Circ-CCDC66 Upregulates REXO1 Expression to Aggravate Cervical Cancer Progression Via Restraining miR-452-5p

Yun Zhang (Doctor8118@163.com)
Wuhan University Renmin Hospital https://orcid.org/0000-0001-7798-531X
Xing Li
Department of Obstetrics and Gynecology, Renmin hospital of Wuhan university
Jun Zhang
Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University
Lin Mao
Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan university

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Abstract

Background

Cervical cancer is one most common cancer types among females around the world. CircRNAs have been revealed to participate in multiple biological functions, and are involved in many diseases' progression. In current study, we aimed to elucidate whether circ-CCDC66 participates cervical cancer progression.

Methods

Real-time quantitative PCR (RT-qPCR) was conducted to measure the expression of circ-CCDC66, miR-452-5p, and REXO1 mRNA. Cell fractionation assay and RNA fluorescence in situ hybridization (FISH) were performed to locate circ-CCDC66 in cells. Cell proliferation ability was detected using cell account kit 8 (CCK-8). TRANSWELL assay was applied to evaluate cell migration or invasion ability. Bioinformatics analysis, biotinylated RNA pull-down, RNA immunoprecipitation, and dual-luciferase reporter assays were conducted to assess the association between miR-452 and circ-CCDC66 or REXO1. Western blot was applied to measure the protein expression of REXO1. Animal tumor model was used to assess the effect of circ-CCDC66 in *vivo*.

Results

Circ-CCDC66 was upregulated in cervical cancer tumor tissues, and correlated with tumor stage and tumor size. Downregulation of circ-CCDC66 inhibited cervical cancer cell proliferation, migration, and invasion. Circ-CCDC66 was an efficient molecular sponge for miR-452-5p, and negatively regulated miR-452-5p expression. MiR-452 directly targeted REXO1. The effects of circ-CCDC66 on cervical cancer cells were functioned through miR-452-5p/REXO1 axis. In animal experiment, downregulation of circ-CCDC66 was found to suppress tumor growth in *vivo.*

Conclusion

Our results demonstrated the effects of circ-CCDC66/miR-452-5p/REXO1 axis in cervical cancer progression, we might provide a novel therapeutic target for cervical cancer.

Highlights

1. Circ-CCDC66 was downregulated in cervical cancer tumor tissues.
2. Circ-CCDC66 downregulation inhibited cervical cancer cell proliferation, migration, and invasion.
3. Circ-CCDC66 regulated cervical cancer progression through upregulating REXO1 expression via sponging miR-452-5p.
4. Circ-CCDC66 downregulation inhibited cervical cancer cell growth in *vivo*.

Background

Cervical cancer is one most common malignancy types of gynecological tumors, and one primary reason for females occurring cancer-related death worldwide [1]. Unfortunately, cervical cancer is the sixth most common cancer type in China, and causes more than 34000 death in 2015[2]. Despite the application of vaccine in the world has been widespread, and the clinical intervention of cervical cancer has made great improvement. Still, 25% of patients diagnosed with cervical cancer fail to recover due to recurrence and distance metastasis[3, 4]. It is imperative to find novel targets for cervical cancer treatment.

Circular RNAs (circRNAs) is one type of non-coding RNAs characterized by its covalently closed loop structures [5]. Due to its stable, conserve, and spatio-temporal specificity futures, circRNAs are abundantly expressed in human tissues[6, 7]. In the past decades, the functions of circRNAs in various biological progressions have been deeply studied, including cervical cancer[8–12]. Gao et al. revealed that has_circ-0018289 mediates cervical cancer cells proliferation, invasion, and migration via acting as a molecular sponge for miR-497 [13], Song T et al. explored the role of hsa_circRNA_101996 in cervical cancer development through miR-8075/TPX2 axis[14], Tang Q et al. demonstrated that hsa_circ_0000515 mediates cervical cancer progression via miR-326/ELK1 pathway[15]. CircRNAs play essential roles in cervical cancer progression, mainly through acting as a sponge for miRNA and regulating gene expression.

CircRNA circ-CCDC66 (hsa_circ_0001313) derives from chr3:56626997-56628056 and consists about 460 nts[16]. It has been revealed that circ-CCDC66 participates in Hirschsprung's disease, Colon cancer, Gastric cancer, and Abdominal Aortic Aneurysm development via regulating multiple cellular progressions[17–20]. However, the role circ-CCDC66 in cervical cancer development is still uncovered.

In this study, we hypothesized that circ-CCDC66 is involved in cervical cancer progression. Firstly, we measured the level of circ-CCDC66 in thirty-six pairs of cervical cancer human samples. Then, by generating circ-CCDC66 knockdown cell models, we assessed the biological functions of circ-CCDC66 in cervical cancer cells. Subsequently, circ-CCDC66 was found to upregulate REXO1 expression via sponging miR-452-5p using bioinformatics analysis. Collectively, our study demonstrated that circ-CCDC66 upregulated REXO1 expression to aggravate cervical cancer progression via miR-452-5p.

Materials And Methods

Clinical human samples

Thirty-six pairs human samples were collected from cervical cancer patients who underwent surgery in the department of obstetrics and gynecology, Renmin Hospital of Wuhan University from 2018 to 2019. All patients were diagnosed with cervical cancer, and approved by two pathologists, respectively. All samples were immediately frozen and stored at -80°C after operation. All patients or their families were informed the approach of sample collection, and informed consent were obtained. All experiment in this study were approved by the Ethics Committee of the Renmin Hospital of Wuhan University.

Cell culture and transfection

All cervical cancer cell lines C33A, HT-3, HeLa, SiHa, normal cervical cell H8, and HEK-293T cells were commercially obtained from American Type Culture Collection (ATCC, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and 1% streptomycin double-antibody at 37°C with 5% CO_2. Sh-circCCDC66 targeting circCCDC66 were constructed by GeneChem (Shanghai, China), circ-CCDC66 or REOX1 sequences were subjected to pcDNA3.1 vector to generate circCCDC66 or REXO1 overexpression vectors by Genepharma (Suzhou, China). MiR-452-5p mimic were constructed and commercially procured from Sangon Biotech (Shanghai, China). Lipofectamine 3000 (Invitrogen, USA) were applied to carry out all transfections.

