

Downregulation of Circ_0000673 Promotes Cell Proliferation and Migration in Endometriosis Via Suppressing PTEN

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Research

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Abstract

Background: Endometriosis is a prevalent gynecologic disease, affecting up to 10% of women at reproductive age and approximately 50% of women with infertility. The function of circRNAs in various diseases has been highlighted. Dysregulated expression of circRNAs in endometriosis has been reported and circ_0000673 was significantly deregulated. However, its explicit role in pathogenesis of endometriosis is yet to be identified.

Methods: circ_0000673 expression was detected in paired ectopic and eutopic endometrium using qPCR and fluorescent *in situ* hybridization. Knockdown of circ_0000673 in eutopic and normal endometrial stromal cells were done by transfection with lentivirus vectors. The proliferation activity of endometrial stromal cells was evaluated by CCK-8 assay and colony formation assay, while the migration capacity was valued by wound healing assay. PTEN, PI3K and p-AKT were detected by qPCR and western blotting. Dual luciferase assay was performed to assess the bonding between circ_0000673, PTEN and miR-616-3p.

Results: The expression of circ_0000673 was reduced in ectopic endometrium. Knockdown of circ_0000673 significantly induced eutopic and normal endometrial cell proliferation and migration. Bioinformatic analysis predicted that circ_0000673 might sponge miR-616-3p. The effect of circ_0000673 knockdown could be recovered by miR-616-3p inhibitor and enhanced by miR-616-3p mimics. Meanwhile, qPCR and western blotting showed that circ_0000673 knockdown inhibited the expression of PTEN, and subsequently activated PI3K and p-Akt. Furthermore, PTEN was confirmed to be a target of miR-616-3p.

Conclusion: The results demonstrated that deregulated expression of circ_0000673 could promote endometriosis progression via sponging miR-616-3p and further regulating PTEN.

1 Introduction

Endometriosis is a debilitating disorder which manifests as endometrial cells outside uterus cavity. It affects up to 10% of women at reproductive age, with an involvement of over 176 million women across the world[1, 2]. Approximately 50–80% of women with endometriosis suffer from pelvic pain and up to 50% from infertility[1, 2]. The exact etiology and pathophysiology of endometriosis are unclear. The most widely accepted hypothesis is that endometriosis is initiated from endometrial pieces into the pelvic by retrograde menstruation. However, the ectopic lesions implantation also depends on other factors that facilitate the viability, proliferation, adhesion, neo-angiogenesis and migration of the reverse endometrial pieces and cells[2]. Until now, the underlying molecular mechanisms need to be clearly defined, which could bring new orientation in exploring targets of endometriosis treatment.

Circular RNAs (circRNAs) are a class of endogenous RNAs with closed loop structure which ascertained the more stability than linear RNAs. With high abundance and specific expression in different tissues or developmental stages, circRNAs have been revealed to participate in the regulation of biological and

pathological processes such as cell proliferation, apoptosis, invasion, angiogenesis and epithelial-mesenchymal transformation, by acting as microRNA (miRNA) sponges[3, 4]. Evidences have demonstrated aberrant circRNA expression in endometriosis[5, 6]. Some studies showed that circRNAs promote the pathogenesis of endometriosis via miRNAs[7–9]. Our further work found that circ_0000673 was significantly deregulated in ectopic endometrium compared to eutopic endometrium[5]. Circ_0000673 is encoded by *RSL1D1* gene and contains 251bp. Studies showed that *RSL1D1* gene can contribute to cell cycle, proliferation, apoptosis and metastasis through down-regulating the expression of *PTEN* gene[10, 11]. It has been demonstrated that dysregulated circ_0000673 in neoplasms may be a novel oncogene by regulating cell proliferation, migration and invasion[12, 13].

Through bioinformatic analysis, we found that circ-0000673 contains miR-616-3p response element (MRE). miR-616-3p is reported as a carcinogenic miRNA (onco-miRNA) in ovarian cancer, breast cancer and gastric cancer by promoting cell proliferation, reducing apoptosis, promoting cell invasion and metastasis, epithelial-mesenchymal transformation and other mechanisms. Evidences have revealed that dysregulated miR-616-3p contributes to abnormal proliferation and apoptosis by targeting PTEN in hepatocellular carcinoma, renal tubular epithelial cells, and cardiomyocytes[14, 15]. However, the functions of circ_0000673 and miR-616-3p in endometriosis have not been defined. The aim of the study was to explore the potential role of circ_0000673 in pathogenesis of endometriosis.

2 Materials And Methods

2.1 Clinical samples

This study was approved and supervised by the Medical Ethics Committee of Xiangya Hospital, Central South University. Written informed consent was obtained from all subjects for participation. Twenty patients with ovarian endometriomas at stage III–IV who provided both eutopic endometrium(EU) and ectopic endometrium(EC) and twenty women without endometriosis as normal control were recruited during the proliferative phase. All subjects were at the age of 20–45 years old, with regular menstrual cycles, who received steroid hormone treatment at least 3 months before specimen collection.

