Novel circular RNA hsa_circ_0077837 suppresses colorectal cancer cell proliferation by regulating the microRNA 21/phosphatase and tensin homolog axis

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Research

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Abstract

Background: Circular RNA (circRNA) is a novel noncoding RNA (ncRNA) that assumes critical roles in tumor occurrence and progression. circRNA functions in many tumors as a kind of microRNA (mRNA) "sponge." The present study sought to investigate the part played by circRNA in colorectal cancer (CRC) progression and to elucidate the relevant action mechanism(s).

Methods: We detected lower hsa_circ_0077837 (circEPB41L2) expression in CRC by data analysis based on previous studies. We demonstrated the antitumor part of circEPB41L2 in CRC cells via a group of biological experiments. We subsequently took advantage of pull-down and dual-luciferase reporter assays to recognize circEPB41L2 downstream microRNA 21 (miR-21). Western blotting was conducted to confirm that the circEPB41L2–miR-21–phosphatase and tensin homolog (Pten)/AKT axis is responsible for CRC tumor growth suppression. The antitumor part of circEPB41L2 *in vivo* was eventually demonstrated by HCT116 xenograft model.

Results: We found that circEPB41L2 overexpression inhibited the proliferation of CRC cells. Further, the miR-21 phenocopy suppressed circNRIP1 overexpression, while its overexpression also inhibited the cancer suppressive function of circEPB41L2. circEPB41L2 was confirmed to be able to inhibit tumor growth by enhancing Pten expression. Finally, the antitumor part of circEPB41L2 was confirmed.

Conclusions: We demonstrated that circEPB41L2 sponges miR-21 to increase the Pten expression level and consequently serve as a cancer suppressor in CRC.

Background

Colorectal cancer (CRC) is the fourth most prevalent malignant tumor and the second most common cause of tumor-related death in the United States, leading to about 50,000 deaths in 2009 [1]. Existing treatment methods include traditional chemotherapy and targeted therapy, which includes monoclonal vascular endothelial growth factor A antibody or EGFR [2]. Even though many advances have been made in therapeutic processes, the overall survival (OS) of patients with CRC remains unfavorable, with the five-year OS reported to be lower than 30% in the majority of countries [3]. The complexity of CRC treatment results from its heterogeneity in tumor tissues because of genetic and epigenetic changes. Hence, it is urgent to improve the knowledge of the molecular mechanisms involved in CRC occurrence and progression.

It was found previously that noncoding RNAs (ncRNAs) are able to modulate CRC occurrence and progression [4]. MicroRNAs (miRs) and long ncRNAs are two kinds of principal ncRNAs present in tumor biology that have been largely explored to date because of their critical functions [5, 6]. Circular RNA (circRNA), which is key in tumor occurrence and progression, is a novel ncRNA [7, 8]. Research suggests that circRNAs fundamentally came from their parental gene exons, even though intronic and exonic–intronic circRNAs might occasionally appear [8, 9]. Exonic circRNAs are linked through covalent bonds and exhibit a heat-to-tail closed loop structure [6]. Because of their circularity, polarity (5' to 3') deficiency,

and lack of polyadenylated tails, circRNAs are steadier and better able to tolerate nuclease degradation compared with their counterparts [10]. Spontaneously resulting endogenetic circRNAs cover selectively conservative miR binding sites (hereinafter abbreviated as miR sites) and consequently exert effects as efficient miR “sponges” and offset miRs [10, 11]. Additionally, circRNAs were found to possess numerous critical aspects such as acting as protein sponges or conducting transcription regulation [12]. In terms of CRC, most of the circRNAs were reported to drive CRC progression by sponging different miRs [13, 14]. Nevertheless, the tumor-suppressive roles of circRNAs in CRC remain to be investigated.

The present study analyzed the RNA sequencing data available from previous publications [14, 15] and found that a novel circRNA, hsa_circ_0077837 (circEPB41L2), was dramatically deregulated in CRC tumors. It was further determined that the enhanced expression of circEPB41L2 suppressed the proliferation ability of CRC cells. Functionally, circEPB41L2 sponged miR-21 to suppress its inhibitory effect on phosphatase and tensin homolog (Pten) expression. As such, our results support that rescuing the expression of circEPB41L2 could be a promising therapeutic method for patients with CRC.

Materials And Methods

Patients and tissue samples

A total of 33 pairs of human CRC and adjacent healthy tissues were obtained from the Zhongshan Hospital, Fudan University, Shanghai, China, between 2014 and 2018. The Ethical Committee of the Zhongshan Hospital, Fudan University approved this study, and all participants signed informed consent forms prior to undergoing surgery.

Cell lines and cell culture

Cell lines used were collected from the Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences in Shanghai, China, and preserved in Dulbecco's modified eagle medium containing 10% fetal bovine serum in a 5% CO_2 condition at 37 °C.

RNA and genomic DNA isolation and quantitative reverse-transcription polymerase chain reaction

RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were conducted as described before [16]. Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed with the ABI Vii7 system (Thermo Fisher Scientific, Waltham, MA, USA), whereas SYBR Green was adopted to stain DNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene. Primers were synthesized as follows: for the human circEPB41L2, 5'-CCTCTGGATCGGAAGACTGA-3' and 5'-TGCCAAGGGACAAGTGTTATTT-3'; for GAPDH, 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CACCCTGTTGCTGTAGCCAAA-3'. The ΔΔCT method was adopted to determine the relative gene expression, wherein the fold alternations were calculated as: $2^{-[\Delta CT\ (sample) - \Delta CT\ (calibrator)]}$.

Plasmids, small interfering RNAs, and cell transfection

The blank plasmid (pLVX-IRES) and circEPB41L2-overexpressing vector (Lv-circEPB41L2) were purchased from Genelily Biotechnology Company (Shanghai, China). The plasmid was packed with the Lenti-X™ HT Packaging System (Takara, Beijing, China) in 293T cells to generate lentivirus. The HCT116, RKO, and DLD1 cells were transferred. The miR-21 mimic and antagonist as well as the miR-NC (negative control with scrambled sequence; 5'- ACUCUAUCUGCACGCUGACUU-3') were prepared by GenePharma (Shanghai, China). The miRs were subjected to reagent transfection as depicted before. After transfection for 2 days, qRT-PCR was adopted to evaluate cell transfection efficiency. The plasmid pcDNA-6.2-miR-21-ASO (termed p-miR-21-ASO) was applied as described before [17]. The AKT expression plasmid (pCDNA3.1-HA-AKT) was purchased from Addgene (#73408; Cambridge, MA, USA).