Animal experiment

The 8-week old nude mice (28 for study) were purchased from Vital River Inc. (Beijing, China). The ethics committee of the Renmin Hospital of Wuhan University approved animal experiments in this study. Firstly, HeLa cells were stably transfected with Sh-circCCDC66 and Sh-NC. Then, about 1×10^7 cells (per tumor) were subcutaneously injected into nude mice. After 28 days, mice were sacrificed, and tumors were harvested for study. The animal experiments were followed with the ethical standard of Helsinki Declaration of 1975 (1983 reversion).

Real-time qPCR

Total RNAs from cervical cancer human samples and cells were collected by Trizol kit (Invitrogen, USA). A PrimerScript RT Reagent kit (TaKaRa, Japan) was used to reversely transcribed RNA into cDNA. The amplification was conducted using SYBR Green dye. GPADH was used as an internal reference. The reliability of PCR results were confirmed by the comparative CT($2^{-\Delta\Delta Ct}$) method.

The information about primers used in study is showed in the supplementary Table 1.

Western blot

Total proteins from cervical cancer tissues and cells were collected by the RIPA lysis buffer (Beyotime, Shanghai, China). Proteins were separated by 10% SDS-PAGE, and then transferred to PVDF membranes (Millipore, USA). Membranes were incubated with primary antibodies over night at 4°C overnight, and then with secondary antibodies at room temperature for 2 hours. Protein bands were visualized by the ECL Western Blotting Detection Kit (PA, USA). Primary antibodies including: REXO1 (0.2 μg/ml, Abcam, #ab243536), GAPDH (1:2000, Abcam, #ab8226).

RNA fluorescence in situ hybridization (FISH)

Fluorescent In Situ Hybridization Kit (RiboBio, China) was used to carry out RNA FISH assay according to manufacturer's instruction. Cy3-labeled circ-CCDC66 probes were procured from GeneChem (Shanghai, China). The results were detected by Fluorescent In Situ Hybridization Kit and visualized with a confocal microscopy.

Cell proliferation experiment

Cell proliferation ability was detected by cell account kit 8 (CCK-8, Dojindo, Japan) according to a previous study [21]. Collectively, HeLa and SiHa cells (1×10^4 per well) were seeded into a 96-well plate and housed for 3 days. CCK-8 solution (10 μl) was added into well, two hours before detection. The absorbance at a wavelength of 450 nm was recorded, and repeated three times.

Cell migration and invasion experiment

Transwell chamber with 8.0 μm pores (Corning, USA) was used to detect cell migration and invasion ability. For migration, the upper chambers were seeded with cells (1×10^4 per well) with DMED, and the lower chambers were added with DMED with 10% FBS. after two days, the cells on the lower surface were fixed with 4% paraformaldehyde, and visualized under a microscope. For invasion, Matrigel (BD, USA) was used to cover upper chambers, cells were incubated on the upper chambers with Matrigel and DMEM, lower chambers were filled with DMEM with 10% FBS. After 2 days, cells attached on lower surface were fixed with 4% paraformaldehyde, and visualized under a microscope. Experiments were carried out three times.

RNA pull-down

The biotinylated RNA pull-down assay was carried out followed by previous studies[22,23]. Biotinylated CCDC66 and miR-452-5p probes were synthesized and commercially obtained from Sangon Biotech (Shanghai, China). Briefly, probe-coated beads were generated using C-1 magnetic beads (Life Technologies, Carlsbad, CA, USA). Then, RNA bands were analyzed by qRT-PCR.

AGO2-RIP

RNA immunoprecipitation assays using anti-AGO2 and anti-IgG antibodies were performed by a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to manufacturer's protocol. Then, RNA bands were subjected to qRT-PCR analysis. Experiments were repeated three times.

Dual luciferase assay

The sequences of CCDC66 and REXO1 3'UTR containing wide-type or mutant-type miR-452-5p binding sites were subjected to pGL3-Basic luciferase vector (Promega, USA). Then, vectors were transfected into 293T and HeLa cells with pRL-TK vector (Promega), miR-452-5p mimic and its normal control mimic. After two days, luciferase activities were detected by Dual-Luciferase Reporter assay system (Promega).

Statistical analysis

All data from experiment were presented as mean± Standard Deviation (SD). SPSS 17.0 software (SPSS, USA) was used to carry out results data. All experiments were repeated at least three times. The

differences between two groups were calculated by student t-test. The differences between multiple groups were analyzed by Analysis of Variance (ANOVA). The statistical correlation between miR-452-5p and circ-CCDC66 or REXO1 were calculated by spearman analysis. $p < 0.05$ was considered statistically significant.

Results

Circ-CCDC66 is upregulated in cervical cancer tissues

The expression of circ-CCDC66 in 36 pairs of cervical cancer human samples were measured. The level of circ-CCDC66 was obviously higher in tumor tissues compared with matched normal tissues (Fig 1.A). Moreover, high expression of circ-CCDC-66 was found to associate with large tumor size and advanced tumor stage (Fig 1.B-C). Then, it was found that circ-CCDC66 was highly expressed in cervical cancer cell lines compared with normal cervical cell line H8, especially in Hela and SiHa cells (Fig 1.D). Moreover, as shown in Fig 1.E and F, circ-CCDC66 was mainly located in cell cytoplasm. Here, our results suggest that circ-CCDC66 may participate in cervical cancer progression.