2.2 Fluorescent *in situ* hybridization

The EU and EC tissues were embedded in paraffin and sliced. Slices were dewaxed in xylene, dehydrated in anhydrous ethanol, and then treated with protease K (20ug/ml). Subsequently,3% methanol-H₂O₂ was added to block endogenous peroxidase. After prehybridization, the hybridization solution with a digoxigenin(DIG)-labeled probe was added and hybridized overnight at 42°C. Sections were then incubated with mouse anti-digoxigenin labelled peroxidase (anti-DIG-HRP, Jackson, USA) at 37°C for 50 minutes. After washing for 3 times, fresh prepared FITC-TSA chromogenic reagent (Servicebio, China) was added reaction was in dark for 5 minutes at room temperature reaction, and then the sections were incubated with DAPI for 5 minutes. Finally, the slices were sealed and observed under Nikon Laser Scanning Confocal Microscope (Nikon, Japan).

2.3 Cell culture and identification

The endometrial tissue was rinsed with Hanks solution, and cut into pieces in F12/DMEM culture medium, followed by collagenase digestion for 60 min and DNase I digestion for 30 min. After centrifugation, the cells were suspended in F12/DMEM with 10% fetal bovine serum, filtered through a cell strainer (70 μ m) to collect endometrial stromal cells. The endometrial stromal cells were inoculated in F12/DMEM with 10% fetal bovine serum in an incubator with 5% CO₂ at 37 °C. The isolated endometrial stromal cells were identified by staining with vimentin using immunofluorescent techniques.

2.4 Cell transfection

Primary eutopic and normal endometrial stromal cells were treated at the third passage, with the cell density at 1×10^4 ml. The lentivirus vectors (Genepharma, China) targeting to the junction region of hsa_circ_0000673 were constructed to knockdown hsa_circ_0000673 expression in endometrial stroma cells. All the lentivirus vectors were transfected into eutopic and normal endometrial stromal cells with transfection enhancers, and the sequences were as follows: LV-1, 5'-GTGGTTCTTGCAGATTATCTC-3'; LV-2, 5'-CTTGCAGATTATCTCCCTCCA-3'; LV- negative control (NC), 5'-TTCTCCGAACGTGTACAGT-3'. The transfection efficiency was confirmed under fluorescence microscopy after 24 hours and cells were harvested for further function experiments after 48 hours. Has-miR-616-3p mimic or inhibitor (Guangzhou RiboBio, China) was transfected using Lipofectamine 2000 reagents (Invitrogen, USA) according to the manufacturer's protocol. All experiments were done three times with triplicates in each assay.

2.5 CCK-8 assay

The CCK8 assay was carried out via CCK8 assay as the manufacturer's protocol (NCM Biotech, China). Briefly, cells were seeded into 96-well plates and divided into four groups with four duplicate wells in each group. After cell attachment, the lentivirus vectors were added and the plates were incubated at 37°C for 24 hours. 10 μ L CCK-8 reagent was added into each well and incubated at 37°C for 2 hours. The cell proliferation was determined by testing the absorbance value at 450 nm.

2.6 Colony formation assay

Cells were paved in six-well plates with 1000 cells per well. LV-circ_0000673 or miR-616-3p mimic/inhibitor was transfected and the cells were cultured for 2 weeks. After washing, the cells were successively treated by 4% paraformaldehyde and 0.1% crystal violet. Then, the colonies in each well were calculated under microscope.

2.7 Wound healing assay

Wound healing assay was performed to assess the migration capability of endometrial stroma cells after knockdown of circ_000673. Briefly, endometrial stromal cells were seeded in six-well plates, and LV-circ_0000673 or miR-616-3p mimic/inhibitor was transfected. Then the monolayer cells were scraped with a micropipette tip to make a straight wound, and cultured with serum-free medium for 24h. The gap width at 0 and 24h was recorded under microscopy.

2.8 qRT-PCR

The cells were collected 72 hours after transfection, and total RNA was extracted using Trizol reagent. The concentration and purity of RNA were measured by NanoVue Plus spectrophotometer (Healthcare Bio-ScienceAB, Uppsala, Sweden). The primers were listed as follows: hsa_circ_0000673, 5'-ATCTGTAAACCTTCTGTCCAAGA-3'(forward) and 5'-TCAAAACTGCTCAGAAGGCG-3'(reverse); *PTEN*, 5'-ACTATTCCCAGTCAGAGGCG-3' (forward) and 5'-TCACCTTTAGCTGGCAGACC-3' (reverse); *PI3K*, 5'-GCACCTGAATAGGCAAGTC-3' (forward) and 5'-TCGCACCACCTCAATAAGT-3' (reverse); *AKT*, 5'-GTGGAGGACCAGATGATGC-3' (forward) and 5'-TGCCCCTGCTATGTGTAAG-3' (reverse); *GAPDH*, 5'-TGCACCACCAACTGCTTAGC-3'(forward) and 5'-GGCATGGACTGTGGTCATGAG-3'(reverse). miR-616-3p stem loop reverse transcription primers and U6 control were designed and synthesized by Guangzhou Ruibo Biological Co., Ltd. The reverse transcriptase kit (TaKaRa, China), was used for reverse transcriptional reaction. Gene expression was performed with SYBR Green qPCR mix (Bio-Rad, USA) using Applied Biosystems 7900 Real-Time PCR system. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.9 Western blot assay