Cell proliferation and colony formation

Cell Counting Kit-8 (CCK8) assay was performed to examine cell growth [14]. Colony formation assay and 5-ethynyl-2´-deoxyuridine (EdU) assay were performed as discussed before [18]. The 5'-bromo-2'-deoxyuridine (BrdU) assay was performed using the BrdU Assay Kit as the protocol provided by the manufacturer.

Western blot

Western blotting was performed with GAPDH and flag antibodies [14]. The protein loading was standardized with GAPDH as the endogenetic control. Among the primary antibodies used, anti-Pten, anti-EPB41L2, and anti-Ki-67 were bought from Abcam (Cambridge, UK), while anti-AKT and anti-p-AKT were purchased from Cell Signaling Technology (Danvers, MA, USA).

RNA-binding protein immunoprecipitation

RNA-binding protein immunoprecipitation (RIPI) assay was performed with a RIPI Kit (17–700) to confirm the linkage of QKI protein and pre-mEPB41L2 using the manufacturer's guidance. qRT-PCR was eventually conducted on the magnetic bead-absorbed RNA mixture relevant to QKI.

Pull-down assay

A total of 1×10^7 HCT116 cells were collected, lysed, and subjected to ultrasound. The circEPB41L2 probe (5'-TGCCAAGGGACAAGTGTTATTT-3'-biotin) was applied to promote C-1 magnetic bead incubation at 25 °C for 120 min to afford beads covered with probe. The resulting lysate with circEPB41L2 or oligo probe was cultivated at 4 °C overnight. The RNA mix containing beads was washed with phosphate-buffered saline supplemented with Tween 20, followed by the completion of elution and separation for PCR.

Dual-luciferase reporter assay

A wild-type circEPB41L2 fragment was established and introduced into a luciferase reporter gene downstream in pMIR-REPORT plasmid. The reporter plasmid was transfected to germinal center cells

using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), followed by the cotransfection of miR-21 mimic and reporter genes to HCT116 cells. Finally, firefly and renilla luciferase activity was examined using the DLR System Kit.

Xenograft models

Six-week-old BALB/c nude mice were fed in our laboratory. The nude mice were inoculated with stably transfected HCT116–empty vector and HCT116–circEPB41L2 cells (4×10^6) to establish a flank xenograft model (n = 6 per group). At 7 days after cell injection, 100 mg of p-Cont or eukaryotic expression vector encoding antisense oligonucleotides against miR-21 (p-miR-21-ASOs) plasmid was locally injected into tumor tissues four times every 3 days. The tumor size was measured dynamically and determined as 0.523 × (length × width × height). Tumor tissues were dissected and preserved at −80 °C for subsequent experiments. The Institutional Animal Care and Use Committee of the Third Military Medical University approved this portion of the study.

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The Kaplan–Meier method and log-rank test were adopted to analyze survival and survival curves, respectively. A p-value of < 0.05 indicated statistical significance.

Results

The expression of hsa_circ_0077837 was lower in colorectal cancer tissues

We analyzed 563 differentially expressed circRNAs in CRC and adjacent normal tissues based on previous studies [14, 15] and found that circEPB41L2 was the most significantly downregulated circRNA (Fig. 1A). To confirm the regulation of circEPB41L2, we measured its expression in the CRC cell lines HCT116, DLD1, RKO, HT29, and SW48 in comparison with normal NCM460 cells. The results here demonstrated that circEPB41L2 was obviously downregulated in all CRC cell lines (Fig. 1B). Furthermore, we affirmed that circEPB41L2 in CRC tissues was dramatically downregulated against healthy tissues (33 pairs; Fig. 1C). The Kaplan–Meier method was adopted to draw disease-free survival and OS curves and the survival information of patients was acquired. Patients with high circNRIP1 levels exhibited a dramatically longer OS (Fig. 1D). circEPB41L2 is derived from the EPB41L2 gene, which exists in chromosome 6 and is composed of exon 2 and 3 head-to-tail splicing (131247744–131277639). To exclude the probability that these products may likewise be resultant from trans-splicing and genome rearrangements, qRT-PCR was conducted and revealed that circEPB41L2 rather than linear EPB41L2 could counteract ribonuclease R digestion (Fig. 1E). Furthermore, we observed that circEPB41L2 acted steadier than linear EPB41L2 when exposed to dactinomycin (Fig. 1F). The results collectively indicate that circEPB41L2 is a steady and crucial diagnosis and prognosis marker that warrants further investigation.

Colorectal cancer cell proliferation was suppressed by hsa_circ_0077837

Since circEPB41L2 is negatively correlated with CRC tumor progression, we surmised that boosting circEPB41L2 expression might attenuate the survival capacity of CRC cells regarding proliferation. To circularize circEPB41L2 in vitro, we transfected HCT116, RKO, and DLD1 cells with circEPB41L2-expressing lentivirus. We succeeded in establishing stable overexpression of circEPB41L2 via lentiviral transfection in HCT116, RKO, and DLD1 cells (Fig. 2A). Furthermore, the EPB41L2 protein levels in HCT116, RKO, and DLD1 cells remained unchanged after circEPB41L2 overexpression (Fig. 2B). We found that circEPB41L2 overexpression dramatically suppressed cell proliferation as displayed by CCK8 assay (Fig. 2C), BrdU assay (Fig. 2D), and colony formation assay (Fig. 2E). Further, circEPB41L2 transfection suppressed DNA synthesis in HCT116 as demonstrated by EdU assay, suggesting a lower proliferation rate (Fig. 2F). We also analyzed the expression of the proliferation marker Ki-67 and found that circEPB41L2 expression also suppressed this marker's protein level (Fig. 2G). Therefore, our results reveal that circEPB41L2 suppressed the proliferation of CRC cells in vitro.