Circ-CCDC66 promotes the proliferation, migration and invasion of cervical cancer cells

To explore whether circ-CCDC66 plays its role in cervical cancer cellular progression, we constructed circ-CCDC66 knockdown cell models by stably transfecting Sh-NC or Sh-circ-CCDC66 into Hela and SiHa cells, the transfection efficiencies were detected (Fig 2.A). By performing CCK-8 and transwell assays, it was found that circ-CCDC66 knockdown significantly suppressed cervical cancer cell proliferation, migration, and invasion abilities (Fig 2.B-D). These results suggested that circ-CCDC66 regulated cervical cancer biological functions.

Circ-CCDC66 acts as an efficient molecular sponge for miR-452-5p

Previous studies indicated that circRNAs play its role via sponging to miRNAs and mediating downstream gene expression[24,25]. In the current study, we identified the downstream factor of circ-CCDC66 by Miranda database. We selected eight miRNAs (CLIP data: strict stringency (>=5)) for investigation. Biotinylated RNA pull-down assay results showed that miR-452-5p was highly enriched in purified bounds (Fig 3.A). Then, by conducting AGO-2 RIP assays, it was found that both circ-CCDC66 and miR-452-5p were enriched in anti-AGO2 bonds (Fig 3.B), suggesting that circ-CCDC66 might interact with miR-452-5p. The wild type (WT) and mutant type (Mut) sequence of circ-CCDC66 binding with miR-452-5p were synthesized (Fig 3.C). Next, Dual luciferase assays were performed, as shown in Fig 3.D, the luciferase activities in vector containing circ-CCDC66 WT sequence and miR-452-5p mimic co-transfected 293T and Hela cells were significantly decreased. Moreover, in Hela and SiHa cells, circ-CCDC66 knockdown obviously increased miR-452-5p expression (Fig 3.E). Collectively, circ-CCDC66 sponged to miR-452-5p and negatively regulated its expression in cervical cancer cell lines. Subsequently, we measured the expression of miR-452-5p in cervical cancer samples, miR-452-5p was lowly expressed in cervical cancer

tumor tissues compared with matched normal tissues and statistically correlated with circ-CCDC66 expression (Fig 3.F and G).

REXO1 is targeted by miR-452-5p

Bioinformatics analysis were used to find putative mRNA targets of miR-452-5p. We found that REXO1 might interact with miR-452-5p. Biotinylated RNA pull-down assays showed that REXO1 mRNA was abundantly enriched in bio-miR-452-5p bonds (Fig 4.A). Next, the binding sites between miR-452-5p and REXO1 were synthesized (Fig 4.B). Next, it was found that miR-452-5p mimic significantly decreased luciferase activity in vectors harboring REXO1 WT sequences infected 293T and SiHa cells (Fig 4.C and D). Furthermore, the expression of REXO1 in Hela and SiHa cells were suppressed by miR-452-5p mimic, but reversed by OE-circ-CCDC66 (Fig 4.E). The above results suggested that miR-452-5p directly targeted to REXO1, and circ-CCDC66 upregulated REXO1 expression in cervical cancer cells via miR-452-5p. Then, REXO1 was found to highly expresse in cervical cancer tumor tissues, and correlated to miR-452-5p expression (Fig 4.F-H).

Circ-CCDC66 mediates REXO1 expression to promote cervical cancer progression via miR-452-5p

Here, we investigated the functions of circ-CCDC66/miR-452-5p/REXO1 axis in cervical cancer cellular progression. Cells models were generated by transfecting Sh-NC, Sh-circ-CCDC66, Sh-circ-CCDC66+OE-NC, Sh-circ-CCDC66+OE-REXO1 into Hela and SiHa cells. The expression of cric-CCDC66 and REXO1 were measured (Fig 5.A). By conducting CCK-8 and transwell assays, it was found that the inhibitory effects of downregulated circ-CCDC66 on Hela and SiHa cells were rescued by REXO1 overexpression (Fig 5.B-F). Sum up, circ-CCDC66 regulated REXO1 expression to promote cervical cancer progression via sponging miR-452-3p.

Downregulation of circ-CCDC66 inhibits cervical cancer cell growth in vivo.

The above experiments demonstrated the role of circ-CCDC66 in vitro. The function of circ-CCDC66 in vivo was assessed through constructing circ-CCDC66 knockdown mouse models. As shown in Fig 6.A and B, circ-CCDC66 knockdown significantly suppressed cervical cancer cell growth in vivo, the representative image of xenotransplantation tumors showed the inhibitory effect of circ-CCDC66 (Fig 6.C).

Discussion

In this study, we revealed that circ-CCDC66 acted as an oncogene in cervical cancer. Previous study has demonstrated that circ-CCDC66 mediates tumorigenesis via regulating multiple cellular functions, such as proliferation, migration, invasion and EMT levels [19, 20, 26]. Here, we found that knockdown of circ-CCDC66 attenuated cervical cancer cell proliferation, migration and invasion abilities in vitro, and inhibited cervical cancer cell growth in vivo. These evidences indicated that circ-CCDC66 might

participate in cervical cancer progression. Subsequently, to uncover the underlying mechanisms, we investigated the downstream factors of circ-CCDC66.

CircRNAs have been widely reported that mediate virous biological progressions through transcriptionally regulating gene expression via sponging to microRNA[24, 25, 27, 28]. In current study, Bioinformatics analysis, RNA-pull down, RIP, and dual-luciferase reporter assays was conducted, and we found that circ-CCDC66 was an efficient molecular sponge for miR-452-5p, and miR-452-5p directly targeted to REXO1 in cervical cancer cells. MiR-452-5p has been revealed that participates in multiple cancer progression, such as: renal cancer, prostate cancer, hepatocellular caner, and lung squamous cell carcinoma[29–32], and showed its specific role in tumorigenesis. But, the function of miR-452-5p in cervical cancer remains unelucidated. Our study found that circ-CCDC66 sponged to miR-452-5p and negatively regulated its expression in cervical cancer cells. Furthermore, it was found that miR-452-5p was downregulated in cervical cancer tumor tissues in comparison with matched normal tissues. Here, we presumed that circ-CCDC66 plays its oncogene role via miR-452-5p in cervical cancer.