Endometrial cells were lysed in RIPA lysis buffer to extract total protein. 50 μ g total protein was taken for 10% SDS-PAGE electrophoresis, transferred onto a 0.45 μ m PVDF membrane and blocked with 5% nonfat milk successively. Primary antibodies of PTEN (1:1000), PI3K (1:1000), pAKT (1:2000), and GAPDH (1:1000) were added and incubated overnight at 4 °C. Then after washing for three times, the secondary antibodies were added incubated at room temperature for one hour. At the end, enhanced chemiluminescence reagent (CST, USA) was added and the reactivity was determined by an enhanced chemiluminescence detection system (Amersham, Pittsburg, PA).

2.10 Dual luciferase assay

Dual luciferase assay was performed to assess the bonding between circ_0000673, PTEN and miR-616-3p. The wild-type (WT) of circ_0000673 and PTEN 3'UTR were inserted into pMIR-reporter plasmids (OBio Technology Corp, China) and the mutant (MUT) type was designed by mutating the binding site of the seed sequence. The WT and MUT luciferase reporter plasmids were respectively co-transfected with miR-616-3p mimics/negative control into 293T cells. Then, the relative luciferase activity was measured by luciferase reporter kits (Promega, Madison, WI, USA).

2.11 Statistical analysis

Data were expressed as mean \pm standard deviation (SD). Two-tailed student's *t* test was performed for pairwise comparisons, and the one-way Variance (ANOVA) Analysis was used for multiple comparisons. All experiments were performed in triplicate and *P* value < 0.05 was set as statistical significance. The statistical analysis was conducted by the SPSS 25.0 and GraphPad Prism 9.0.

3 Results

3.1 Declined expression of circ_0000673 in endometriosis

qPCR confirmed that circ_0000673 were significantly downregulated in ectopic endometrium compared with eutopic endometrium (Fig. 1A). Fluorescent *in situ* hybridization also showed that circ_0000673 was low or non-expressed in ectopic endometrium, whereas it was upregulated in eutopic endometrium and mainly distributed in the cytoplasm (Fig. 1B).

3.2 Knockdown of circ_0000673 promotes endometrial cell proliferation and migration

Endometrial stromal cells presented a fusiform cell morphology under light microscopy, and the positive expression of vimentin by immunofluorescence stain confirmed the endometrial stromal cells (Fig. 2A). Transfection efficiency was observed by fluorescence of green fluorescent protein, which demonstrated that the cells were successfully transfected with circ_0000673 knockdown lentiviral vectors (Fig. 2B). qPCR confirmed that circ_0000673 was significantly down expressed after transfection with LV-circ_0000673-1/2 in endometrial stroma cells (Fig. 2C). The CCK-8 and cell clone formation assay were performed to assess the function of circ_0000673 in endometrial cell proliferation. Interestingly, cell proliferation rate and colony formation capacity were significantly increased in the knockdown LV-circ group compared with the negative control. was significantly increased by circ_0000673 knockdown in eutopic endometrial stromal cells (Fig. 2D, E). The results indicated that downregulation of circ_0000673 may play important roles in cell proliferation in endometriosis. Wound healing assays showed that the numbers of migrating cells after transfection with circ_0000673 knockdown lentiviral vectors were significantly increased compared with those in the control group (Fig. 2F).

3.3 circ_0000673 sponged miR-616-3p

circRNAs have been known to involve in regulating gene expression by serving as a miRNA sponge. We predicted circRNA-miRNA interactions using TargetScan and miRanda databases, which revealed that miR-616-3p has a binding site to circ_0000673 (Fig. 3A). The expression of miR-616-3p was significantly increased after circ_0000673 knockdown (Fig. 3B). Compared with negative control group, luciferase intensity significantly reduced in the group co-transfected with circ_0000673-WT plasmids and miR-616-3p mimics; whereas no significant differences were observed on luciferase intensity in those co-transfected with mutant plasmids or negative control (Fig. 3C). The data verified the binding between circ_0000673 and miR-616-3p.