hsa_circ_0077837 functions as a microRNA 21 sponge

Numerous studies have indicated that circRNA could modulate tumor progression and downstream gene expression as an miR sponge. A cross-analysis of three databases revealed that the target miR of circEPB41L2 was possibly the Circular RNA Interactome (Fig. 3A). Subsequently, we chose the eight most common miRs on the basis of conjugation scores, which might have displayed the latently functional circEPB41L2-assimilated miRs in CRC, including miR-21, miR-1178, miR-1236, miR-1248, miR-502, miR-515, miR-936, and miR-942. circEPB41L2-relevant miRs were identified via pull-down assay using a specific biotin-marked circEPB41L2 probe. We subsequently conducted qRT-PCR to analyze the expression levels of these miRs. Consequently, we demonstrated the high enrichment of circEPB41L2 and miR-21 (Fig. 3B). To deeply examine the absorption of miR-21 and circEPB41L2, we investigated the miR-21 expression level in circEPB41L2-overexpressing HCT116 and found that circEPB41L2 expression suppressed the miR-21 level (Fig. 3C). A specific biotin-marked miR-21 probe successfully captured circEPB41L2 against the miR-NC group (Fig. 3D). Furthermore, dual-luciferase reporter assay was performed to confirm the linkage of circEPB41L2 and miR-21 with complementary sequences as a basis. A circEPB41L2 fragment was established and introduced into the downstream luciferase reporter gene, which was subsequently used for cotransfecting a miR-21 mimic to HCT116 cells. A marked decrease in luciferase reporter activity was detected at this point (Fig. 3E), thereby demonstrating the linkage of circEPB41L2 and miR-21. FISH was conducted in HCT116 and revealed the colocalization of miR-21 and circEPB41L2 in cytoplasm (Fig. 3F). As such, it was suggested thus that circEPB41L2 could function as an miR-21 sponge, but whether it could affect miR-21 degradation remained to be determined. HCT116 cells transfected with control or circEPB41L2 plasmid were exposed to dactinomycin. At 0, 12, and 24 h after treatment, total RNA was isolated and miR-21 abundance was determined. The findings indicated

that the miR-21 level in the circEPB41L2 group was notably reduced compared with that in the control group (Fig. 3G). In brief, circEPB41L2 can function as an miR-21 sponge in CRC cells.

hsa_circ_0077837 suppresses colorectal cancer cell proliferation by sponging microRNA 21

After demonstrating that circEPB41L2 could function as an miR-21 sponge, we attempted to determine the part of this miR in circ-mediated CRC proliferation, even though several studies have already suggested that miR-21 promotes tumor progression. First, we detected the miR-21 expression in CRC and NCM460 cells. As expected, miR-21 expression in CRC cells was obviously decreased (Fig. 4A). Further, the miR-21 expression level presented a negative correlation with the circEPB41L2 expression level in 33 CRC tumors (Fig. 4B). To investigate the function of miR-21 in CRC cell proliferation, we synthesized an miR-21 antagonist and transfected it into HCT116 cells. By performing CCK8, BrdU, and colony formation assays in HCT116 cells, we determined that the transfection of miR-21 antagonist inhibited HCT116 cell proliferation (Figs. 4C–4E), which phenocopied the effect of circ overexpression. To further confirm the effect of circ on CRC proliferation through miR-21, we transfected the miR-21 mimic into HCT116 with circ overexpression. Our results demonstrated that enhanced miR-21 expression abolished the suppressive effect of circ overexpression on CRC cell proliferation (Figs. 4F–4H). The findings ultimately suggested that circ sponges miR-21 to suppress the proliferation of glioma.

hsa_circ_0077837 overexpression rescued phosphatase and tensin homolog expression

miR-21 was reported to be able to promote cell proliferation through acting on the Pten/AKT pathway. On the basis of the presumption that circEPB41L2 assumes a tumor promotor role in the germinal center via the typical competing endogenous RNA mechanism, we attempted to recognize the endogenetic RNAs of circEPB41L2, which was likewise the miR-21 downstream objective gene. Enhanced expression of circEPB41L2 increased the expression of Pten in messenger RNA and the protein level, which was abolished by miR-21 cotransfection (Figs. 5A and 5B). Dual-luciferase reporter assay revealed that circEPB41L2 transfection increased the Pten luciferase reporter activity, while cotransfection with miR-21 mimic suppressed the reporter acidity (Fig. 5C), suggesting that circEPB41L2 mediated Pten expression by sponging miR-21. To further confirm Pten in circEPB41L2 mediated the suppression of CRC proliferation, we cotransfected Pten small interfering RNA with circEPB41L2 plasmid in HCT116 cells. Here the absence of Pten abolished the suppressive effects of circEPB41L2 in HCT116 cell proliferation (Figs. 5D–5G). Akt is a well-known Pten downstream target in the suppression of cancer progression. We therefore tested whether the Pten/Akt pathway was involved in the circEPB41L2 function. Accordingly, the enhanced expression of Akt in HCT116 cells also compromised the suppressive role of circEPB41L2 in HCT116 proliferation (Figs. 5H–5J). Therefore, it can be concluded that circEPB41L2 suppressed the CRC cell proliferation through the Pten/Akt pathway.

circEPB41L2 suppressed CRC cell growth in vivo

After determining the influence of circEPB41L2 in vitro, we attempted to explore whether circEPB41L2 affects the biological performance of tumors in vivo. Hence, steadily transfected HCT116–empty vector and HCT116–circEPB41L2 cells were used for constructing a flank xenograft model. Our observations confirmed that the tumor growth rate and sizes in the circEPB41L2 group were dramatically reduced compared with those in the EV group (Figs. 6A and 6B). qRT-PCR was performed in xenografted tumors to study miR-21 regulation in vivo and revealed that miR-21 expression level was suppressed in circEPB41L2-overexpression tumors (Fig. 6C). Also, Pten and Ki-67 expression levels were observed in xenografted tumors (Fig. 6D). To further verify that miR-21 is sponged by circEPB41L2, miR-21 mimic was continuously injected intraperitoneally into nude mice xenografted with HCT116-circEPB41L2 cells. The administration of the miR-21 mimic increased the tumor growth of circEPB41L2-overexpressed tumors (Figs. 6E and 6F). Collectively, these results suggested that circEPB41L2 suppressed tumor growth in vivo by sponging miR-21.

Discussion

In our study, we performed database mining based on previous studies conducted on the subject of circRNA expression in CRC tumors and normal tissues and found that a novel circRNA, circEPB41L2, was dramatically deregulated in tumor tissues. Our results further confirmed that circEPB41L2 was downregulated in our cohort of patients as well as in multiple CRC cell lines. The enhanced expression of circEPB41L2 significantly suppressed CRC cell proliferation, which indicated that circEPB41L2 may be relevant as a CRC tumor suppressor. Functionally, we revealed that circEPB41L2 sponged miR-21 to elevate the expression of Pten, which is a well-known tumor suppressor. Our in vivo xenograft data also support the thought that boosting the expression of circEPB41L2 can suppress tumor growth, which provides a novel therapeutic target for CRC treatment.

Currently, circRNAs have attracted interest around the world as a critical kind of endogenetic RNA. Two characteristics of spontaneously resulting circRNAs—namely, exonuclease resistance and conservative miR sites—make them attractive tools for realizing miR function loss [10, 19]. More and more studies have implied that some spontaneously resulting circRNAs can function as effective miR sponges and assume crucial roles in tumor occurrence. For instance, the overexpression of endogenetic ciRS-7 (consisting of 63 conservative miR-7 target sites) was relevant to miR-7 inhibition and EGFR and RAF1 downstream activation, leading to a more invasive phenotype [20]. Elsewhere, circCCDC66 [21], circFoxo3 [22], and circHIPK3 [23] were reported as other spontaneously resulting circRNAs that act as miR sponges and possess miR sites. Nevertheless, most of the known circRNAs were reported oncogenes, which promote cancer proliferation, migration, and invasion. With improvements in the understanding of circRNAs, a number of investigators have realized that several circRNAs can sponge oncogenic miR to suppress tumor growth. The present study suggested that circEPB41L2 was obviously decreased in CRC tumors, which might make it a potential tumor suppressor. circEPB41L2 was also found to be downregulated in lung adenocarcinoma and squamous cell carcinoma, supporting its status as a

possible useful biomarker and tumor suppressor in both conditions [24]. Here, our additional experiments further confirmed the tumor-suppressive role of circEPB41L2 and revealed the function of circEPB41L2, which is exerted by sponging miR-21.

The overexpression of miR-21, a characteristic oncomiR, was observed in CRC [25, 26]. Increased miR-21 expression is involved in crucial CRC-relevant processes through suppressing PDCD4, TIMP3, and PTEN [27, 28]. Hence, miR-21 is an attractive therapeutic target. In our study, we further confirmed that miR-21 expression in CRC suppressed the Pten/AKT pathway and promoted CRC cell proliferation. Recently, linear sponge RNAs including miR sites were confirmed to cause miR-21 function loss [29, 30], which was considered as the basis of a promising strategy to suppress miR-21 expression. In our study, we confirmed that circEPB41L2 was dramatically increased and could sponge miR-21 to suppress CRC proliferation. We also observed that suppressing miR-21 can hinder the pernicious performances of CRC via the Pten/AKT signaling pathway. circEPB41L2 proved able to exert effects as an miR sponge and it is thought that circEPB41L2 enhancement may become a potential CRC treatment option in the future.

Conclusions

Several notable conclusions were gathered in the present study. First, in this research, the first man-made circRNAs including diverse bulged oncomiR miR-21 sites were prepared through in vitro enzyme ligation and synthesis. Subsequently, this circRNA is better able to tolerate nuclease degradation. Next, this circRNA is an miR-21 sponge, inhibiting miR-21 modulatory activity after transcription and CRC proliferation and enhancing miR-21 downstream protein expressions. Further, circRNA sponges can be easily prepared in great numbers. Therefore, these findings imply that this circRNA exhibited immense potential for becoming a new tool for antitumor research and future molecular treatment.

Declarations

Ethics approval and consent to participate: The Ethical Committee of the Zhongshan Hospital, Fudan University approved this study, and all participants signed informed consent forms prior to undergoing surgery.

Consent for publication: Not applicable.

Availability of data and material: The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests: The authors declare that they have no competing interests

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Small molecule compounds induce the iPS cells of diabetic patients to differentiate into pancreatic β cells.

Authors' contributions

Study design/planning: WZS, TGD, BZ, YDG, WDG

Data collection/entry: WZS, TGD, BZ, YDG, WDG

Data analysis/statistics: WZS, TGD

Data interpretation: WZS, TGD, WDG

Preparation of manuscript: WZS, TGD, BZ

Literature analysis/search: WDG

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Abbreviations

circRNA: Circular RNA

ncRNA: noncoding RNA

mRNA: microRNA

CRC: colorectal cancer

miR-21: microRNA 21

OS: overall survival

Pten: phosphatase and tensin homolog

qRT-PCR: quantitative reverse-transcription polymerase chain reaction

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

p-miR-21-ASO: plasmid pcDNA-6.2-miR-21-ASO

pCDNA3.1-HA-AKT: AKT expression plasmid

CCK8: Cell Counting Kit-8

EdU: 5-ethynyl-2´-deoxyuridine

BrdU: 5'-bromo-2'-deoxyuridine

RIPI: RNA-binding protein immunoprecipitation

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Figures

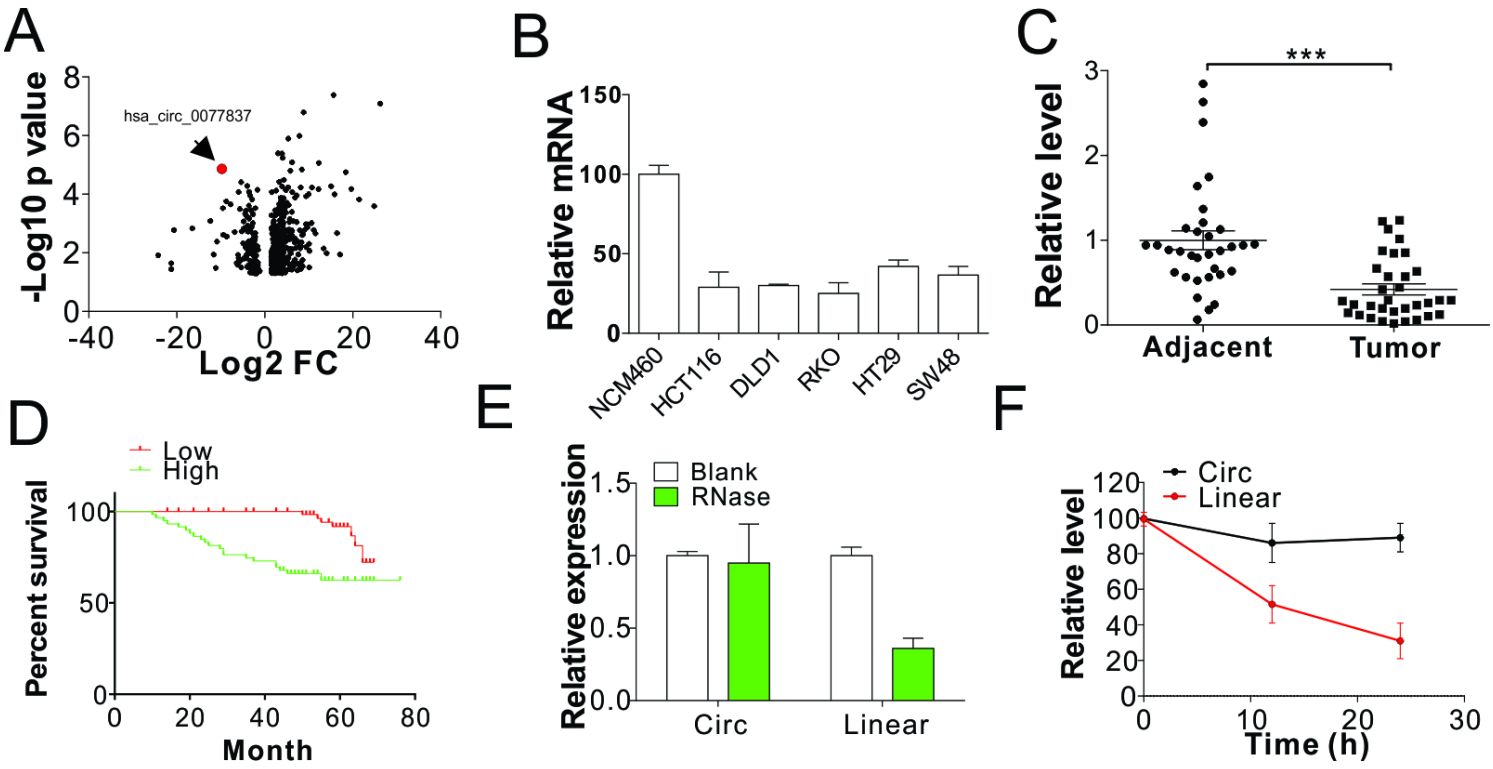

Figure 2

circEPB41L2 is downregulated in CRC tumors and cells. (A) The expression changes of circRNA transcripts in tumor tissues were reflected using a cluster heat map. (B) The messenger RNA level of circEPB41L2 in multiple CRC cells relative to that in NCM460 cells. (C) circEPB41L2 expression in 33 pairs of CRC tumors and adjacent tissues. (D) Patients with a high circEPB41L2 level exhibited a dramatically longer OS. (E) qRT-PCR was conducted and revealed that circEPB41L2 instead of linear-circEPB41L2 could counteract ribonuclease R digestion. (F) circEPB41L2 remained steadier than linear NRIP1 when exposed to dactinomycin.

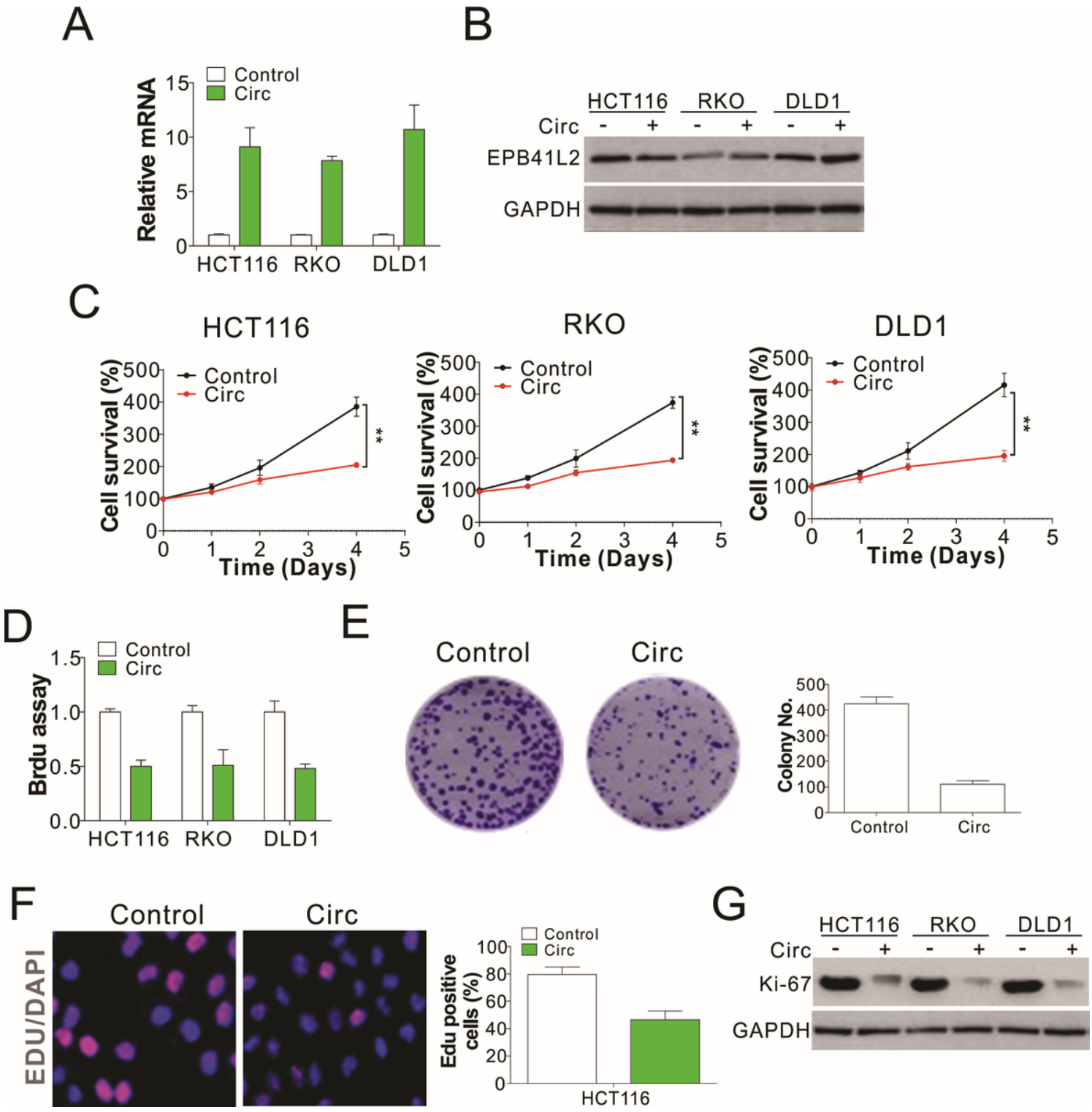

Figure 4

circEPB41L2 suppressed CRC cell proliferation in vitro. (A) The messenger RNA levels of circEPB41L2 in HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2. (B) The protein levels of EPB41L2 in HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2. (C) The CCK8 assay of cell viability results in HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2. (D) The BrdU assay of cell proliferation results in HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2. (E) The colony formation assay results of HCT116, RKO, and DLD1 cell stably transfected

with lenti-circEPB41L2. (F) The DNA synthesis outcomes of HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2 as analyzed by EdU assay. (G) The Ki-67 expression findings in HCT116, RKO, and DLD1 cells stably transfected with lenti-circEPB41L2.