CircRNAs paly its role in tumorigenesis mainly through transcriptionally regulating mRNA expression and mediating protein levels. Our study found that miR-452-5p directly targeted to REXO1, and regulated REXO1 expression in cervical cancer cells. By performing CCK-8, transwell assays, we found that overexpression of REXO1 reversed the inhibitory effects of circ-CCDC66 on cell proliferation, migration, invasion abilities. Moreover, REXO1 was highly expressed in cervical cancer tissues. Sum up, circ-CCDC66 was found to upregulate REXO1 expression to promote cervical cancer development via miR-452-5p. However, the downstream factors of REXO1 in cervical cancer progression need further exploration.

Conclusions

In conclusion, our study revealed that the expression of circ-CCDC66 was upregulated in cervical cancer tumor tissues, and circ-CCDC66 mediated the proliferation, migration, and invasion abilities of cervical cancer cells. Moreover, our results demonstrated that circ-CCDC66 promoted cervical cancer progression via miR-452-5p/REXO1 axis, indicating that circ-CCDC66 might be a novel therapeutic target for cervical cancer.

Declarations

Availability of data and materials

The data performed and analyzed during the present study are available from the corresponding author on reasonable request.

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N.A.

Author information

Affiliations

Department of Obstetrics and Gynecology, Renmin Hospital of Wuhan University, Wuhan, China.

Yan Zhang, Xing Li, Jun Zhang, Lin Mao

Contributions

YZ conceived and leading performed the experiments, contributed reagents/materials/analysis tools, and wrote the manuscript. XL performed the experiments and analyzed data. JZ reviewed drafts of the manuscript, and analyzed data. LM performed the experiments and participated data correction. All authors read and approved the final manuscript.

Corresponding author

Correspondence to Yan Zhang

Ethics declarations

Ethics approval and consent to participate

All experiments in this study were approved by the Ethics Committee of the Renmin Hospital of Wuhan University.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interests to declare in the current study.

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Figures

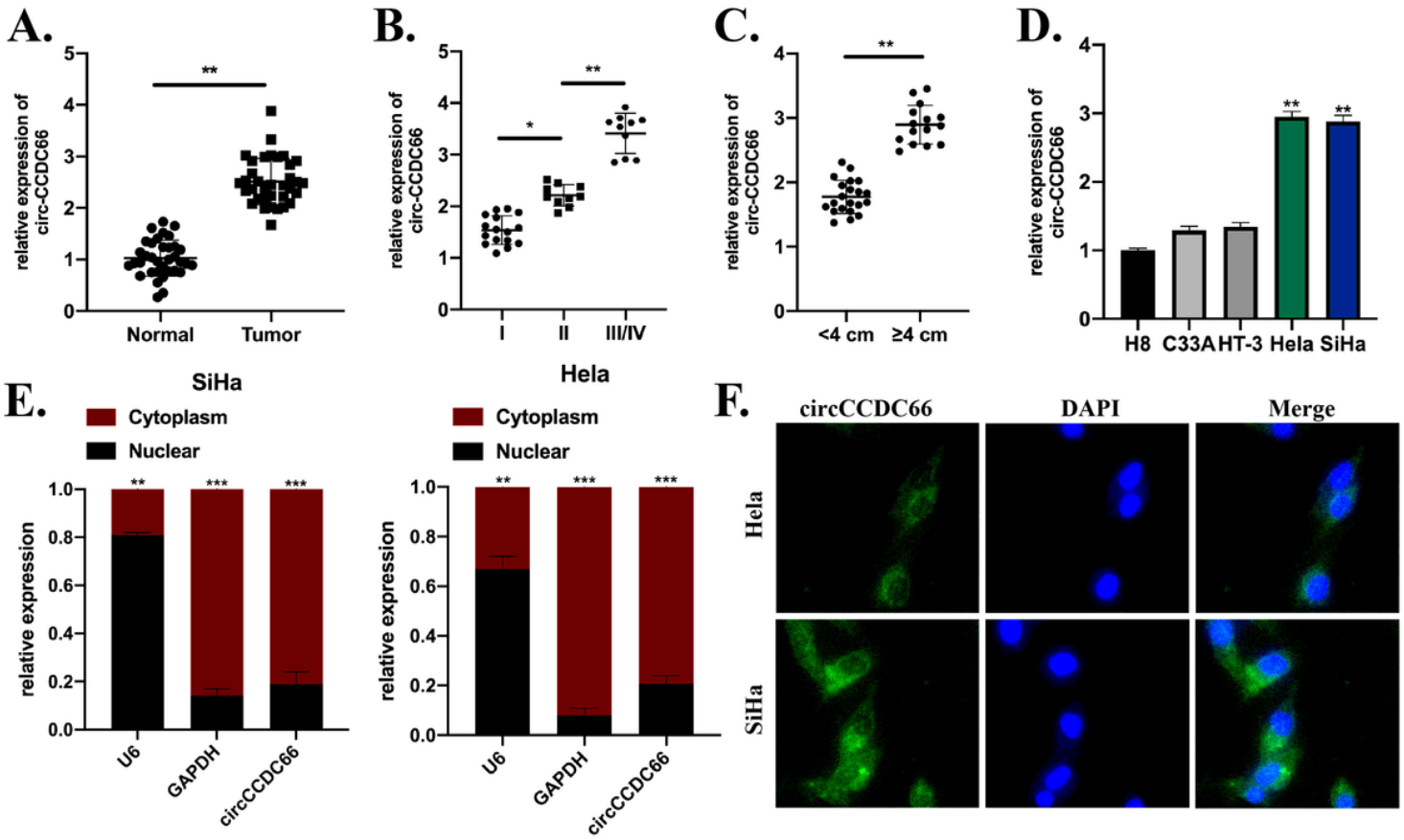

Figure 1

Circ-CCDC66 is upregulated in cervical cancer tissues. A: The expression of circ-CCDC66 in 36 pairs of cervical cancer tissues and its matched normal tissues. B and C: relative expression of CCDC66 was analyzed according to TNM stage and tumor size. D: relative expression of circ-CCDC66 in cervical cancer cell lines HeLa, SiHa, C33A, HT-3, and normal cervical cell line H8. E: nuclear-cytoplasmic fractionation assays were conducted to detect the expression of CCDC66 in the nuclear or cytoplasmic of cervical cells. F: The location of CCDC66 in cervical cells were detected by FISH assay. Data were presented as mean ± SD, all experiments were repeated at least three times. *P < 0.05, **P < 0.01, ***P < 0.001.