3.4 circ_0000673/ miR-616-3p induced endometrial cell proliferation and migration via PTEN

To further clarify the mechanisms of circ_0000673/miR-616-3p on endometrial cell proliferation and migration, cells were transfected with circ_0000673 lentivirus knockdown vectors and/or miR-616-3p mimics/inhibitors, respectively. Cells transfected with knockdown LV-circ_0000673 or miR-616-3p mimic

showed significantly increased capacity of proliferation by CCK8 and colony formation assays (Fig. 4A, B), and also increased migration capacity by wound healing assays (Fig. 4C). Furthermore, the increased capacity of proliferation and migration was recovered by adding the miR-616-3p inhibitor (Fig. 4A, B, C). qPCR and western blot assay revealed that circ_00007673 knockdown or miR-616-3p mimic reduced the expression of PTEN and increased PI3K and pAkt, and the expression of PTEN, PI3K and pAkt were recovered by adding miR-616-3p inhibitor (Fig. 4D, E). These data showed that circ_00007673 and miR-616-3p play converse roles in endometriosis, and downregulation of circ_0000673 increased the expression of miR-616-3p. The promotion of circ_00007673/miR-616-3p on endometrial cell proliferation and migration may be related with PTEN pathway.

3.5 PTEN was a target of miR-616-3p

miRNAs were known to be important post-transcriptional repressors by binding to 3'-UTR. Then, we conducted luciferase reporter assay to assess whether the expression of PTEN was directly regulated by miR-616-3p. miR-616-3p mimics significantly reduced the luciferase activity when co-transfected with PTEN-WT plasmid, while no effect on luciferase activity when co-transfected with PTEN-MUT plasmid. The data confirmed that miR-616-3p targeted PTEN (Fig. 5).

4 Discussion

Endometriosis is an enigmatic disease affecting mainly reproductive-aged women because of pain and declined fertility. The treatment effect is dissatisfied on account of uncertainty of its pathogenesis[16, 17]. Exploring the subtle mechanism and identifying new molecular targets are needed to bring promise for more effective treatment. circRNAs have been reported to be important molecules in regulating gene expression and biological processes[18, 19]. Differential expression of circRNAs in endometriosis has been identified[5, 6]; while the subtle mechanism has not been clearly defined. In this study, we explored the function of a down-regulated circRNA(circ_0000673) on proliferation and migration in endometriosis.

The proliferation and migration of endometrial tissue outside of the uterus is a vital character of endometriosis; whereas the exact pathological progression remains largely unknown[20]. CircRNAs has been recognized as important molecules involving in various biological processes and its abnormality may contribute to many diseases[3]. Studies have showed abnormal expression of circRNAs in endometriosis and indicated their diagnosis value in in discerning diseases at earlier time[21, 22]. Our previous study showed the profile of differentially expressed circRNAs, which revealed that circ_0000673 was attenuated in ovarian endometrioma[5]. In this study, we confirmed downregulation of hsa_circ_0000673 ovarian ectopic endometrium by fluorescent in situ hybridization, which implied the underlying roles in ovarian endometriosis. Moreover, our findings uncover that loss of circ_0000673 could promotes proliferation and migration of endometrial cells. Recent studies suggested that circATRNL1 promotes epithelial-mesenchymal transition(EMT) by regulating Yes-associated protein 1[23], and high expression of circ_0007331 play a vital role in the proliferation and invasion in endometriosis[9]. The studies suggested the regulation effects of circRNAs in the progression of endometriosis. Recently, dysregulated circ_0000673 were reported to participate in tumor invasion, differentiation in

cholangiocarcinoma, which indicated it as a novel oncogene[12]. We supposed that the potential functions of circ_0000673 in endometriosis, which was need to be further clarified.

The function of circRNAs has been identified as a miRNAs sponge, which exerts an inhibitor of miRNAs. We found circ_0000673 could bind to miR-616-3p, which may target *PTEN*. *PTEN* was reduced in endometriosis and associated with the severity stage[24]. Wu et al revealed that miR-616-3p could facilitate migration and EMT of gastric cancer cell lines[14]. Thus, we hypothesized that downregulated circ_0000673 may promotes progression of endometriosis by sponging miR-616-3p. We found miR-616-3p was increased after circ_0000673 knock-down. Additionally, dual luciferase assay identified miR-616-3p was a target of circ_0000673. The miR-616-3p mimic has a similar promoting effects on proliferation and migration of endometrial cells as downregulation of circ_0000673, while the miR-616-3p inhibitor rescued the promoting phenomenon. The data confirmed circ_0000673 involved in regulating the proliferation and migration of endometrial cells via miR-616-3p.

The role of miRNAs depends on the gene translational inhibition or degradation by binding to the 3'-UTR of target gene. We found *PTEN* was predicted to be the target of miR-616-3p and the direct binding relationship was confirmed by dual luciferase assay. *PTEN* is a tumor-suppressor gene with specificity phosphatase activity, and plays important roles in cell growth, proliferation and migration via PI3K-AKT pathway[25, 26]. Studies showed *PTEN* expression was decreased in both endometriosis tissue and primary cultured endometrial stromal cells, which contributed to cell proliferation[27]. Aberrant *PTEN* expression in endometriotic stromal cells reduced cell apoptosis via AKT/mTOR signaling[28]. In our study, knockdown of circ_0000673 or adding the mimic of miR-616-3p reduced mRNA and protein level of *PTEN* in both eutopic and normal endometrial stromal cells, accompanying with promoted cell proliferation and migration. Inhibition of miR-616-3p in endometrial stromal cells with circ_0000673 alleviated the phenomenon of cell proliferation and migration. Activation of PI3K and AKT by abnormal *PTEN* expression plays important roles in cell cycle and establishment of endometriosis[29, 30]. Our results showed increased expression of PI3K and p-AKT with reduced expression of *PTEN* in endometrial stromal cells that was knocked down of circ_0000673, which demonstrated circ_0000673 involved in progression of endometriosis by promoting cell proliferation and migration of endometrial stromal cells through *PTEN*/PI3K/AKT signaling pathway.

