A complementary sequence was observed between circRNA_000166 and miR-330-5p predicted using CircInteractome database (Figure 3A). Luciferase reporter assay indicated that miR-330-5p expression was reduced in WT circRNA_000166 transfected cells (Figure 3A). Furthermore, RNA pull-down assay demonstrated that circRNA_000166 were enriched in miR-330-5p group (Figure 3B). Inhibition of circRNA_000166 induced upregulation of miR-550-3p (Figure 3C).

qRT-PCR assay was conducted to examine miR-330-5p expression in 10 pairs of human CC tissue and their adjacent normal tissue. The data showed that miR-330-5p expression was decreased in CC tissue compared with

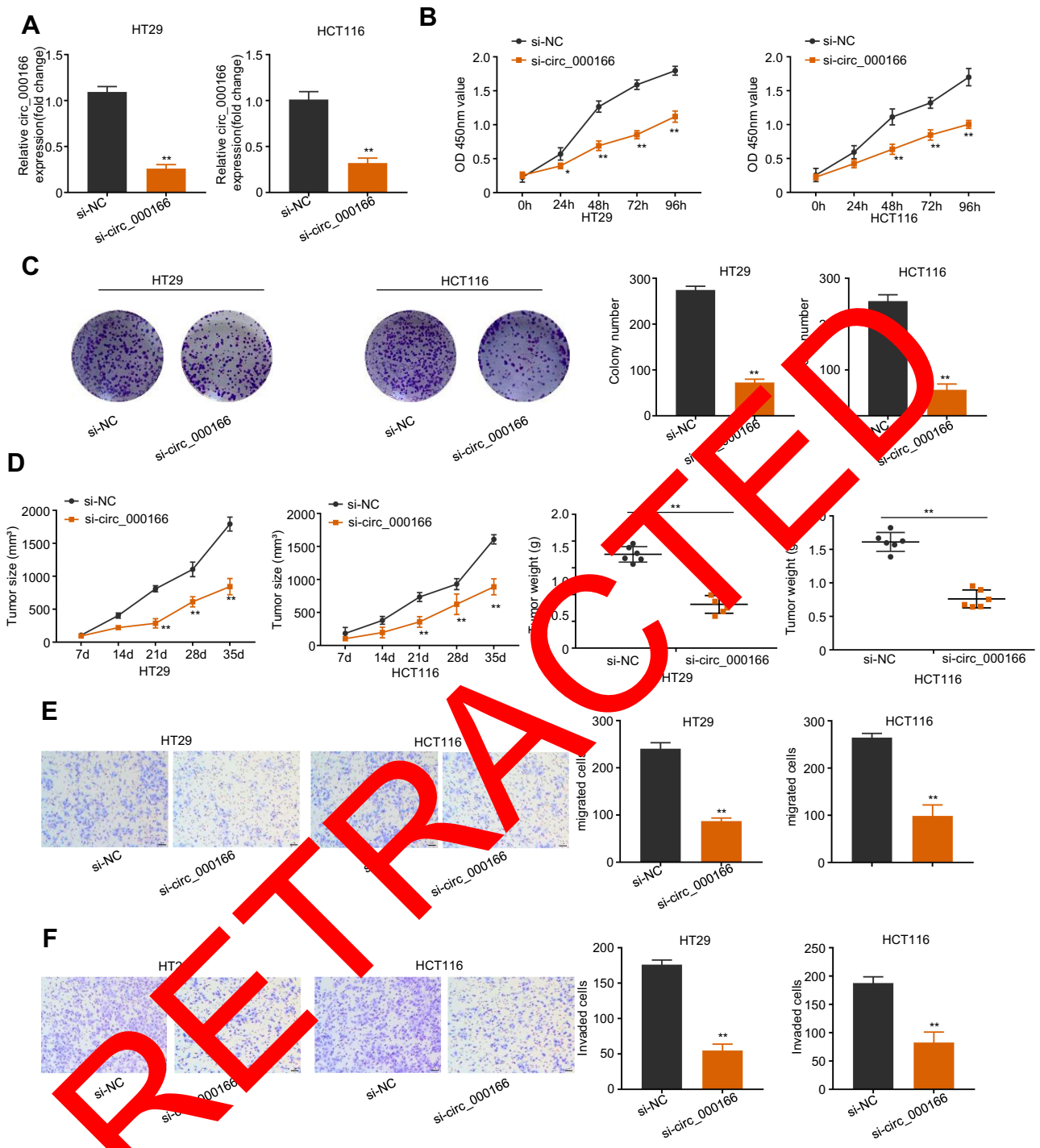

Figure 2 circRNA_000166 knockdown inhibited colon cancer proliferation, migration and invasion. (**A**) qRT-PCR analysis of knockdown efficiency of hsa_circ_000166 in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166. (**B**) CCK-8 assay results of cell viability in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166; (**C**) Colony formation in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166; (**D**) Tumor size and weight in nude mice implanted subcutaneously with 1×10^6 of HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166 (**E**) HT29 and HCT116 cell migration using transwell assay; (**F**) HT29 and HCT116 cell invasion using transwell assay. *$p < 0.05$; **$p < 0.01$.

Abbreviations: si-NC, Scramble siRNA; si-circRNA_000166, siRNA of circRNA_000166.

normal tissue (Figure 3D). Consistently, TCGA database demonstrated miR-330-5p expression was lower in CC tissue than that in normal tissue (Figure 3E). A negative correlation was observed between the expression of circRNA_000166 and miR-330-5p human CC tissue (Figure 3F).