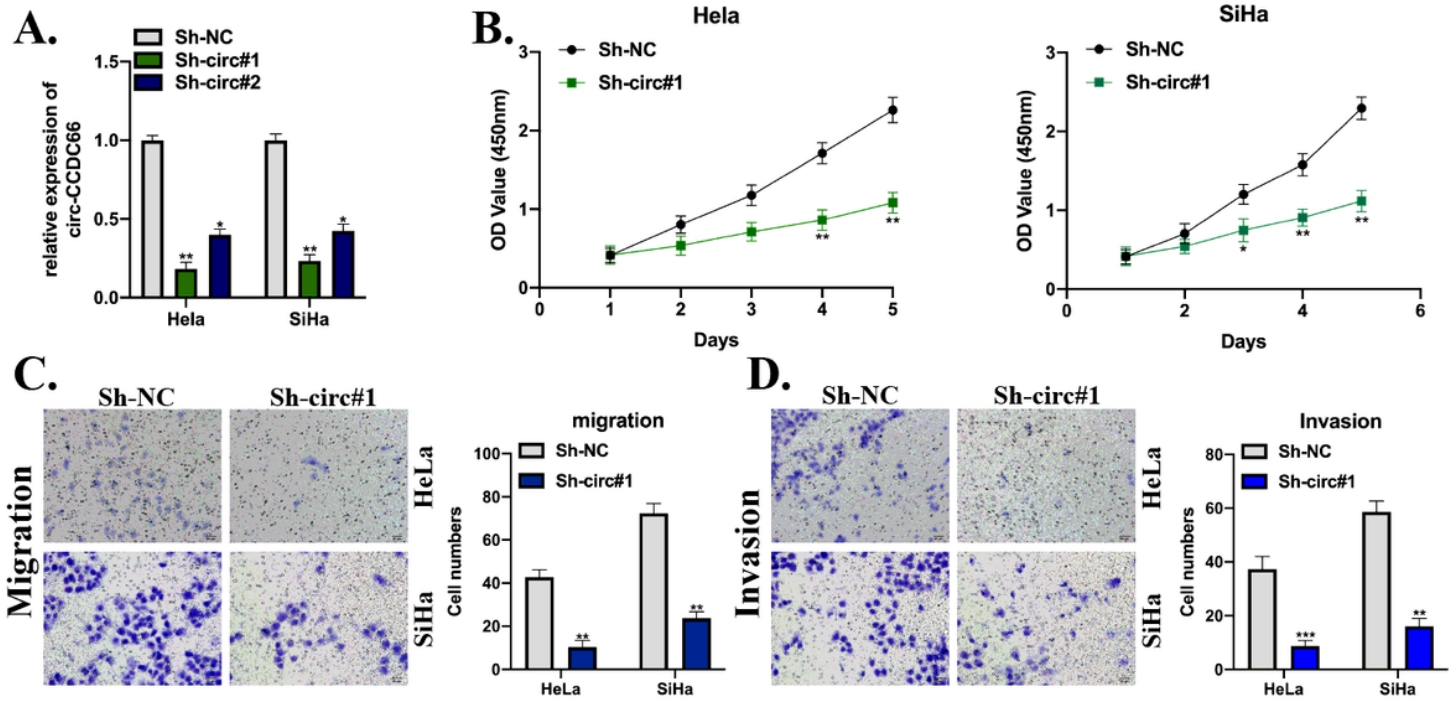

Figure 2

Circ-CCDC66 promotes the proliferation, migration and invasion of cervical cancer cells. A: Cervical cancer cell lines HeLa and SiHa were stably transfected with Sh-NC or Sh-circ-CCDC66#1, Sh-circ-CCDC66#2, relative expression of circ-CCDC66 was analyzed by qRT-PCR assay. B: Cell proliferation abilities of treated HeLa and SiHa were detected by CCK-8 assays. C: Transwell migration assays were conducted to evaluate cell migration ability. D: Transwell invasion assays were performed to measure cell invasion ability. Data were presented as mean ± SD, all experiments were repeated at least three times. *P < 0.05, **P < 0.01, ***P < 0.001.