Conclusion

In summary, our results showed that downregulated circ_0000673 inhibited *PTEN* by sponging miR-616-3p, which then activated PI3K and p-AKT and further promoted proliferation and migration of endometrial stromal cells. The study revealed the roles of circRNAs in endometriosis, which may bring new sights on exploring novel therapeutic targets for endometriosis.

Declarations

Authors' contributions

WY performed the experiments and prepared the original draft. DB and CZ collected the samples and designed the experiments. LS acquired the funding, designed the project, and reviewed and edited the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All participants provided informed consent in accordance with the ethical guidelines of (Protocol No. 37923/2-3-2012). The study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (201910255).

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Figures

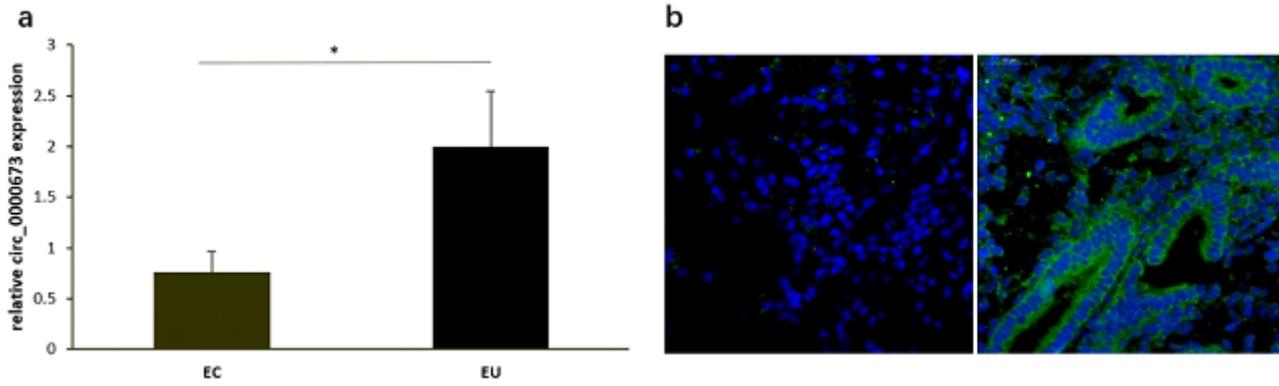


Figure 1

Hsa_circ_0000673 was deregulated in ectopic endometrium compared to eutopic endometrium. a hsa_circ_0000673 expression in paired EC and EU was detected by qPCR analysis. b hsa_circ_0000673 expression was examined by fluorescent in situ hybridization. Blue is the nucleus and green represents the positive expression. * P < 0.05.