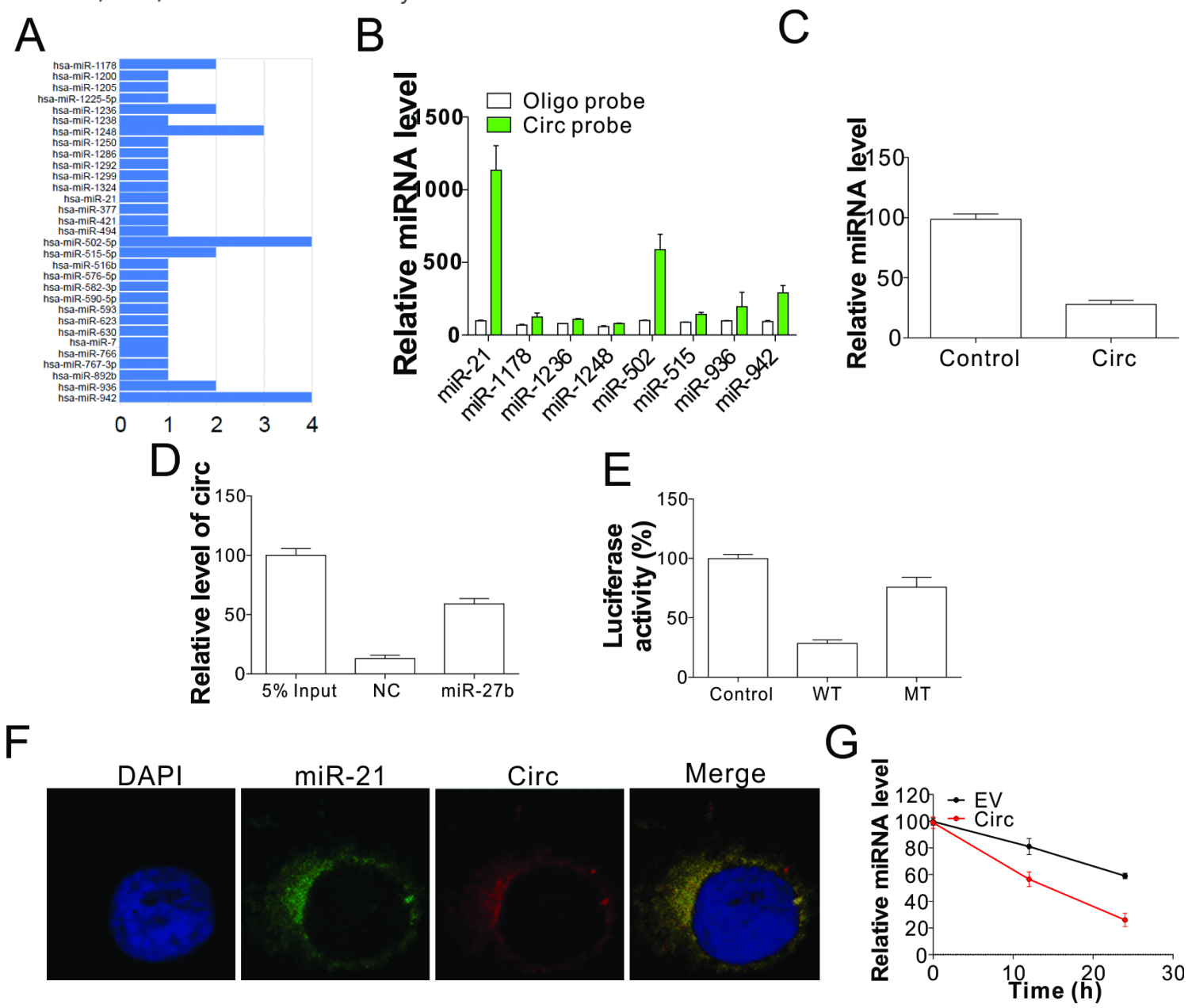

Figure 6

miR-21 is the sponging target of circEPB41L2. (A) The interacting miRs of circEPB41L2 as predicted by Circular RNA Interactome. (B) The enrichment of miRs in HCT116 cells by circEPB41L2 probe pull-down. (C) The miR-21 expression in HCT116 cells stably transfected with lenti-circEPB41L2. (D) A specific biotin-marked miR-149-5p probe captured circEPB41L2 against the miR-NC group. (E) The linkage of circEPB41L2 and miR-21 with their complementary sequences as a basis was determined through dual-luciferase reporter assay. (F) FISH displayed the colocalization of miR-21 and circEPB41L2. (G) The miR-21 expression level in HCT116 cells stably transfected empty vector or circEPB41L2 and was exposed to 2 μg/mL of dactinomycin at a specific time.

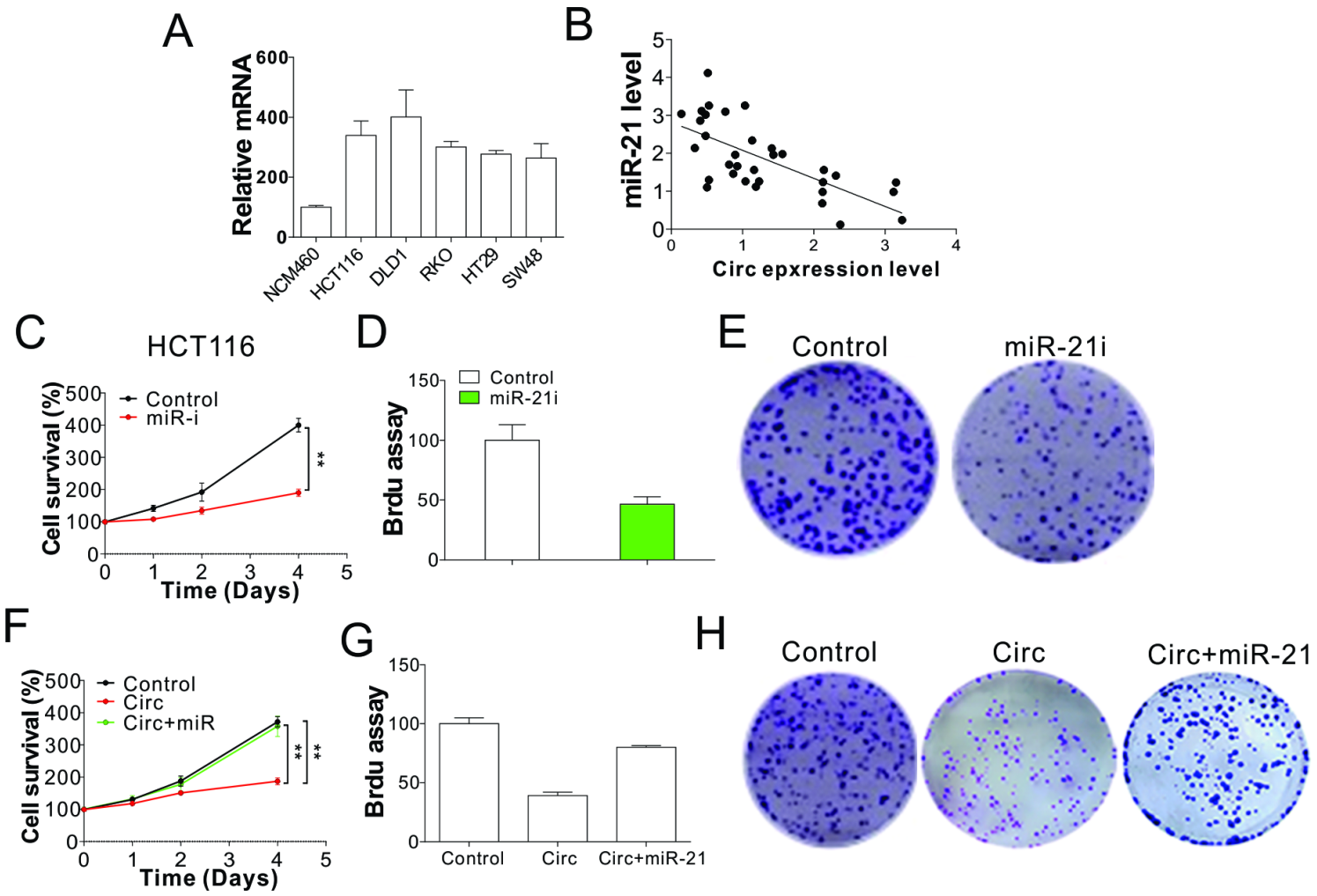

Figure 8

miR-21 mediated the tumor-suppressive role of circEPB41L2. (A) The expression level of miR-21 in CRC cell lines and NCM460 cells. (B) The expression level of miR-21 and circEPB41L2 in 33 CRC tumors. (C–E) The CCK8 assay (C), BrdU assay (D), and colony formation assay (E) in HCT116 cells treated with control or miR-21 antagonist. (F–H) The CCK8 assay (F), BrdU assay (G), and colony formation assay (H) in HCT116 cells stably transfected with control empty vector (control) or circEPB41L2 with or without treatment of miR-21 mimic (miR).

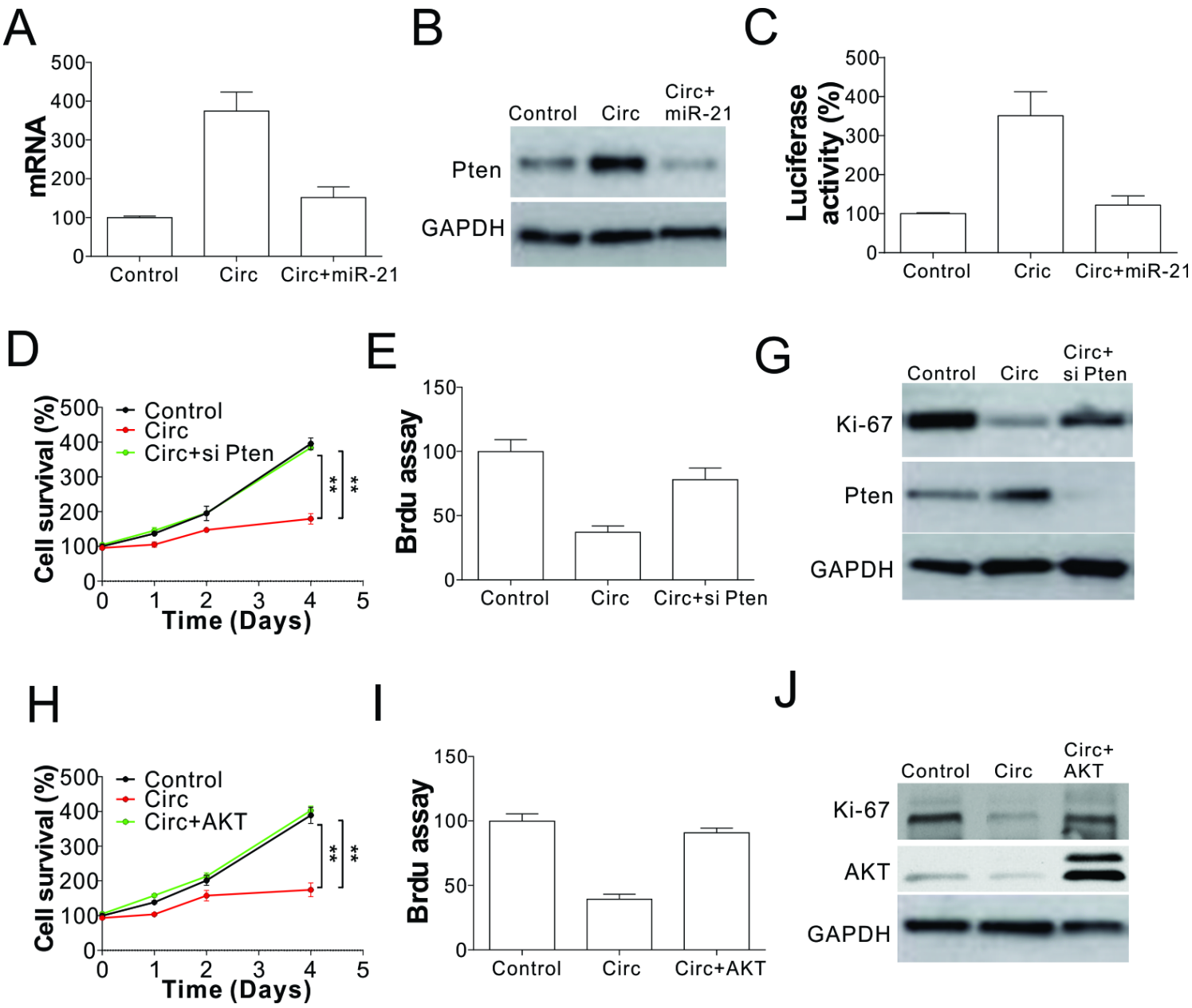

Figure 9

Pten is the target of miR-21 upon circEPB41L2 overexpression. (A, B) The RNA level (A) and protein (B) of Pten in HCT116 stably transfected with control empty vector (control) or circEPB41L2 with or without treatment of miR-21 mimic. (C) The Pten luciferase reporter activity in HCT116 cells stably transfected with control empty vector (control) or circEPB41L2 with or without treatment of miR-21 mimic. (D, E) The CCK8 assay (D) and BrdU assay (E) in HCT116 cells stably transfected with control empty vector (control) or circEPB41L2 with or without treatment of Pten siRNA. (E) The expression of Ki-67 and Pten in HCT116 cells treated as reported in (D). (H-J) The CCK8 assay (H) and BrdU assay (I) in HCT116 cells stably transfected with control empty vector (control) or circEPB41L2 with or without the transfection of AKT plasmid. (J) The expression of Ki-67 and AKT in HCT116 cells treated as reported in (H).

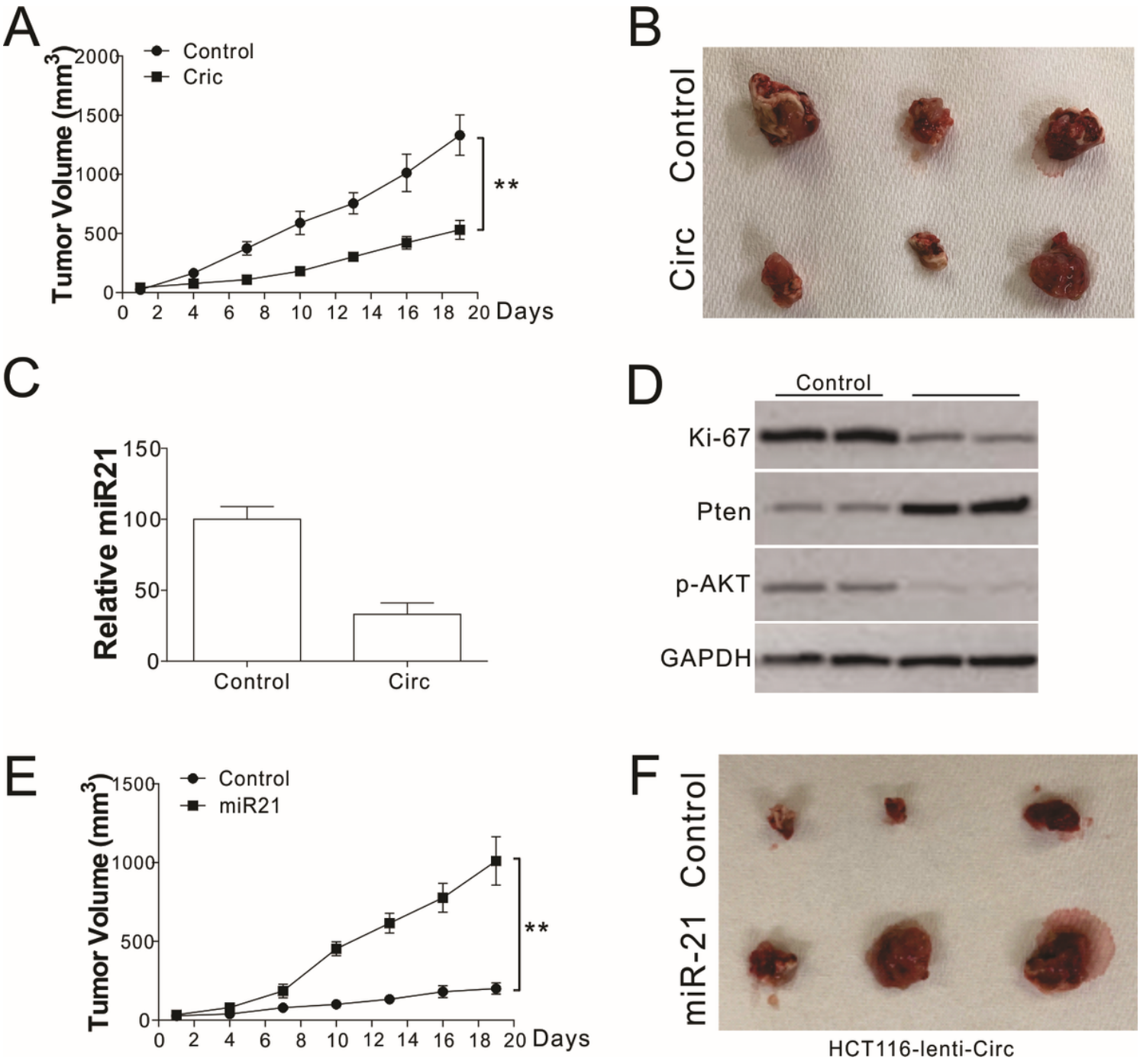

Figure 11

CircEPB41L2 suppressed tumor growth in vivo. (A) The tumor growth curves of nude mice xenografted with HCT116 cells stably transfected with control empty vector (control) or circEPB41L2. (B) The representative tumors. (C) miR-21 levels in different groups of tumors. (D) The expression of indicated proteins in different groups of tumors. (E) The tumor growth curves of nude mice xenografted with HCT116 cells stably transfected with circEPB41L2 and treated with miR-21-ASO plasmids. (F) The representative tumor.