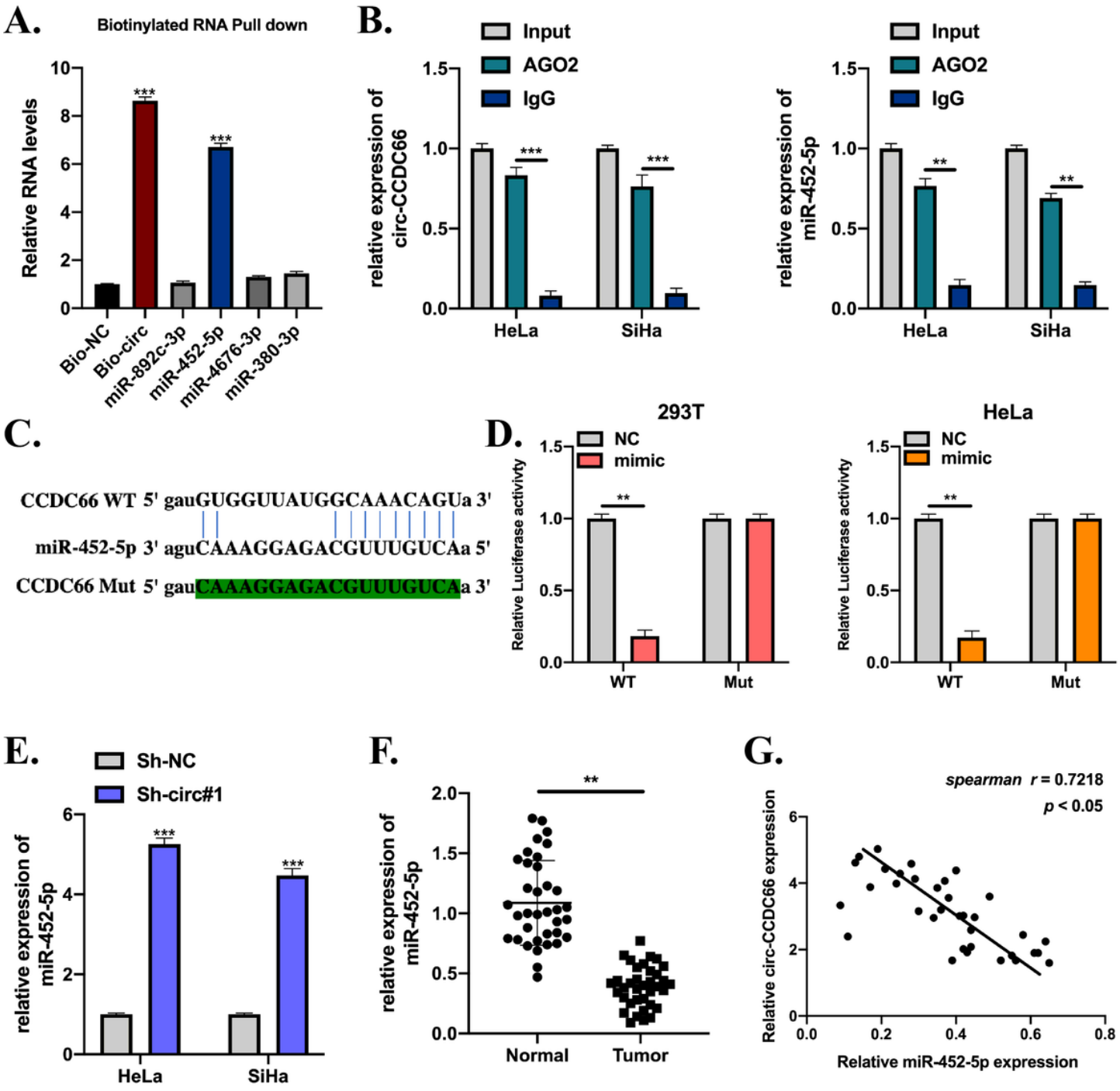

Figure 3

Circ-CCDC66 acts as a molecular sponge for miR-452-5p. Putative downstream miRNAs of circ-CCDC66 were predicted by Miranda (http://miranda.org.uk/) database, eight miRNAs were selected for our further study. A: Full length of circ-CCDC66 were biotinylated, and the complexes were subjected to qRT-PCR assays. Relative miRNAs expression were presented. B: AGO-2 RIP assays were conducted, and the expression of circ-CCDC66 and miR-452-5p in protein bonds were detected. C: The binding sites between circ-CCDC66 and miR-452-5p were showed. D: Luciferase reporter assays were used to determine the interaction between circ-CCDC66 and miR-452-5p in 293T and Hela cells. E: The expression of miR-452-5p in Sh-NC or Sh-circCCDC66 transfected Hela and SiHa cells were detected by qRT-PCR. F: the

expression of miR-452-5p in cervical cancer tissues were measured. G: Spearman analysis was employed to assess the correlation expression between circ-CCDC66 and miR-452-5p. Data were presented as mean ± SD, all experiments were performed at least three times. **P < 0.01, ***P < 0.001.