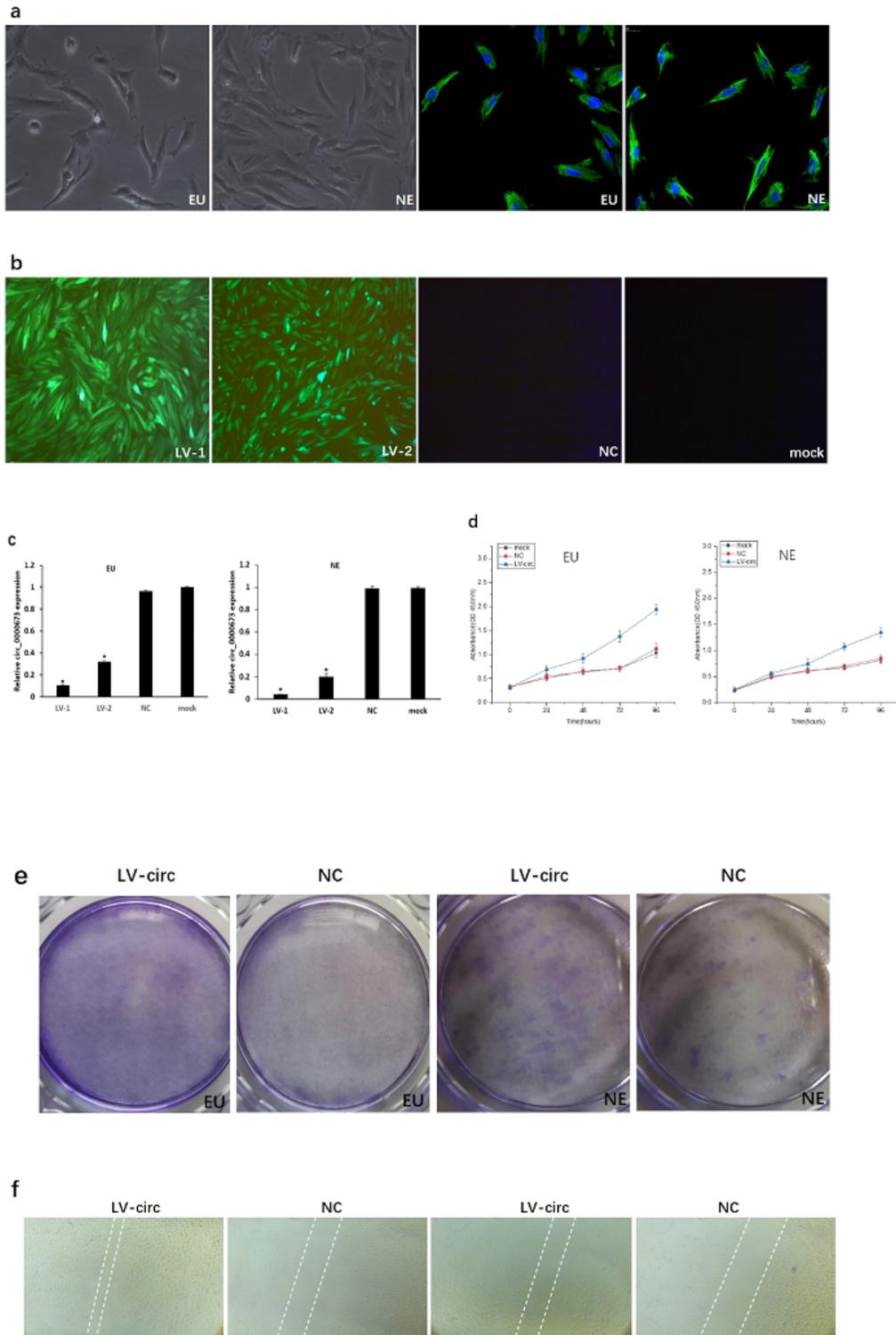


Figure 2

Knockdown of circ_0000673 promoted endometrial cell proliferation and migration. a Identification of endometrial stromal cells. Endometrial stromal cells presented a fusiform cell morphology. Vimentin-positive expression by immunofluorescence stain confirmed the endometrial stromal cells. b The fluorescence of green fluorescent protein was observed to display transfection efficiency, which showed that the cells were successfully transfected by circ_0000673 lentiviral vectors(green). Blue represents

nuclei. c qPCR confirmed significantly down expression of circ_0000673 after transfected with LV-circ_0000673-1/2 in endometrial stroma cells. d The CCK8 experiment showed that cell proliferation abilities of endometrial stroma cells were significantly increased after circ_0000673 knockdown. e Clone formation assays demonstrated that cell vitality in the group transfected with circ_0000673 lentiviral vectors was higher than control group. f Wound healing assays showed the cell migration capacity was obviously enhanced with circ_0000673 knockdown. * P < 0.05.