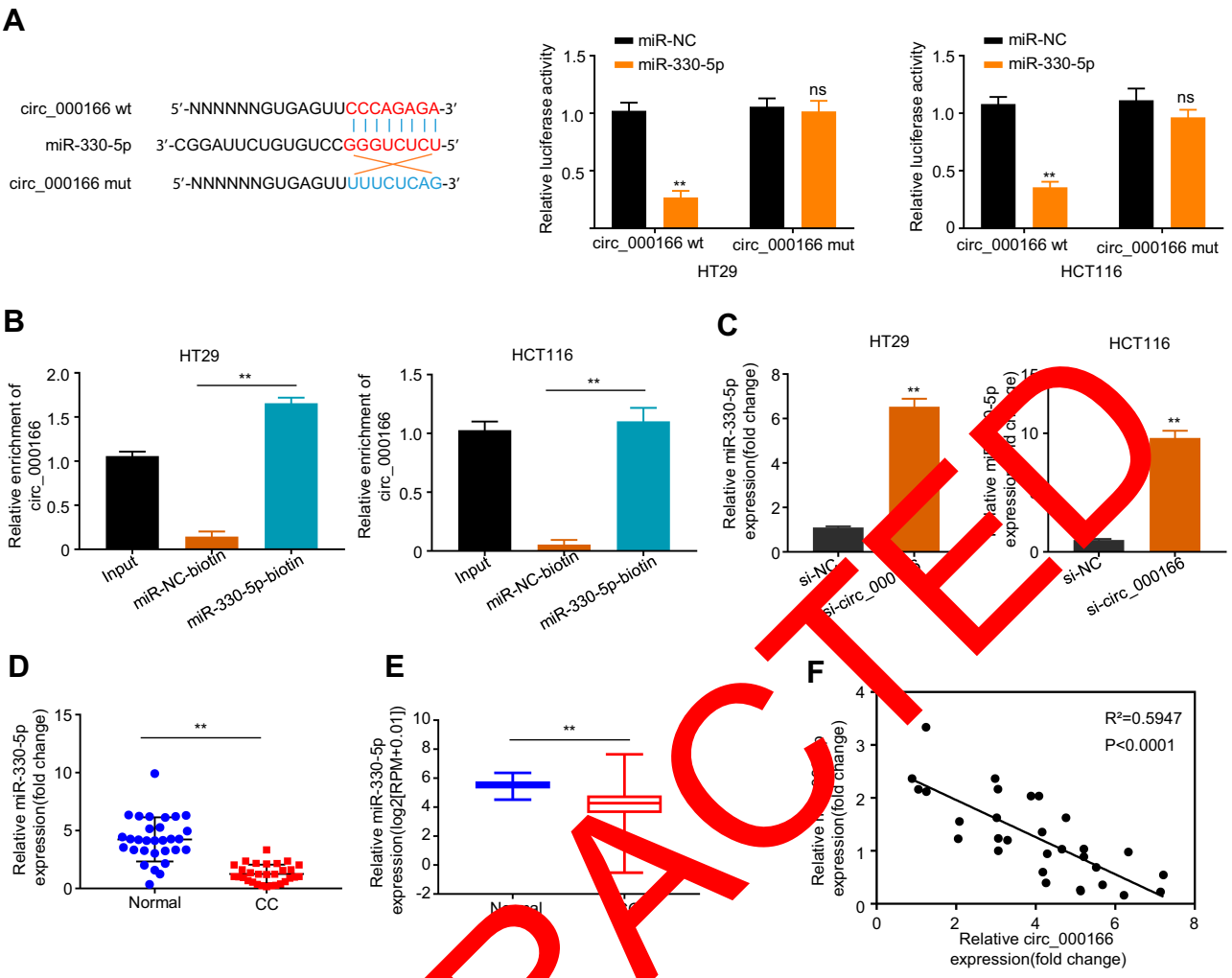

Figure 3 circRNA_000166 sponged miR-330-5p. (**A**) The putative binding sites between circRNA_000166 and miR-330-5p. Relative miR-330-5p expression in cells co-transfected with wt or mut circRNA_000166 and miR-330-5p using luciferase reporter assay; (**B**) Enrichment of circRNA_000166 using RNA pull-down experiments; (**C**) Relative miR-330-5p expression in circRNA_000166 knockdown cells; (**D**) Relative miR-330-5p expression in CC tissue and their adjacent normal tissue; (**E**) Relative miR-330-5p expression from TCGA database; (**F**) Spearman's rank-order correlation between miR-330-5p and circRNA_000166. $^{**}p < 0.01$.
Abbreviations: ns, no significance. miR-NC, negative control of miR-330-5p; wt, wild type; mut, mutant.

circRNA_000166 Regulated Colon Cancer Progression by Sponging miR-330-5p

The results of qRT-PCR showed that miR-330-5p expression was significantly upregulated in si-circRNA_000166 group compared with si-NC group and was reduced in si-circRNA_000166 + miR-330-5p inhibitor group compared with si-circRNA_000166 group (Figure 4A). CCK-8 assay indicated that knockdown of circRNA_000166 decreased the proliferation of HT29 and HCT116 cells at 24h, 48h, 72h and 96h, and that cell viability was increased in si-circRNA_000166 + miR-330-5p inhibitor group compared with si-circRNA_000166 group (Figure 4B). Similar results were also observed in colony growth of HT29 and HCT116 cells (Figure 4C). Cell migration and invasion data revealed that both cell migration and invasion were inhibited by si-circRNA_000166 and restored dual inhibition of circRNA_000166 and miR-330-5p (Figure 4D and E).

circRNA_000166 Promotes ELK1 Expression via Sponging miR-330-5p

The miRNA target prediction website http://www.targetscan.org (TargetScan) was used to predict the direct target mRNA of miR-330-5p. The results demonstrated that there were miR-330-5p binding sites in the 3′-untranslated region (3′UTR) of ELK1 (Figure 5A). The results of luciferase assay demonstrated that overexpression of miR-330-5p reduced the luciferase activity of wt ELK1

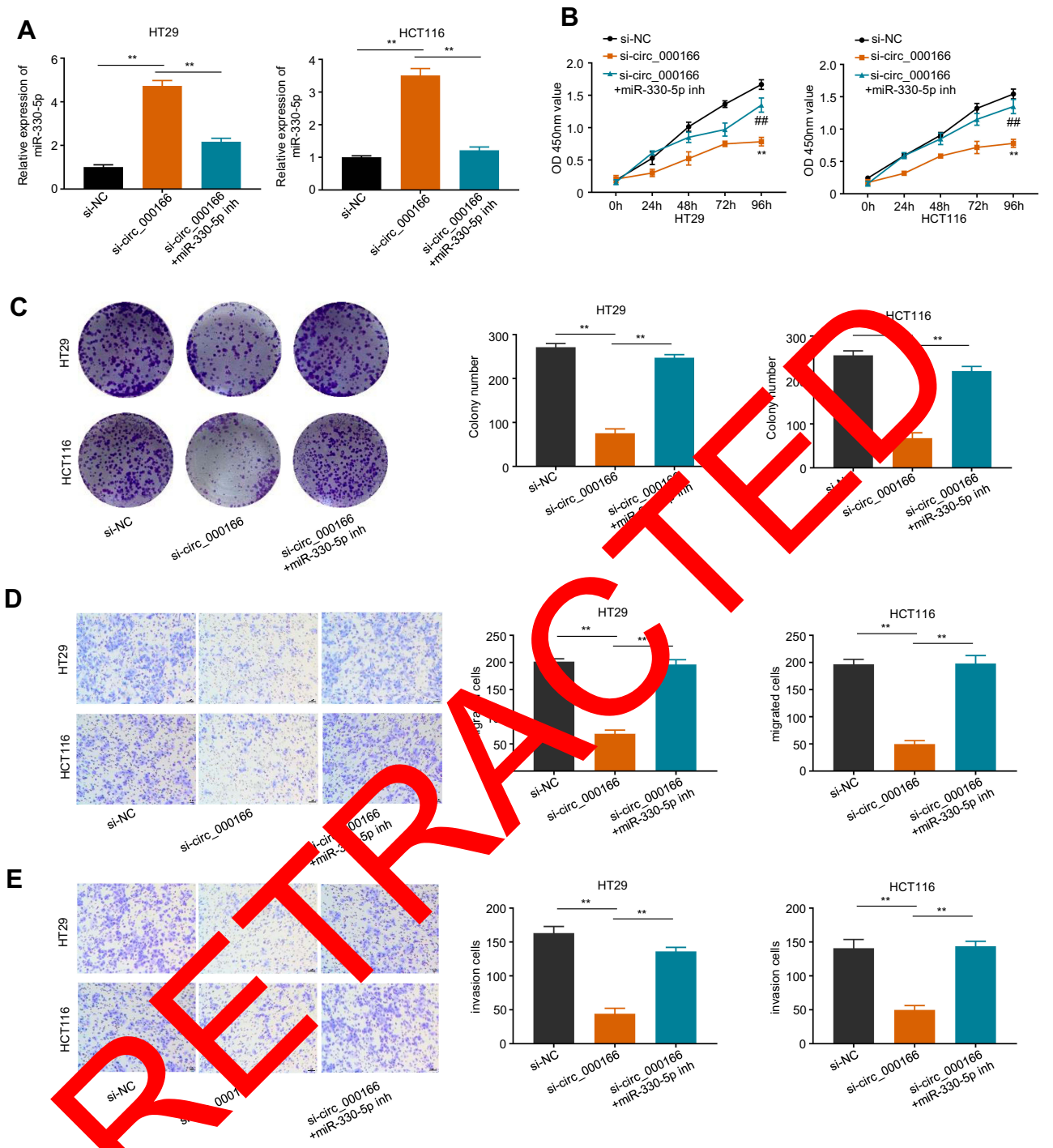

Figure 4 circRNA_000166 accelerated colon cancer progression by targeting miR-330-5p. (**A**) qRT-PCR analysis of relative miR-330-5p expression in HT29 and HCT116 cells transfected with si-NC, si-circRNA_000166 and si-circRNA_000166 + miR-330-5p inhibitor; (**B**) CCK-8 assay of cell viability in HT29 and HCT116 cells transfected with si-circRNA_000166 + miR-330-5p inhibitor; (**C**) Colony formation in HT29 and HCT116 cells transfected with si-circRNA_000166 + miR-330-5p inhibitor; (**D**) HT29 and HCT116 cell migration using transwell assay; (**E**) HT29 and HCT116 cell invasion using transwell assay. $^{**}p < 0.01$ versus (vs) si-NC; $^{\#\#}p < 0.01$ vs si-circ_000166.

transfected cells while reduction of luciferase activity was not observed in the 3′-UTR of ELK1 mutant group (Figure 5A).

mRNA and protein expression of ELK1 was examined by qRT-PCR and Western blotting, respectively. In the cells transfected with miR-330-3p inhibitor and si-circRNA_000166, both mRNA level and protein expression of ELK1 was upregulated in HT29 and HCT116 cells (Figure 5B and C). On the contrary, in the cells transfected with miR-330-3p mimic and circRNA_000166 + miR-330-5p mimics, both

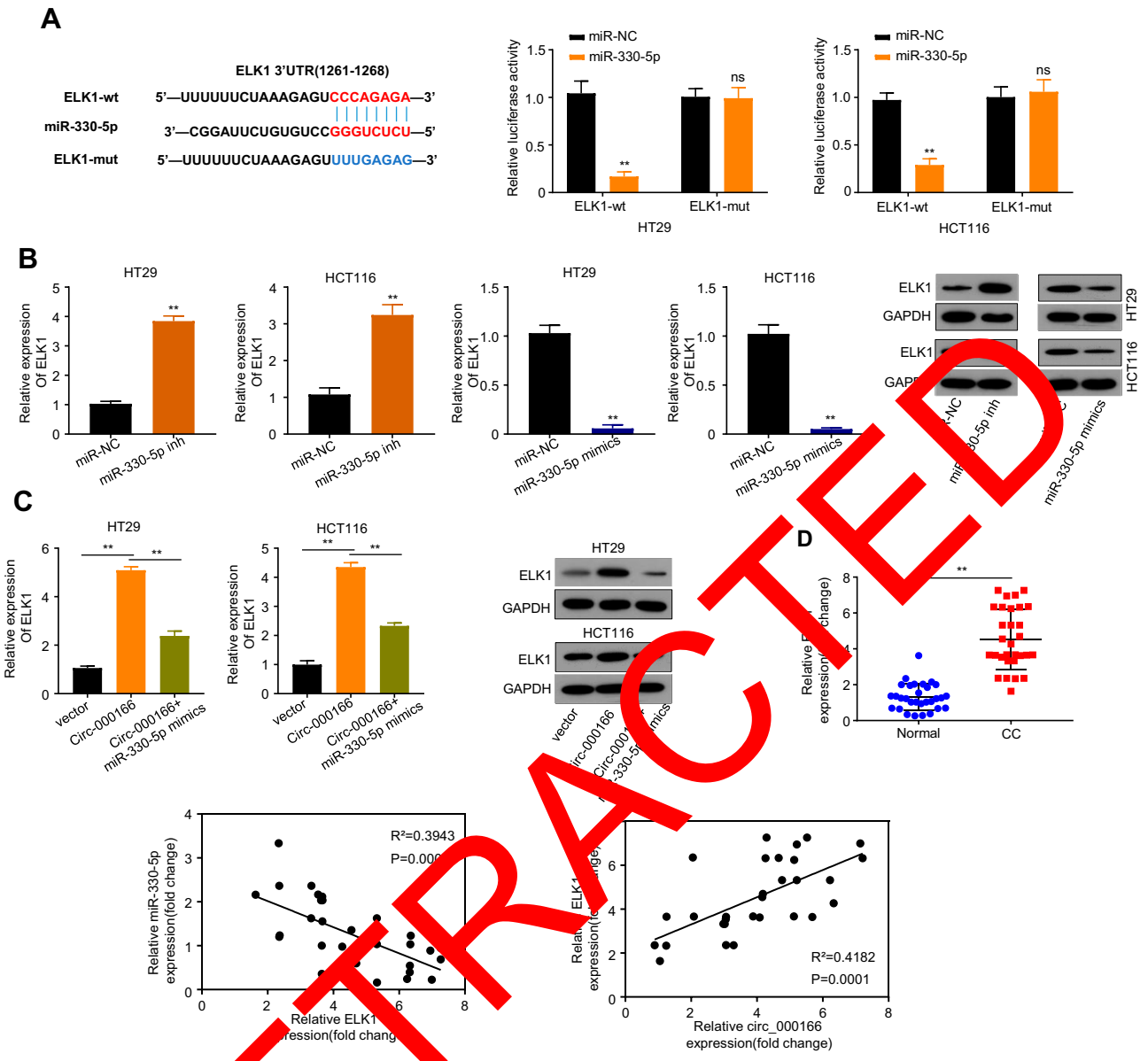

Figure 5 circRNA_000166 p[illegible]oted ELK[illegible]expression through sponging miR-330-5p. (**A**) The putative binding sites between ELK1 and miR-330-5p. Relative ELK1 expression in cells transfected wi[illegible]t an[illegible]ut circRNA_000166 using luciferase reporter assay; (**B**) Relative ELK mRNA and protein expression in HT29 and HCT116 cells transfected with miR-NC, miR-330[illegible]mimics and [illegible]nhibitors; (**C**) Relative ELK mRNA and protein expression in HT29 and HCT116 cells transfected with vector, circRNA_000166 and [illegible]ELK[illegible]000166[illegible]miR-33[illegible]5p mimics; (**D**) Relative ELK1 expression in 30 pairs of CC tissue and their adjacent normal tissue. Spearman's rank-order correlation [illegible]ween mi[illegible]30-5p an[illegible], and between circRNA_000166 and ELK1. **p < 0.01.
Abbreviation[illegible]s, no signifi[illegible]nce. miR-NC, miRNA negative control.

mRNA level and p[illegible]tein expression of ELK1 was downregulated compared with the cells transfected with miR-330-3p inhibitor and circRNA_000166 (Figure 5B and C). ELK1 mRNA expression was elevated in CC tissue (Figure 5D). Spearman's rank-order correlation results manifested a negative correlation between the expression of ELK1 expression and miR-330-5p, and a positive correlation between ELK1 expression and circRNA_000166 in 30 pairs of human CC tissue (Figure 5D).

Discussion

circRNAs are stable in cells because of their covalent closed-loop structure without a 5′end and a poly-A tail, which protected circRNAs from ribonuclease degradation.[34] Many studies have shown that circRNAs may be involved in miRNA inhibition and tumorigenesis, including CC.[34–36] For example, circular RNA PIP5K1A promotes CC cell invasion and migration via sponging miR-1273a.[37] Overexpression of circCCDC66 accelerated CC cell proliferation, migration and

invasion.[38] As a sponge of miR-6778-5p, CircRNA CBL.11 could regulate YWHAE expression, resulting in suppressing cell growth of CRC.[39] All of these evidences suggest that circRNAs play an important role in CC development. However, the role of circRNA_000166 in CC has not been reported. The present study is the first to indicated that hsa_circRNA_000166 was upregulated both in CC tissue and cell lines, suggesting that hsa_circRNA_000166 contributed to CC progress. Inhibition of hsa_circRNA_000166 reduced CC cell proliferation, migration and invasion, resulting in colon tumor growth arrest, demonstrating hsa_circRNA_000166 might be a therapeutic target of CC.

ELK1, characterized by a conserved DNA-binding domain, or Ets domain, was regarded as a transcription factor engaged mainly in the regulation of cell growth, differentiation, and migration.[40,41] It is reported that tumor-derived CXCL5 promotes CC cell migration via activation of ERK/ELK1/Snail pathway, suggesting that activation of ELK1 might contribute to human CC metastasis.[42] In cervical cancer cells, upregulation of ELK1 enhanced cell proliferation, migration and invasion.[43] All these publications proved that increased expression of ELK1 promoted cancer progress and metastasis. In this study, ELK1 expression was higher in CC tissue, positively correlated with circRNA_000166, and high level of circRNA_000166 in CC accelerated cell proliferation, migration and invasion both in vitro and in vivo, indicating that ELK1 might play an important role in CC development and metastasis, which was indirectly regulated by circRNA_000166.

miR-330-5p has been reported to play a role in many cancers, such as cervical cancer, and pancreatic cancer.[44–46] In this study, TargetScan and luciferase assay proved that ELK1 was the direct target of miR-330-5p, which is coincident with previous study of miR-330-5p in cervical cancer.[44] Overexpression of miR-330-5p suppressed cell growth of non-small cell lung cancer (NSCLC).[47] The present study showed that miR-330-5p expression was lower in CC tissue than that in normal tissue and inhibition of miR-330-5p increased CC cell proliferation, migration and invasion. These findings revealed that high level of miR-330-5p expression could inhibit CC cancer progress.

Accumulated studies have revealed that circRNAs can act as a sponge of miRNAs to regulate gene expression.[26–28] miR-330-5p was also regulated by circRNAs. For example, increasing expression of circPTN rescued the inhibition of proliferation and downregulation of SOX9/ITGA5 in glioma cells through binding to miR-330-5p.[48] In NSCLC, circFARSA targeted miR-330-5p and miR-326 to relieve their inhibitory effects on oncogene fatty acid synthase.[49] This is the first study to report the interaction between circRNA_000166 and miR-330-5p, and the interaction between 3′UTR of ELK1 and miR-330-5p. Upregulation of ELK1 by circRNA_000166 was mediated by miR-330-5p to promote CC cell proliferation, migration and invasion, thereby accelerating CC progress.

In conclusion, our data demonstrated that the hsa_circRNA_000166 upregulated the expression of ELK1 as sponge of miR-330-5p, promoting CC cell proliferation, migration and invasion. These findings may contribute to a better understanding of between the regulatory miRNA network and CC pathogenesis. The hsa_circRNA_000166 may be a potential biomarker and future therapeutic target of CC.

Disclosure

The authors report no conflicts of interest in this work.

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