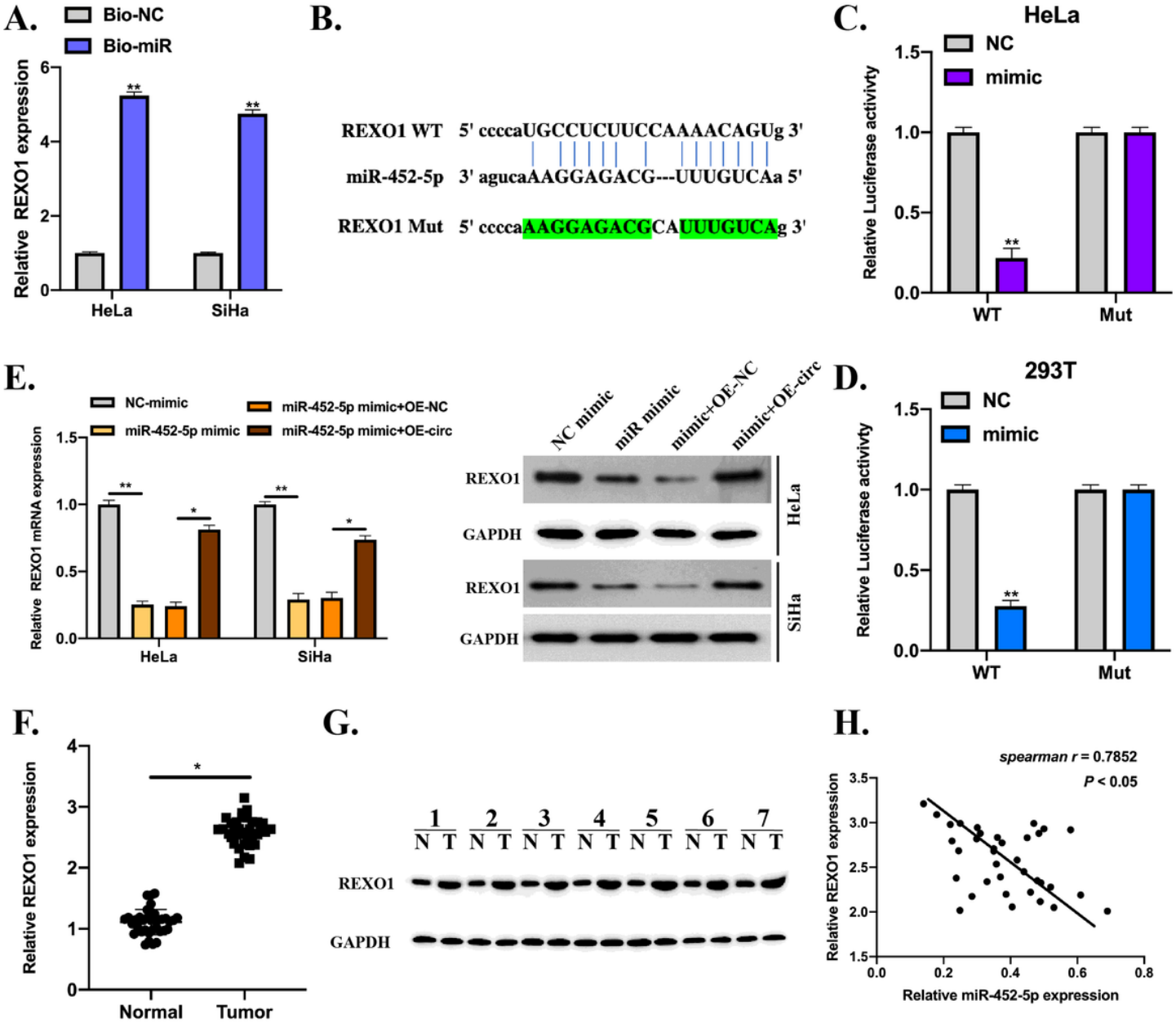

Figure 4

REXO1 is targeted by miR-452-5p. Potential mRNA targets of miR-452-5p were predicted by PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_exe.html), RNA22V2 (https://cm.jefferson.edu/rna22/), miRmap (https://mirmap.ezlab.org/downloads/mirmap201301e/), TargetScan Human 7.2 (http://www.targetscan.org/vert_72/) dataset. A: Bio-miR-452-5p and Bio-NC probes were constructed, and qRT-PCR was applied to analyze the expression of REXO1 mRNA in the purified bonds. B: the binding sites between miR-452-5p and REXO1, mutant sequence of REXO1 was synthesized. C and D: Luciferase reporter assays were performed to determine the correlation between miR-452-5p and REXO1 in 293T and Hela cells. E: Hela and SiHa cells stably transfected with NC mimic, miR-452-5p mimic, miR-452-5p mimic

with OE-NC, and miR-452-5p mimic with OE-circ-CCDC66 as indicated, the protein and mRNA levels of REXO1 were measured. F: The expression of REXO1 mRNA in cervical cancer tissues were evaluated. G: The protein expression in randomly selected seven pairs of cervical cancer human samples were detected by western blot. H: The expression of miR-452-5p and REXO1 in tumor tissues were analyzed by spearman analysis. Data were presented as mean ± SD, all experiments were performed at least three times. *P < 0.05, **P < 0.01.