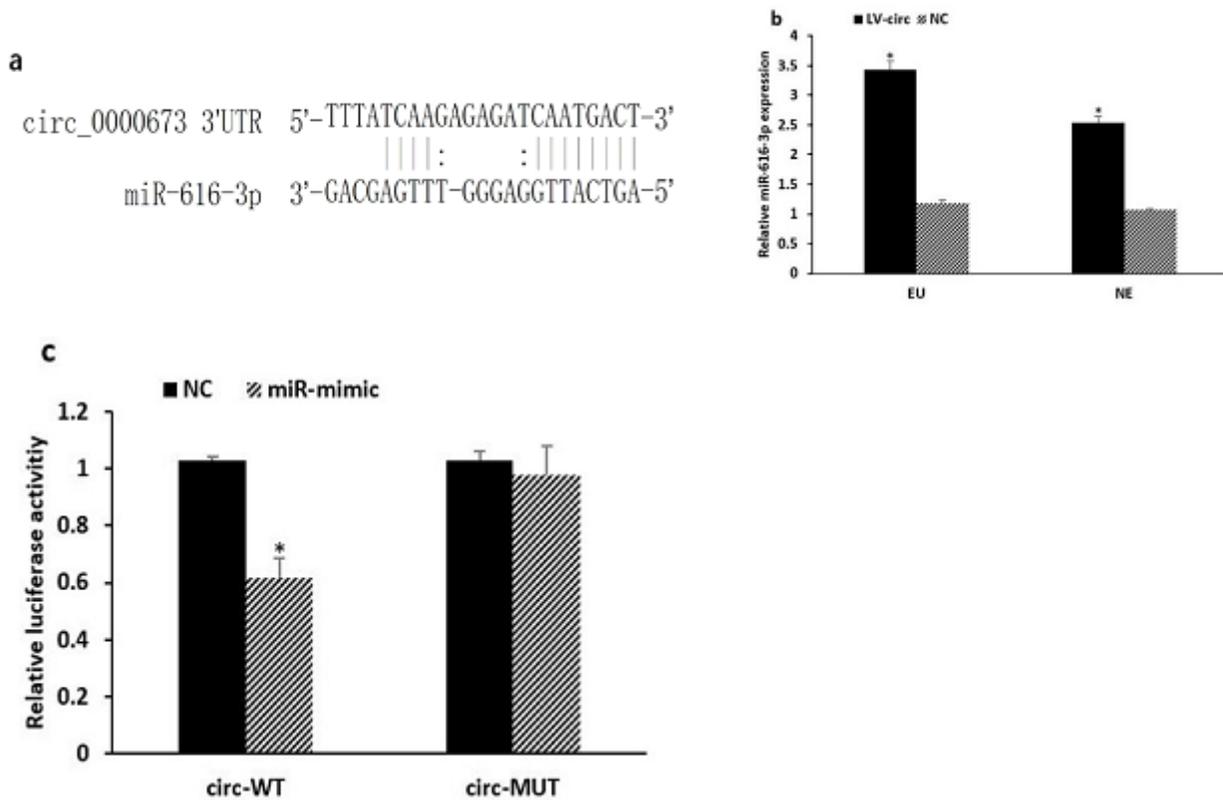


Figure 3

Circ_0000673 suppressed the miR-616-3p expression. a The binding site of circ_0000673 and miR-616-3p. b qPCR showed that the miR-616-3p expression was significantly increased after circ_0000673 knockdown in endometrial cells. c Relative luciferase activity was obviously attenuated the group transfected with circ-WT and miR-mimic; while no differences were observed in those co-transfected with mutant plasmids or negative control. Data showed as mean \pm SD. *Mean compared with negative control group, P < 0.05.

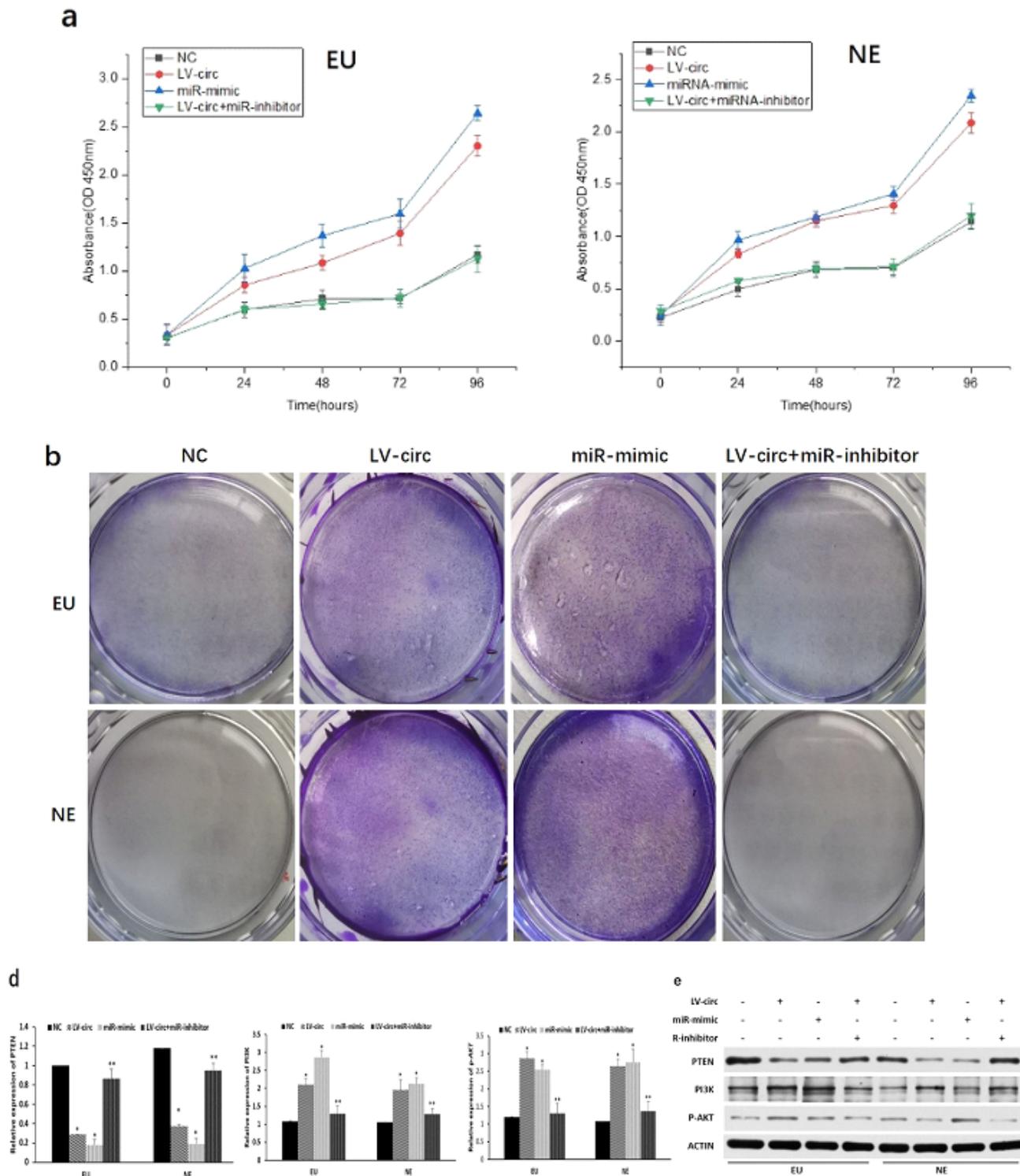


Figure 4

Effect of circ_0000673/miR-616-3p on cell proliferation and migration via PTEN. a CCK8 experiments were performed to assess the proliferation capacity of EU/NE cells treated with negative control, LV-circ, miR-mimic and LV-circ+miR-inhibitor. b Colony formation assays were done to detect the cell vitality of EU/NE cells treated with negative control, LV-circ, miR-mimic and LV-circ+miR-inhibitor. c Wound healing assays were conducted to identify the migration capacity of EU/NE cells treated with negative control, LV-

circ, miR-mimic and LV-circ+miR-inhibitor. d qPCR was done to examine the relative mRNA level of PTEN, PI3K and p-AKT in EU/NE cells treated with negative control, LV-circ, miR-mimic and LV-circ+miR-inhibitor. e Western blot assay was used to detect the protein expression of PTEN, PI3K and p-AKT in EU/NE cells treated with negative control, LV-circ, miR-mimic and LV-circ+miR-inhibitor. *Means compared with negative control group, $P < 0.05$. **Means compared with LV-circ group, $P < 0.05$.

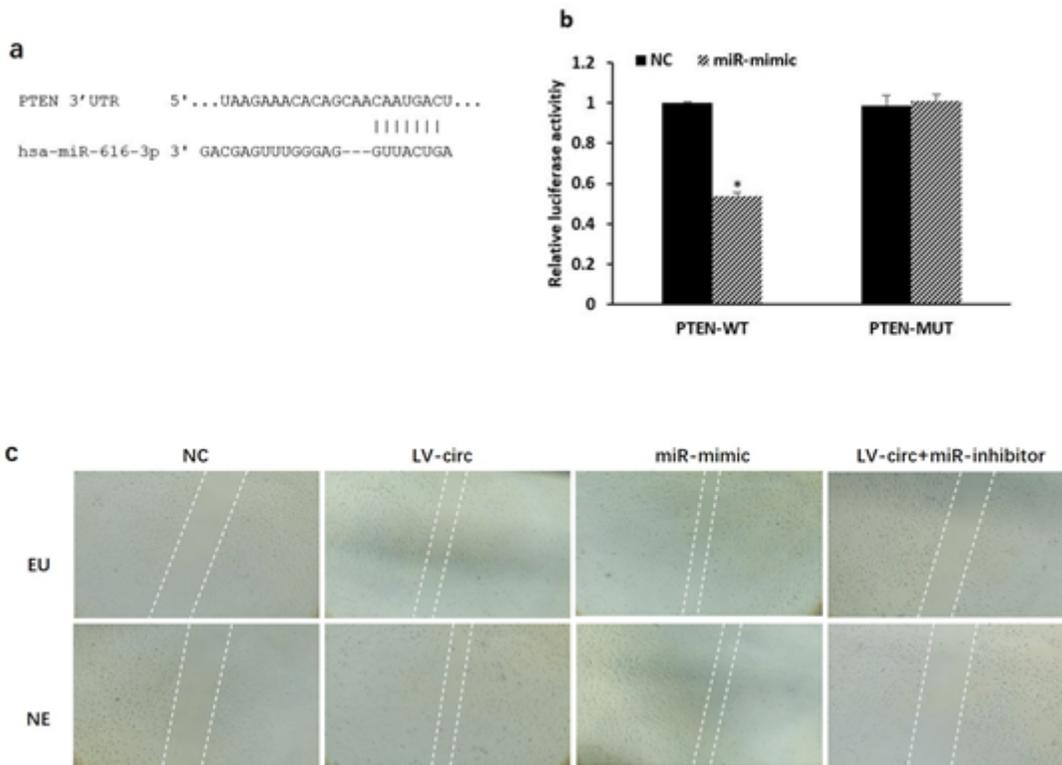


Figure 5

PTEN was a target of miR-616-3p. a The binding site of miR-616-3p and PTEN. b Relative luciferase activity was statistical reduced in the group transfected with PTEN-WT and miR-mimic, and no changes were observed in those treated with PTEN-MUT or negative control. Data showed as mean \pm SD. *Mean compared with negative control group, $P < 0.05$.