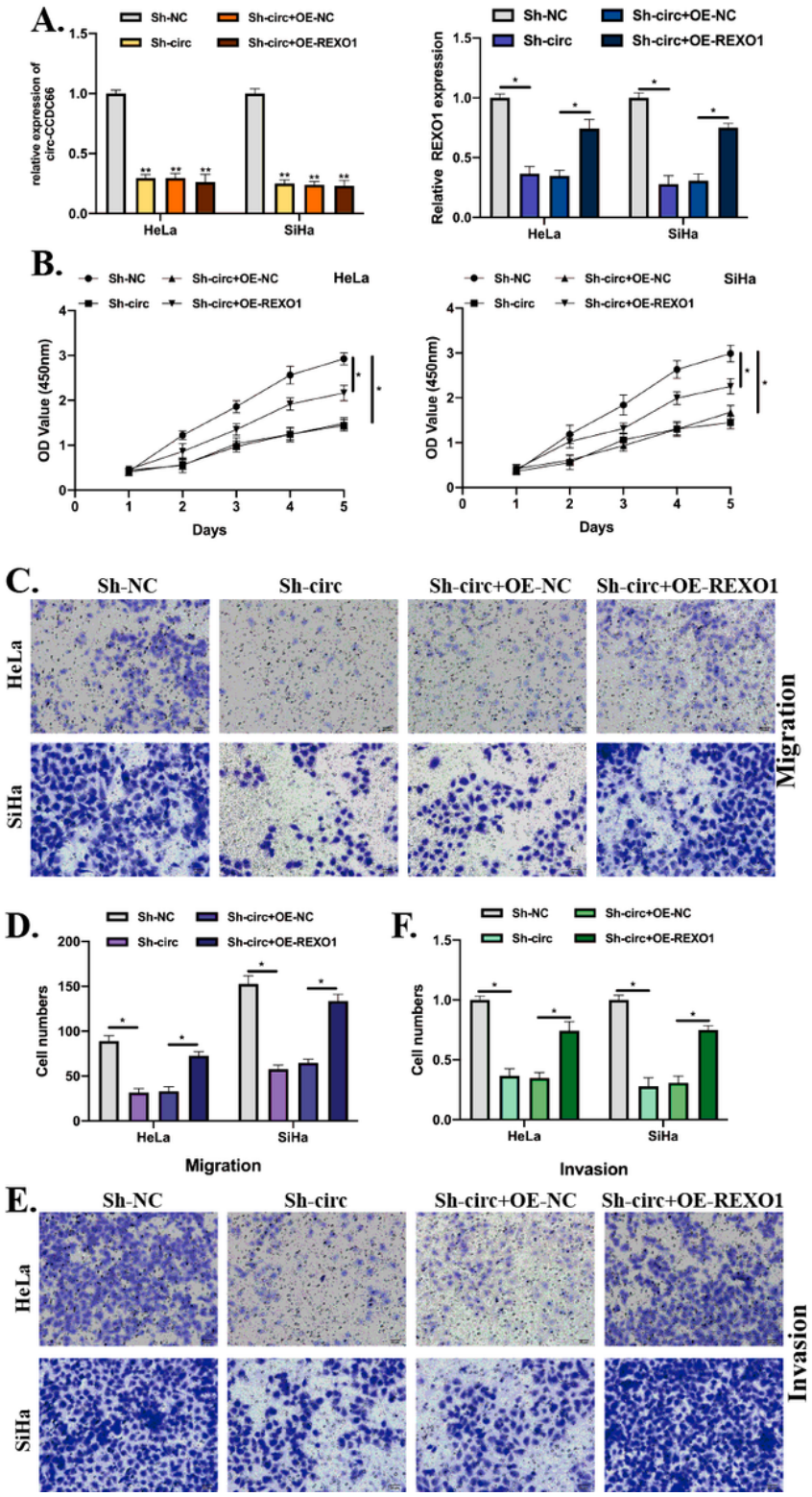

Figure 5

Circ-CCDC66 mediates REXO1 expression to promote cervical cancer progression via miR-452-5p. Cell models were constructed by transfecting Sh-NC, Sh-circ-CCDC66, Sh-circ-CCDC66+OE-NC, Sh-circ-CCDC66+OE-REXO1 into Hela and SiHa cells. A: transfection efficiencies were detected. B: Cells were subjected to CCK-8 assays for cell proliferation detection. C and D: Transwell migration assays were performed to evaluate cell migration abilities, number of migrated cells were recorded. E and F: Cell invasion abilities were determined by transwell invasion assays, invasion cells were recorded and comparative statistics were presented. Data were presented as mean ± SD, all experiments were performed at least three times. *P < 0.05, **P < 0.01.

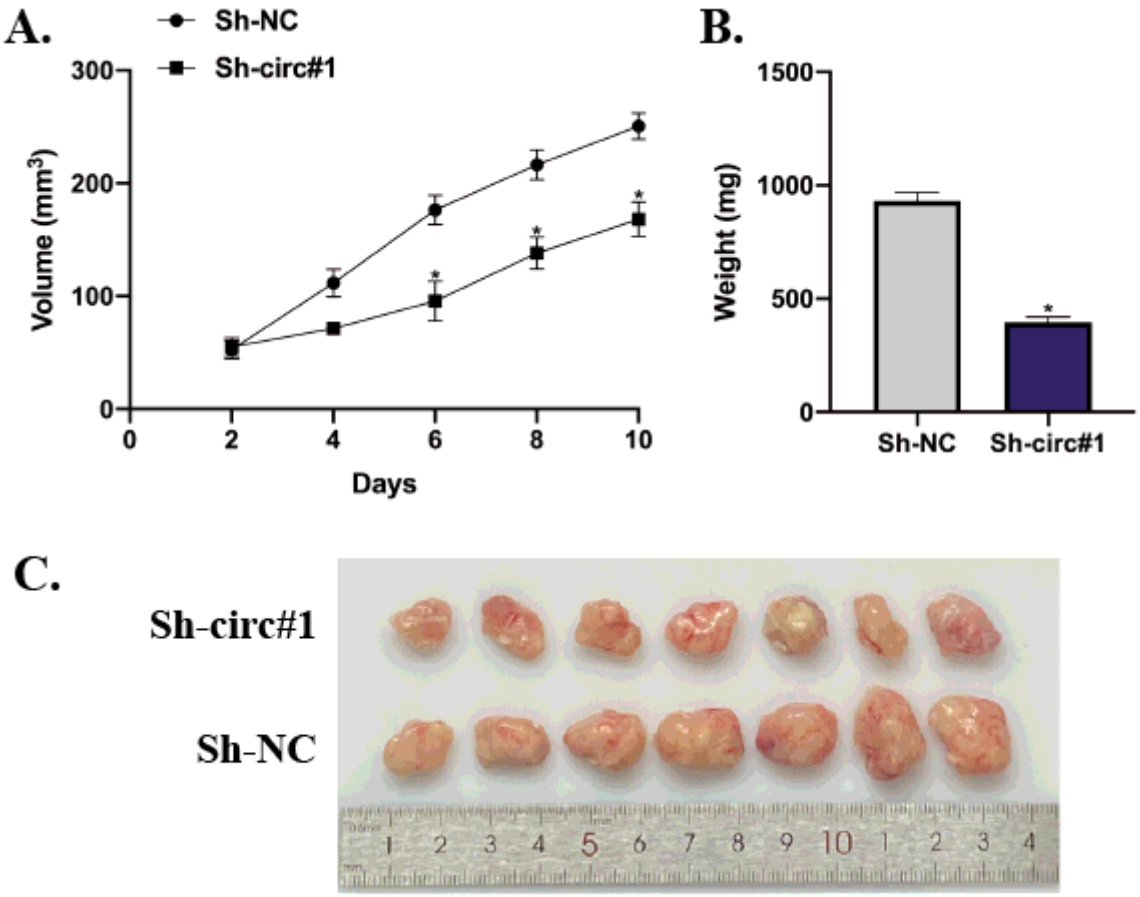

Figure 6

Downregulation of circ-CCDC66 inhibits cervical cancer cell growth in vivo. Hela cell (106 per tumor) pre-transfected with Sh-NC or Sh-circCCDC66 were subcutaneously injected into node mouse (7 mice for each group). After 28 days, tumors were harvested after mice were sacrificed. A and B: tumor volumes and tumor end weights were recorded. C: Representative image of xenotransplantation tumors were showed. Data were presented as mean ± SD, all experiments were performed at least three times. *P < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx