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miR-21 regulates autophagy and apoptosis of ectopic endometrial stromal cells of adenomyosis via PI3K/ AKT/ mTOR pathway

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Adenomyosis (AM) is a common and challenging disease in gynecological clinics, which adversely affects women's physical and mental health. Despite the growing number of studies, the mechanisms associated with the growth of the lesion are poorly understood. Studies show that abnormal proliferation, apoptosis, and migration in ectopic endometrial stromal cells (EESc) of AM may contribute to the development and progression of AM. Understanding the underlying molecular mechanisms can significantly contribute to diagnosing and treating AM. In the present study, EESc was isolated and cultured from the ectopic endometrium of patients with AM. These cells were treated with a PI3K/AKT activator (740 Y-P) and an inhibitor (LY294002), while the expression of microRNA-21 (miR-21) was interfered with. The effects of miR-21 on the apoptosis and autophagy of EESc, as well as the associated mechanisms, were investigated from multiple perspectives. Here, we found that 740 Y-P could significantly promote proliferation, inhibit apoptosis of EESc, and increase the expression of mTOR and p-mTOR proteins in EESc. Moreover, activating miR-21 enhanced the pro-migration effect of 740 Y-P and reversed the pro-apoptotic effect of LY294002, reducing the apoptosis rate and increasing the migration ability of EESc. Our investigation revealed that miR-21 can inhibit apoptosis and autophagy and promote migration of EESc. This effect is likely mediated via the PI3K/AKT/mTOR pathway.

Keywords Adenomyosis, microRNA-21 (miR-21), Ectopic endometrial stromal cells, Apoptosis, Autophagy, PI3K/ AKT/ mTOR pathway

Adenomyosis (AM) is the invasion of abnormal endometrium into the myometrium, resulting in the formation of lesions, accompanied by proliferation and fibrosis of the surrounding muscle fiber tissue, resulting in localized or diffuse lesions within the myometrium leading to enlargement of the uterus and thickening of the uterine walls[1]. While there have been advancements in the development of therapeutic drugs for AM in recent years, the condition remains one of the main causes of dysmenorrhea, menorrhagia, chronic pelvic pain, and infertility in women[2]. The pathogenesis of AM is complex. AM is regarded as an estrogen (E2)-dependent disease among the widely accepted theories. E2 exerts its biological effects by binding to its receptor, thereby forming a hormone-receptor complex that plays a crucial role in the onset and progression of AM, as well as in the mechanisms underlying dysmenorrhea[3]. In recent years, AM has gained significant attention in gynecology, and its pathogenesis continues to evolve[4]. It has been shown that abnormal proliferation, autophagy, apoptosis, and migration in ectopic endometrium may cause the growth of the ectopic lesion, which may contribute to the development and progression of AM[5,6]. Understanding the underlying molecular mechanisms can significantly contribute to the diagnosis and treatment of AM.

MicroRNA-21 (miR-21) is a widely studied small non-coding RNA (ncRNA) that regulates the expression of several target genes. It participates in various intercellular signal transductions, including cellular proliferation, apoptosis, autophagy, and differentiation[7]. miR-21 has been implicated as a cancer-promoting factor for diverse malignant tumors such as endometrial, ovarian, and gastric cancers[8,9]. Downregulation of miR-21 expression inhibits abnormal proliferation and invasion of cancer cells, which can be a potential clinical therapeutic strategy for cancer treatment. However, there is a limited report addressing the role of miR-21 in the pathogenesis of AM.

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To observe the expression of miR-21 in ectopic endometrial tissues of AM, the expression of miR-21 in both the eutopic and ectopic endometrium of AM was assessed through organizational detection methods in previous experiments conducted by our research group. Ectopic and eutopic endometrium of the AM, along with normal uterine endometrium and uterine myometrium were detected. The results showed that the expression of miR-21 in the ectopic endometrium was significantly higher than that in the control groups ($P < 0.05$). Additionally, the expression of miR-21 in ectopic endometrial stromal cells (EESc) under E2-activated and E2-deprived conditions indicated that E2 activation significantly upregulated the expression of miR-21 in EESc[10]. Current research focuses on understanding the processes for autophagy and apoptosis in the ectopic endometrium, as key roles in AM onset and progression, which leads to abnormal cell proliferation[2,5,11], and ncRNA can affect the progression and prognosis of AM by participating in the regulation of these processes[12]. The mechanism behind this needs further exploration.

Phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is one of the key intracellular signaling pathways controlling essential functions of cells including, but not limited to the regulation of cell growth, differentiation, and metabolism[13]. Inhibiting this pathway is one of the major approaches for managing malignancy[14]. Studies show that miR-21 inhibits cell autophagy and apoptosis by regulating the PI3K/AKT/mTOR pathway, thereby promoting tumor cell proliferation and metastasis, which is closely related to the occurrence and prognosis of various tumors[15–17]. However, the relationship between miR-21 with the PI3K/AKT/mTOR signaling pathway remains unclear in the molecular mechanisms underlying AM. Therefore, in the present study, primary EESc derived from patients with AM were cultured in vitro. The PI3K/AKT signaling pathway within these cells was either inhibited or activated, while simultaneously the expression of miR-21 was interfered with. The effects of miR-21 and the PI3K/AKT/mTOR signaling pathway on AM were investigated from multiple perspectives. The effect of autophagy and apoptosis in EESc provides a robust theoretical basis for understanding the mechanism of AM, enabling the identification of potential new targets for the treatment of AM.

Materials and methods

Ethics approval and consent to participate

The project was approved by the ethics committee of Guangdong Provincial Hospital of Chinese Medicine (number: BF2020-123–01) and the study was conducted following the 1964 Declaration of Helsinki and its later amendments, and the Ethics Guidelines for Medical Research Involving Human Subjects. Written informed consent was obtained from all patients for participation in this study.

Patient selection and tissue collection

Six ectopic lesions samples from patients with AM who underwent surgery at Guangdong Provincial Hospital of Chinese Medicine during February 2023 and June 2023 were chosen for EESc culture. Two experienced pathologists examined all the samples and provided comprehensive pathological reports.

Participants selected for EESc culture were non-menopausal and had not received hormonal therapy, including contraceptive pills, implants, or hormonal intrauterine devices, for at least three months before surgery. The exclusion criteria for all participants encompassed postoperative pathological results that were inconsistent with the diagnosis of AM. No participants were suffering endometrial diseases and other gynecological conditions—such as uterine fibroids, endometrial cancer, and cervical cancer, among others. All participants gave informed consent.

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM, Servicebio, China), Fetal Bovine Serum (FBS, GIBCO,USA), Trypsin-ethylene diamine tetra acetic acid (EDTA) Solution (Biosharp, China), phosphate-buffered saline (PBS [1X], Biosharp, China), streptomycin (100X, Procell), collagenase type I (0.1%; Sigma, USA), PI3K/AKT activator (740 Y-P, MedChemExpress, USA), PI3K/AKT inhibitor (LY294002, MedChemExpress, USA), Annexin V-APC/7-AAD Apoptosis Detection Kit (Nanjing KeyGen Biotech, China), OPTI-MEM (GIBCO, USA); lipofectamine 2000 (Invitrogen, USA), mimics negative control (NC), miR-21-5p mimics, inhibitor NC, miR-21-5p inhibitor (JTS scientific company, Wuhan, China), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, MedChemExpress, USA), Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Polyclonal Antibody from Rabbit, Hangzhou Goodhere Biotech, China), The mouse anti-human mammalian target of rapamycin (MTOR), phosphorylated (p)-MTOR, the horseradish peroxidase (HRP)-conjugated secondary antibody (Proteintech Group , Wuhan, China), and enhanced chemiluminescence (ECL, servicebio, China), and others.

Culture, passage, and identification of EESc

AM tissue was collected aseptically in the operating room in pre-cooled PBS and transported to the laboratory within 30 min. After washing the tissue thrice with PBS, they were cut into approximately 1 mm^3 pieces with ophthalmic scissors, and two volumes of 0.1% Type I collagenase were added and incubated in a water bath at 37 °C for 4–6 h, shaking once every hour. Following termination of digestion, the solution was filtered through a strainer, the filtrate was collected and centrifuged at 1000 rpm for 8 min. The supernatant was discarded and the EESc were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, at 37℃ with 5% CO_2. The culture medium is replaced every 2–3 days. When the cell density reaches 90%, the cells are digested with trypsin–EDTA for passage. According to the previous protocol used by our research group[19], the 3rd to 7th-generation cells were selected for analysis. Immunocytochemistry used vimentin antibodies, keratin antibodies (ZSGB-BIO, China), and blank control liquid to identify cells cultured in the first generation.

Effect of PI3K/AKT pathway on autophagy, apoptosis, and proliferation of EESc

LY294002 is a documented inhibitor of the PI3K signaling pathway, and 740 Y-P is a potent PI3K activator. EESc were cultured according to the method described in the previous section. EESc in the logarithmic growth phase and in good growth condition were taken and inoculated at a density of 3×10^5 cells per well in a 6-well cell culture plate and then cultured overnight in a 37℃ with 5% CO2 incubator. EESc was divided into three groups: blank group, PI3K/AKT inhibitor group (LY294002), and PI3K/AKT activator group (740 Y-P).

Effect of miR-21 on apoptosis and autophagy of EESc through regulation of PI3K/AKT signaling pathway

EESc was cultured and processed as described in the previous sections. miR-21 mimic, a chemically synthesized mature miRNA double-strand, can cause overexpression of miR-21. Conversely, miR-21 inhibitors can suppress the expression of miR-21. EESc in the logarithmic growth phase and good growth condition were taken and inoculated at a density of 3×10^5 cells per well in a 6-well cell culture plate and then cultured overnight in a 37℃ with 5% CO2 incubator. EESc were divided into seven groups: miR-21 mimic + 740 Y-P group, miR-21 mimic + LY294002 group, miR-21 mimic NC group, miR-21 inhibitor + 740 Y-P group, miR-21 inhibitor + LY294002 group, miR-21 inhibitor NC group, and blank control group. Two hours before transfection, the medium was changed to serum-free DMEM.

For transfection experiments, 10 μL siRNA was mixed with 100 μL serum-free opti-MEM and allowed to stand at 25℃, for 5 min. Lipofectamine™ 2000 (5 μL in 100 μL of opti-MEM), was added and mixed well and allowed to stand at room temperature for 5 min. The two solutions were mixed gently and again allowed to stand at room temperature for 20 min; following which, 20 μL of this mix was added to each culture-plate well, and cultured in a shaker incubator for 6 h. The medium was aspirated and a normal culture medium was added. PI3K/AKT inhibitors or agonists were added according to the pre-defined groups and the plates were again incubated for 48 h.

Detection of the expression of miR-21 in EESc after transfection by Quantitative real-time polymerase chain reaction (qRT-PCR).

After transfection, the expression of mir-21 was detected by *qRT-PCR*. The mRNA sequence of the target gene was found on GenBank, and specific primers were designed using Primer Express 2.0 software. The primer probes were synthesized by Invitrogen Company. The primer sequences are as follows: miR-21 forward primer: GCTGTGGTAGCTTATCAGACTG; reverse primer: GTGCAGGGTCCGAGGT; H-U6 forward primer: CTGC GCAAGGATGACACG; reverse primer: GTGCAGGGTCCGAGGT.

Total RNA extraction was conducted in accordance with the TRIzol user manual provided by Invitrogen. The reaction conditions were 50 °C for 15 min, 85 °C for 5 s, and 4 °C for 10 min. cDNA was synthesized with 4μL of total RNA per sample. PCR reactions were performed with SYBRTM Premix Dimer Eraser(2 ×) 10 μL, 0.4μL of 5'and 3'primers, and 5.2μL PCR-grade distilled water. The reaction conditions were started at 95 °C for 10 min for initial denaturation and then followed by 40 cycles of 10 s at 95 °C and 60 s at 60 °C.

Detection of EESc proliferation by MTT assay

After treating the EESc according to the above grouping, 10 μL MTT solution was added to each well and incubated for 2 h at 37 °C with 5% CO2. The optical density (OD) was measured by a microplate reader at 450 nm. Cell proliferation rate (%) = (OD of test group—OD of control) / (OD of NC group—OD of control).

Detection of EESc apoptosis by flow cytometry

Cells in the logarithmic growth phase (3×10^5/mL) were seeded in a 6-well plate until they grew with adherence. Cultured cells were digested with trypsin, washed twice with PBS, and centrifuged at 1200 rpm for 5 min. After this, Following the instructions of the AnnexinV-APC/7-AAD Apoptosis Detection Kit (KeyGEN Bio TECH, China), the cells were resuspended in 500 μL Binding buffer, 100 μL of suspension was pipetted into the flow tube, and stained with 5 μL fluorescein isothiocyanate (FITC)-Annexin V and 5 μL 7-AAD, then, incubated for 10 min in the dark followed by the analysis of cell apoptosis by flow cytometry.

Western blotting for protein expression

After processing the cells according to the above grouping, the cells in each group were collected by centrifugation. Radioimmunoprecipitation assay (RIPA) lysis buffer was added to cells to obtain the protein. Total protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Gbs, China). Then, 40 μg protein was loaded into 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) gel, transferred onto PVDF membrane, blocked with 5% skim milk, and incubated with primary antibodies against GAPDH (1:1000), MTOR (1:10000), and p-mTOR (1:1000). After washing with the PBS three times, the membrane was incubated with HRP-conjugated secondary antibody (1:10000 dilution). The protein band was visualized after the addition of the ECL reagent. Finally, Image-J software (https://imagej.net/software/imagej/) was used to analyze the gray value of each group of target proteins.

Detection of EESc migration by wound-healing assay

Cells stably-infected for 48 h were plated in a 6-well plate and cultured at 37 °C with 5% CO_2 until full confluence. Sterile 200-mL micropipette tips were used to scratch the cell layers and the plate was washed three times with PBS to remove the excised cell debris, then incubated in DMEM for 48 h. The cell-free wound area was photographed with an inverted microscope.

Observation of autophagy in EESc by transmission electron microscopy (TEM)

A total of 3 samples were used for TME analysis, and three different fields of view were collected for each sample. Stably infected cells for 48 h were collected and suspended with an equal volume of 2.5% glutaraldehyde, centrifuged and the supernatant discarded. A mixture containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1% phosphate buffer was slowly added for fixation for 30 min and washed three times with PBS. After re-fixing with 1 g/L osmium tetroxide for 30 min, the fixed sample was washed three times with PBS. After that, the samples were dehydrated with ethanol gradient, embedded, and polymerized in an oven for 72 h, following which the specimens were sectioned, double stained with uranyl acetate and lead citrate, and observed under a TEM (ZEPTOOLS, China) and filmed.

Statistical analyses

SPSS26.0 software (https://kk.msc23.cn/spss/) was used for statistical analyses of data. First, Levene's homogeneity of variance test was used to analyze the measurement data. If the data satisfied normality and homogeneity of variance, it was expressed as mean ± standard deviation (mean ± SD), and the overall difference was compared using the analysis of variance method. When the differences between populations were statistically significant, the Least Significant Difference (LSD) method was used for pairwise comparisons. If the data did not meet the homogeneity of variances, it was expressed as the median (interquartile range), and the Wilcoxon score was used, followed by the Kruskal–Wallis test. $P < 0.05$ was considered as a statistically significant difference.

Results

Culture and identification of EESc

In this study, four cases of EESc from ectopic lesions of AM were successfully cultured. The primary cultivation of EESc is shown in Fig. 1A. Immunocytochemistry was performed with the cultured cells of each case, and the results are shown in Fig. 1B. Vimentin, as a marker protein of stromal cells, shows positive expression, while the marker protein of epithelial cells, keratin, is negatively expressed. These results indicate that the purity of EESc is relatively high.

Effects of PI3K/AKT pathway on EESc

PI3K/AKT pathway promotes proliferation of EESc

After culturing EESc with 740 Y-P or LY294002 for 48 h, the cell proliferation rate measured by MTT is shown in Fig. 2. Compared with the black group, the proliferation rate of the 740 Y-P group was significantly higher while the cell proliferation rate of the LY294002 group was significantly lower (both $P < 0.001$).

PI3K/AKT pathway inhibited apoptosis of EESc

The analysis of cell apoptosis via flow cytometry, following the culture of EESc with 740 Y-P or LY294002 for a duration of 48 h, is presented in Fig. 3. The results show that the total apoptosis rates in the black, 740 Y-P, and

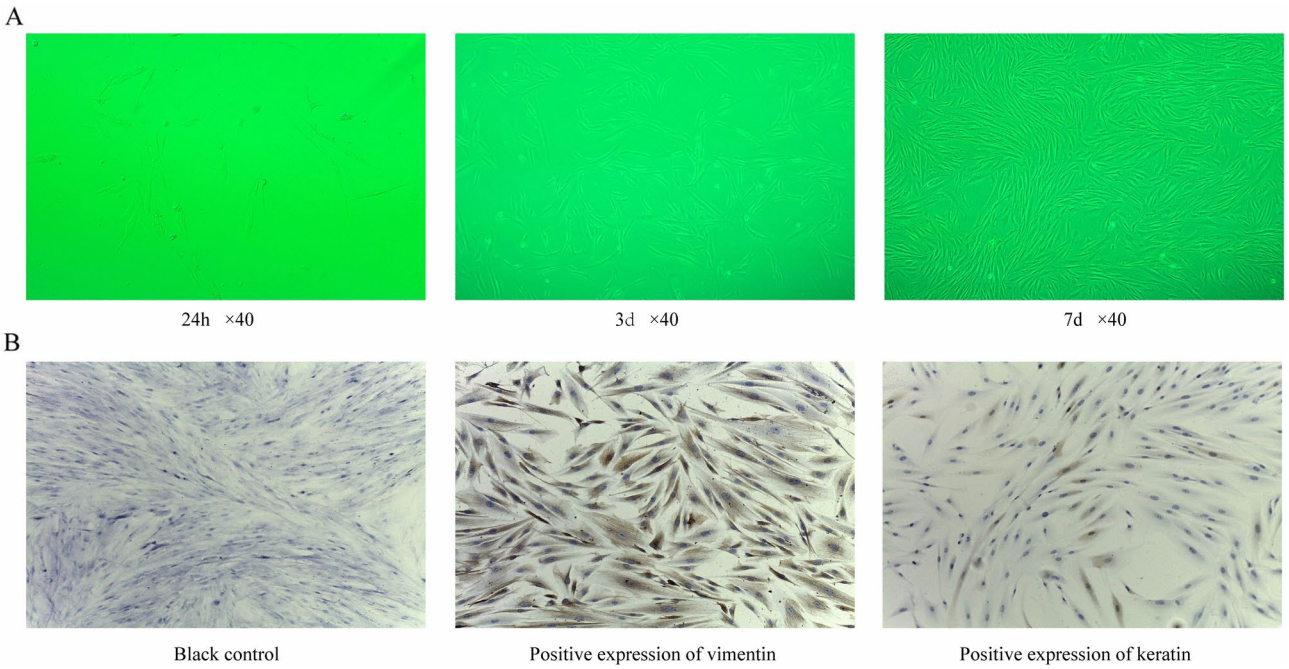

Fig. 1. Cultivation and identification of EESc. (**A**) Primary EESc cultivation (inverted microscope × 40): adhesion after 24 h of culture; The cells grew vigorously after 3 d–5 d; After 7 days of culture, the cells almost covered the entire bottle wall. (**B**) Immunocytochemistry of EESc (inverted microscope × 200).

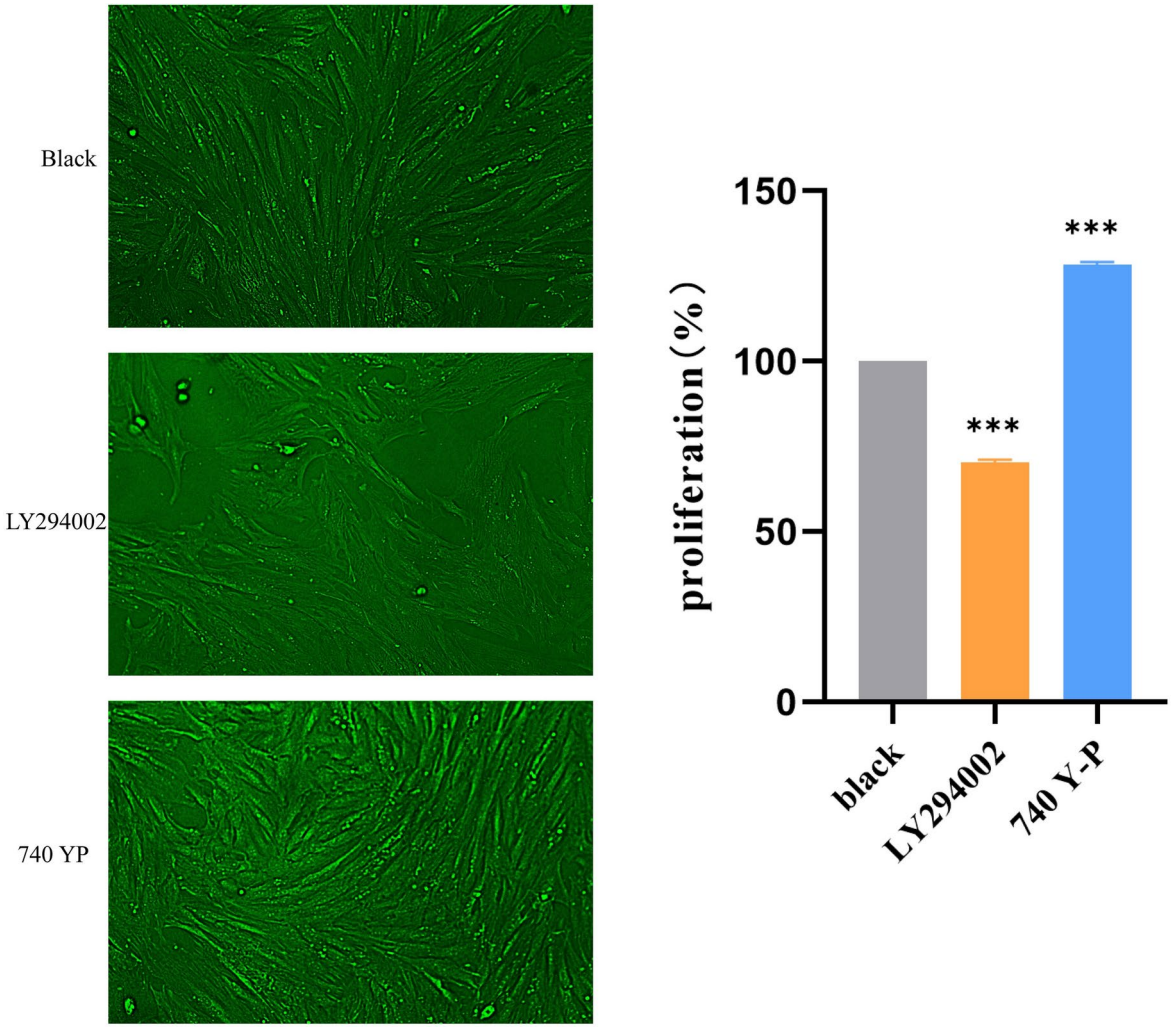

Fig. 2. PI3K/AKT pathway promotes proliferation of EESc. *: Compared with the blank group, ***, $P < 0.001$. Abbreviations: LY294002, PI3K/AKT inhibitor group; 740 Y-P, PI3K/AKT activator group

LY294002 groups were 5.88, 4.54, and 23.26%, respectively. In comparison to the black group, the total apoptosis rates in the LY294002 group were significantly elevated. ($P = 0.000$).

PI3K/AKT pathway increased the expression of mTOR and p-mTOR proteins in EESc

Western blot analysis of mTOR and p-mTOR expression is shown in Fig. 4 and Table 1. Compared to the black group, the expression of p-mTOR in the LY294002 group was significantly reduced ($P = 0.047$), whereas the expression of p-mTOR in the 740 Y-P group showed a notable increase ($P = 0.015$). In comparison to the LY294002 group, the expression of p-mTOR in the 740 Y-P group was significantly elevated ($P = 0.01$).

PI3K/AKT pathway reduces autophagy of EESc

The results of TEM are shown in Fig. 5. In the black group, irregular nuclear morphology can be observed, the mitochondria in the cytoplasm have no obvious swelling, a small number of autophagic vesicles and lysosomes can be seen in the cytoplasm, and the endoplasmic reticulum is abundant and normal in morphology. In the LY294002 group, the nuclear morphology was notably pyknotic, and the organelles in the cytoplasm were disordered. Normal mitochondria were reduced, and more abnormal mitochondria with vacuolation changes were seen. Lysosomes increased and more autophagosomes appeared. The nuclei of the 740 Y-P group were plump., mitochondria in the cytoplasm were abundant and had normal structure, and no autophagosomes or lysosomes were found.

Effects of miR-21 on migration and apoptosis of EESc via PI3K/AKT/mTOR pathway

Expression of miR-21 in each group after transfection

The results of transfection of EESc with miR-21 are shown in Fig. 6**.** After the EESc were transfected for an equivalent duration and subsequently observed under a microscope, the number of EESc in the miR-21-mimic group was significantly higher compared to the other three groups. Compared to the control group, the

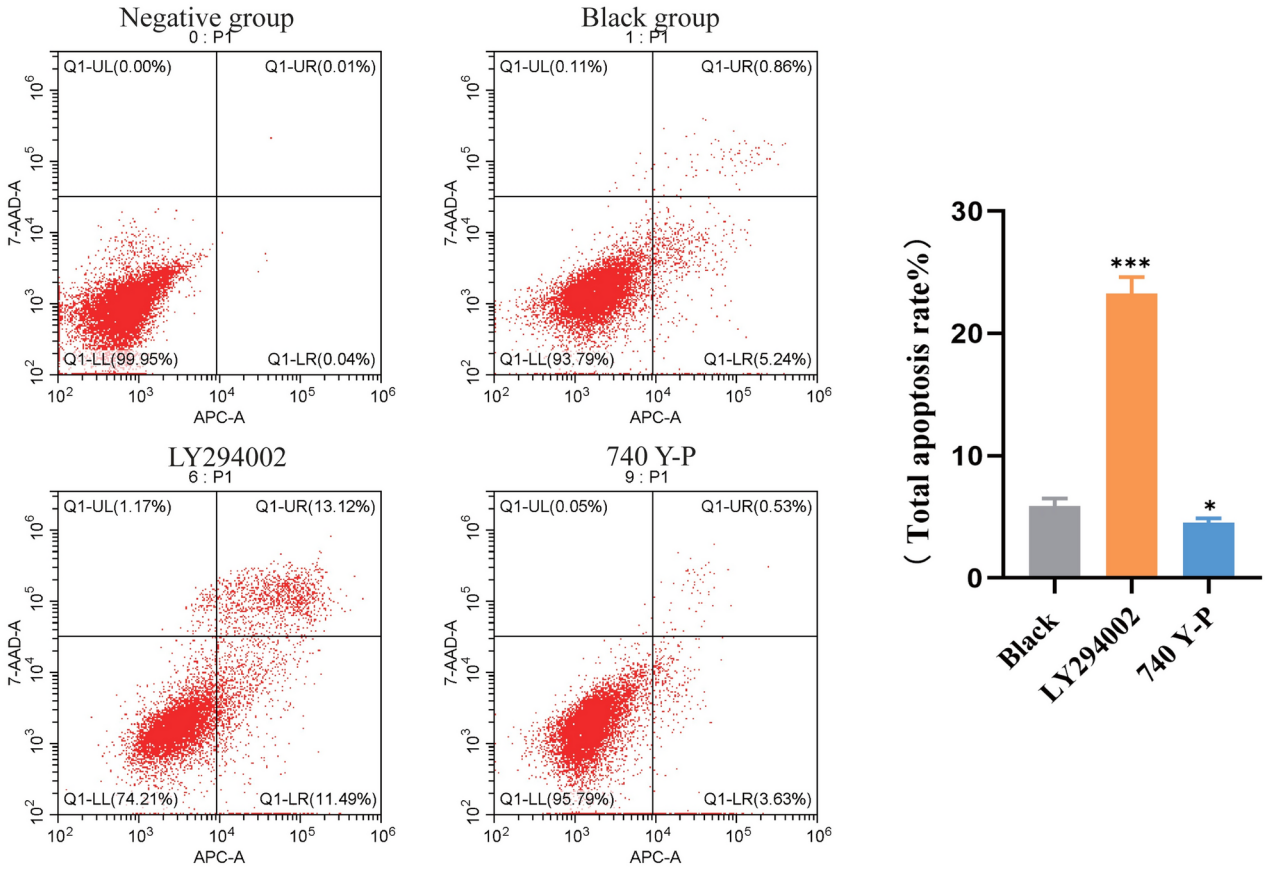

Fig. 3. PI3K/AKT pathway inhibited apoptosis of EESc. *: Compared with the blank group, *** $P<0.001$, * $P<0.05$

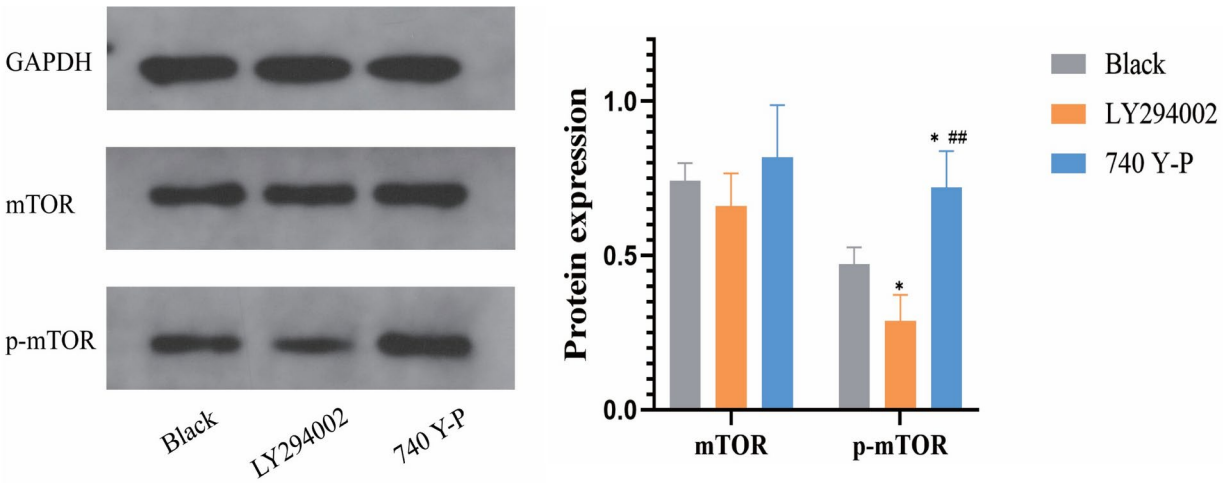

Fig. 4. Expression of mTOR and p-mTOR in EESc by western blotting.

expression levels of miR-21 in the miR-21 mimic group were significantly elevated ($P<0.001$), whereas those in the miR-21 inhibitor group were markedly reduced ($P<0.001$).

miR-21 inhibits apoptosis of EESc via PI3K/AKT/mTOR pathway

After transfection with miR-21, the EESc were categorized into seven distinct groups. Apoptosis was assessed using flow cytometry, as illustrated in Fig. 7 **(A, B, C)**. The results show that the miR-21-inhibitor + 740 Y-P group had the lowest total apoptosis rate of 4.3%, while the miR-21-mimic + LY294002 group had the highest

Group	mTOR	p-mTOR
Black	0.742 ± 0.058	0.471 ± 0.056
LY294002	0.660 ± 0.106	0.287 ± 0.085*
740 Y-P	0.819 ± 0.168	0.720 ± 0.119*#

Table 1. The expression of mTOR and p-mTOR in EESc. *: Compared with the black control group, $P < 0.05$; #: Compared with the LY294002 group, $P < 0.01$ *: Compared with the black group, $P < 0.05$; #: Compared with the LY294002 group, $P < 0.01$.

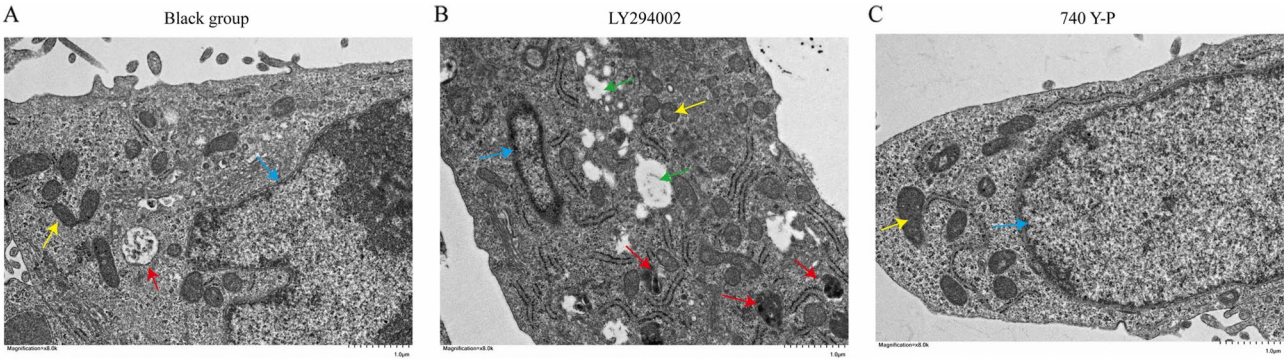

Fig. 5. Effect of PI3K/AKT pathway on the ultrastructure of EESc observed under TEM. The red arrow represents autophagosomes and lysosomes, the yellow arrow represents normal mitochondria, The green arrow represents abnormal mitochondria, and the blue arrow represents the nucleus

total apoptosis rate of 41.46%, as illustrated in Fig. 7D. When EESc were transfected with the miR-21 mimic, a comparison with the black group revealed no significant difference in the LY294002 treatment group ($P = 0.202$). However, the total apoptosis rates in the 740 Y-P group were significantly reduced ($P = 0.026$). When EESc were transfected with the miR-21 inhibitor, a comparison with the black group revealed that the total apoptosis rates in the LY294002 group were significantly increased ($P = 0.000$). In contrast, the total apoptosis rates in the 740 Y-P group showed a significant decrease ($P = 0.000$), As shown in Fig. 7E.

miR-21 promotes the migration of EESc via the PI3K/Akt pathway

The wound-healing assay of EESc after transfection with miR-21 is shown in Fig. 8 **(A, B, C).** Compared to the control group, the migration distance in the miR-21 inhibitor + 740 Y-P group and the miR-21 inhibitor + LY294002 group was significantly reduced ($P = 0.000$), with a more pronounced decrease observed in the latter. Conversely, the migration distance in the miR-21 mimics + 740 Y-P group and the miR-21 mimics + LY294002 group showed a significant increase ($P = 0.000$), with a more significant enhancement noted in the former, as shown in Fig. 8D. It is worth noting that Simultaneously activated the PI3K/AKT signaling pathway, the migrating ability of EESc in the miR-21-mimic group was improved while in the miR-21-inhibitor group were reduced ($P < 0.001$), as shown in Fig. 8E**.**

miR-21reduces autophagy of EESc through PI3K/AKT/mTOR pathway

The results of TEM in seven groups are shown in Fig. 9. It can be seen that the nucleus and the mitochondria of the black group have a more regular shape and a small amount of autophagosomes and lysosomes can be seen. In the miR-21 inhibitor combined with the LY294002 group, the nuclear morphology of EESc exhibited shrinkage. A significant number of autophagosomes and lysosomes were observed in the cytoplasm, while the quantity of normal mitochondria decreased and was replaced by vacuolated mitochondria. Additionally, the endoplasmic reticulum displayed abnormal swelling. However, no autophagosomes were observed in the miR-21 mimics + LY294002 group or the miR-21 mimics + 740 Y-P group. In the latter group, the nucleus appeared regular and well-defined, the mitochondria within the cytoplasm were notably more plump and normal, and the endoplasmic reticulum was abundant and remained undisturbed.

The red arrow represents autophagosomes and lysosomes, the yellow arrow represents normal mitochondria, the green arrow represents abnormal mitochondria, and the blue arrow represents the nucleus.

Discussion

AM is a common and challenging gynecological condition. It is more commonly seen in women who have had multiple pregnancies and are over the age of 40, although it can occur in women of any age 18. Since only one-third of patients with AM show noticeable clinical symptoms, a significant number of women are identified with AM only after undergoing a hysterectomy[18]. Consequently, research on AM lags behind that of other gynecological conditions[19]. The pathogenesis of AM is intricate, encompassing a variety of theories that pertain to the onset

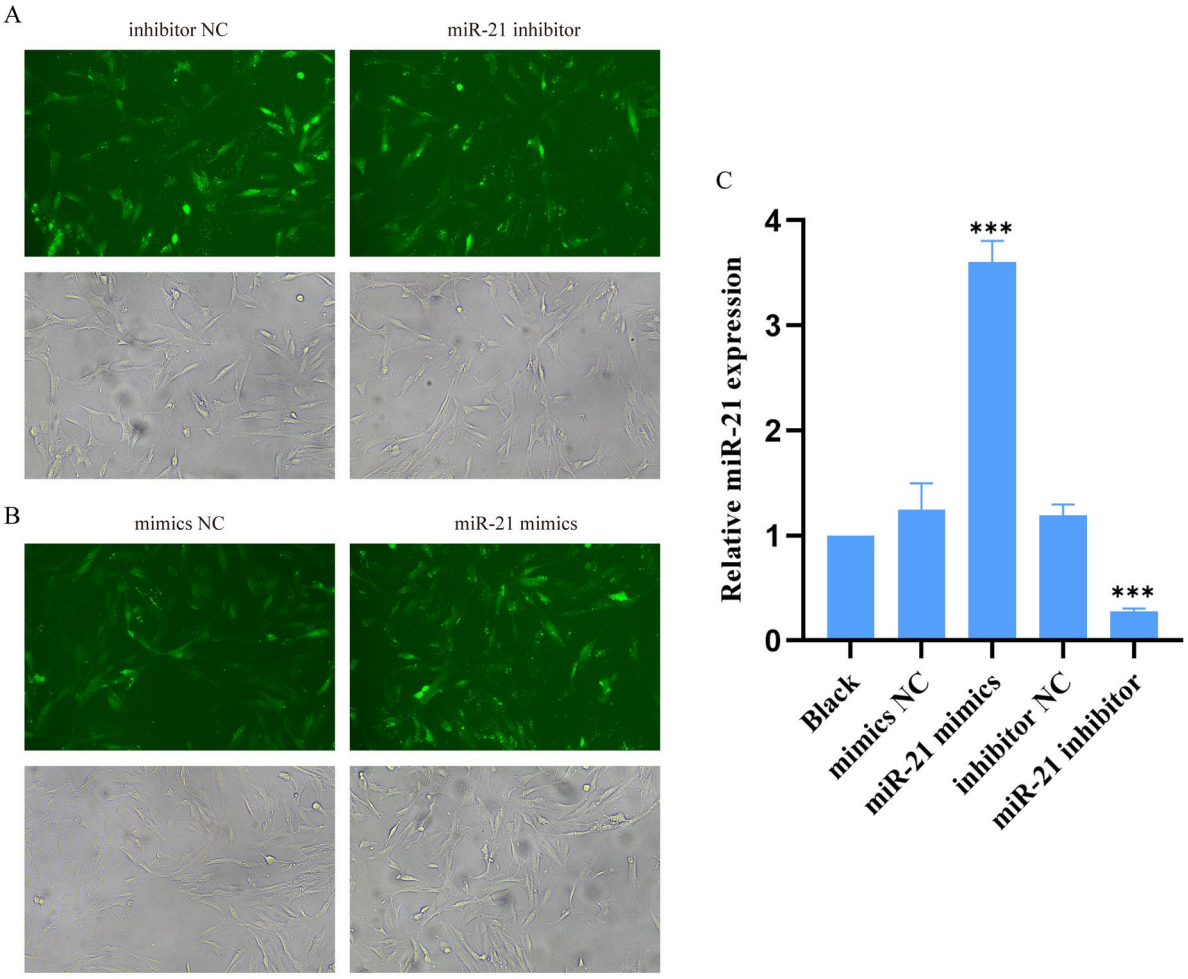

Fig. 6. The results of EESc after transfection. (**A**) Transfection in the miR-21 inhibited group (**B**) Transfection in the miR-21 activated group, (**C**) the expression of miR-21 after transfection. *: compared to the black group, ***, $P < 0.001$.

and progression of the disease[3]. Autophagy and apoptosis are widely researched cellular mechanisms and are regarded as a "double-edged sword" engaged in the regulation of body metabolism[20,21]. Under the influence of modulation of related genes, autophagy, and apoptosis can play complex dual roles in the survival and death of tumor cells and influence the occurrence, development, and prognosis of various diseases[22]. Autophagy induction, as a new approach to tumor treatment, exerts anti-tumor effects by increasing the autophagy level of tumor cells[23–25]. Abnormalities in the processes of autophagy and apoptosis within ectopic endometrium have also been observed to play a significant role in the pathogenesis of AM. Argent[5] found that the autophagy-related protein LC3B was significantly reduced in the ectopic endometrium of AM lesions compared with the eutopic endometrium. However, Zhou[26] showed that autophagy in the ectopic endometrium of AM patients is activated through Hedgehog signaling, which means that induced autophagy may promote the survival of ectopic endometrium. Though the roles of autophagy and apoptosis in the occurrence and development of AM have been continuously explored, it remains a debatable topic with a need for further detailed study.

The PI3K/Akt/mTOR signaling pathway is extensively involved in cell growth, survival, and proliferation. Inhibiting this pathway is an important way to combat cancer progression[13]. As a serine/threonine kinase, mTOR can be directly activated by activated AKT, associated with a variety of growth factors and signal transduction processes, and is closely related to autophagy[13,27]. Liu[28]showed that the activated PI3K/AKT/mTOR pathway promotes the proliferation of endometrial cancer cells by inhibiting autophagy, and mTOR inhibitors can induce autophagy and reduce the proliferation of endometrial cancer cells that are progesterone-resistant. In this study, We observed that the activation of the PI3K/AKT pathway led to a reduction in apoptosis and significantly enhanced the proliferation rate of EESc. Additionally, there was an elevation in the expression levels of mTOR and p-mTOR proteins within EESc. On the contrary, inhibiting the PI3K/AKT pathway significantly increased the apoptosis rate of EESc while markedly decreasing its proliferation rate. Additionally, a reduction in p-mTOR protein expression was observed in EESc, accompanied by an increase in autophagy as evidenced by TEM. This indicates that the PI3K/AKT/mTOR signaling pathway can influence the biological processes of EESc, leading to a reduction in apoptosis and autophagy while promoting the proliferation of EESc.

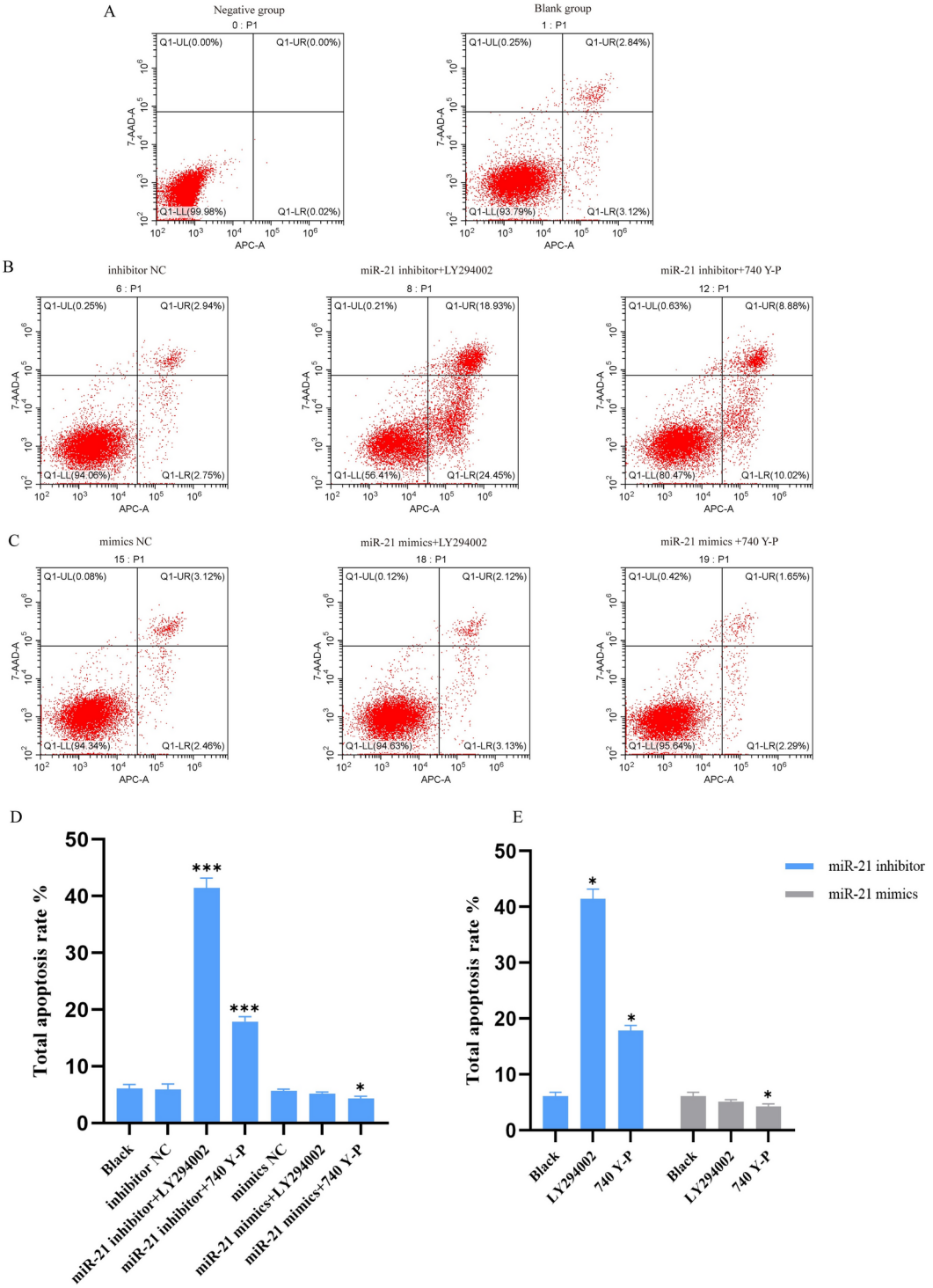

Fig. 7. Effects of miR-21 on apoptosis of EESc by regulating PI3K/Akt pathway. (**A**) The apoptosis of EESc in each group by flow cytometry. Apoptosis of EESc in the control group. (**B**) Apoptosis of EESc in miR-21 inhibitor group. (**C**) Apoptosis of EESc in miR-21 mimics group; (**D, E**) Histogram of total apoptosis rate in each group.*compared with the black group, *, $P<0.05$; ***, $P<0.001$.

To enhance our understanding of the regulatory mechanisms governing the PI3K/AKT/mTOR pathway in EESc, we undertook an in-depth investigation. MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that play a crucial regulatory role in various biological processes, including migration, proliferation, and invasion[12,29–31]. Guo[32] confirmed 10 differentially expressed miRNAs in normal endometrium and ectopic endometrium of AM and showed that downregulation of miR-10b can inhibit the phosphorylation of Akt and upregulate E-cadherin, which reduces the invasiveness of ectopic endometrium. miR-21, recognized as a cancer-

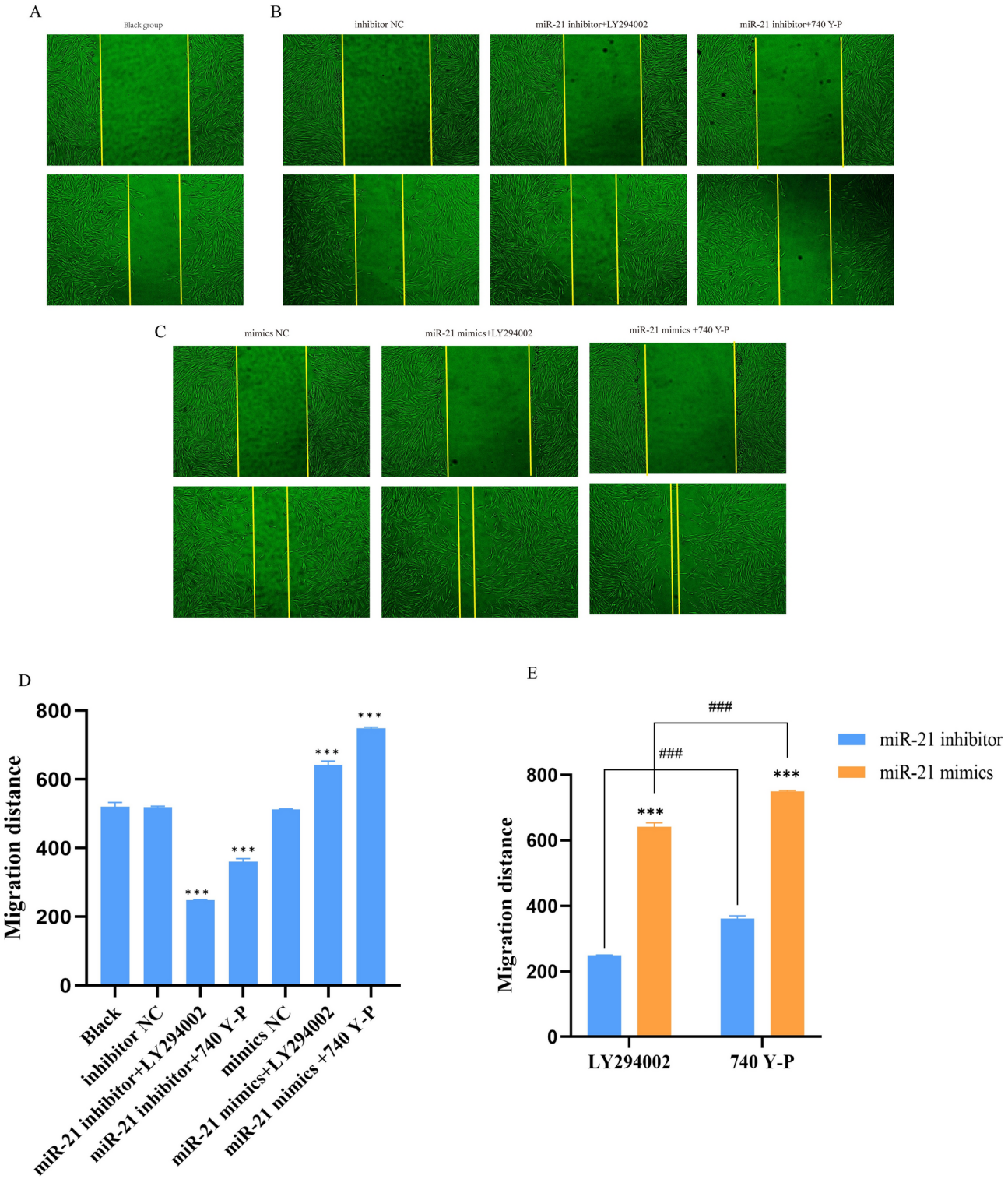

Fig. 8. Effect of miR-21 regulating PI3K/AKT signaling pathway on migration of EESc. (**A**) Wound-healing assay of EESc in the black group; (**B**) Wound-healing assay of EESc in miR-21 inhibitor group; (**C**) Wound-healing assay of EESc in miR-21 mimics group; (**D, E**) Histogram of migration distance of EESc in each group, * compared with the black group, *** $P < 0.001$.

promoting factor in numerous malignant tumors, is currently among the most extensively studied miRNAs due to its significant roles in tumor efficacy and prognosis[15,33].

Numerous studies have indicated that miR-21 plays a significant role in abnormal cell proliferation and metastasis, while also reducing apoptosis by modulating the PI3K/AKT signaling pathway. This dysregulation

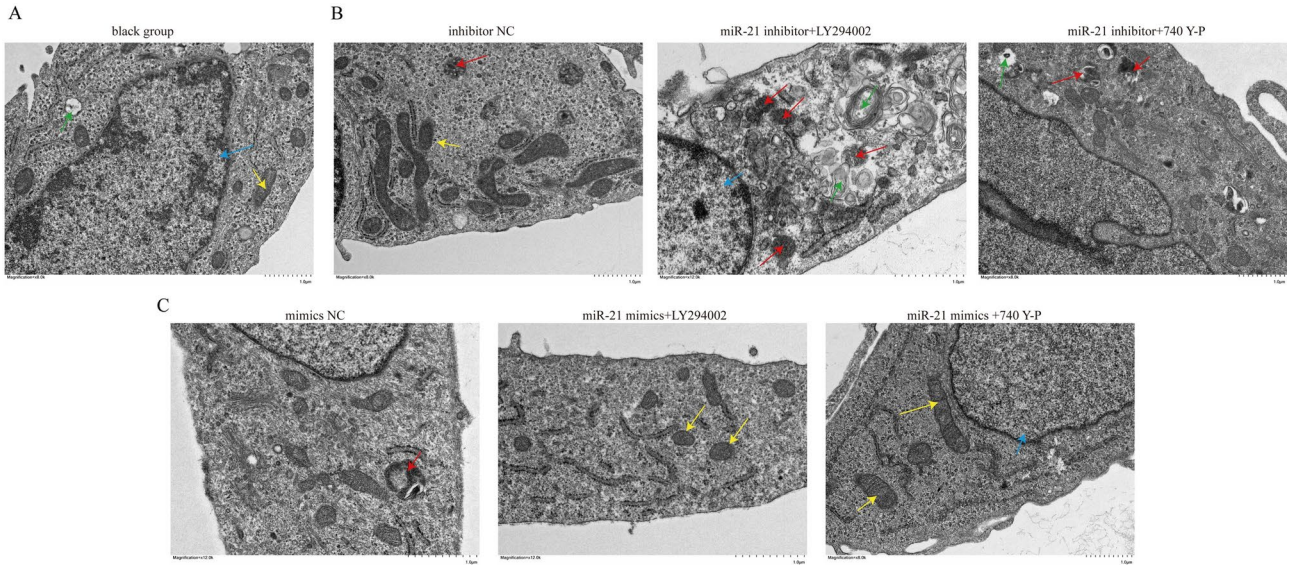

Fig. 9. Effects of miR-21on autophagy of EESc by PI3K/AKT signaling pathway.

ultimately contributes to the development of various diseases[34,35]. Liu8 found that the expression of miR-21 is upregulated in ovarian cancer cells. Reducing the expression of miR-21 can inhibit the activity of the PI3K/AKT signaling pathway and promote apoptosis of ovarian cancer cells. This finding provides new insights for the treatment of ovarian cancer. By comparing the expression of miR-21 in four different tissues, we observed a significant upregulation of miR-21 in the ectopic endometrium of AM. This finding suggests that miR-21 may play a regulatory role in the pathogenesis of AM lesions Furthermore, through transfection experiments involving miR-21 mimic and miR-21 inhibitor, we found that the overexpression of miR-21 significantly enhanced the proliferation and migration of EESc. Conversely, the inhibition of miR-21 led to increased apoptosis and autophagy[10]. In the present study, If miR-21 is activated, the apoptosis of EESc can be diminished through the regulation of the PI3K/AKT pathway, while the migratory capacity of EESc can also be significantly enhanced. When both miR-21 and the PI3K/AKT pathway are concurrently inhibited, the intracellular autophagy was more obvious, and the apoptosis rate was also highest among all groups. Furthermore, The findings suggest that the activation of miR-21 significantly enhanced the pro-migration effects of 740 Y-P while simultaneously reversing the pro-apoptotic effects of LY294002. This resulted in a reduction in apoptosis rates and an increase in the migratory capacity of EESc. It is important to note that the inhibition of the PI3K/AKT signaling pathway can lead to an increased apoptosis rate in EESc. However, if the expression of miR-21 is activated during this process, a significant reduction in the apoptosis rate of EESc occurs instead. These findings indicate that miR-21 not only regulates apoptosis and migration of EESc through the PI3K/AKT pathway but also engages in modulating related biological processes via other signaling pathways, thereby influencing the progression of AM.

Overall, as a tumor-promoting factor, miR-21 not only regulates malignant tumor cells but also plays a crucial regulatory role in the EESc concerning autophagy and apoptosis inhibition, as well as abnormal proliferation. The mechanism of action may be associated with the activation of the PI3K/AKT/mTOR signaling pathway by miR-21. Although our results provide a new perspective and theoretical support on the abnormal autophagy of AM ectopic endometrium for understanding the mechanisms underlying AM, the limitations of this project should not be ignored. Firstly, The cell culture experiments involve very limited samples, which may undermine the reliability of the conclusions drawn. Secondly, We focused on the effects of miR-21 on autophagy and apoptosis of EESc, and only a wound-healing assay has been conducted to assess the migratory ability of ASCs in the present study. In the subsequent experiments, we will increase the sample size and adopt more comprehensive research methods to further verify our research results. Thirdly, the mechanism of AM is intricate, necessitating further in-depth research to elucidate the relationship between miR-21 and AM from various perspectives. This exploration could provide additional avenues for the treatment of AM.

Data availability

The Primer sequences generated during the current study are available in the miRBase database [https://mirbase.org/mature/MIMAT0000076].

Received: 7 August 2024; Accepted: 28 February 2025
Published online: 04 March 2025

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Acknowledgements

We would like to acknowledge the women affected by AM from whom we continue to learn and for whom we strive to discover. We would like to acknowledge all the founders who used the software in the study. We would like to acknowledge all fundings for financial support of this study.

Author contributions

Yu-yan Zeng designed of the research. Yu-yan Zeng and Jin-jin Jia wrote the main manuscript text. Jie Lu and Hui-jie Lai performed the experiments.Bo-Wen Sun , Jin-jin Jia , and Jie Lu collected the data and performed the statistical analysis.All authors reviewed the manuscript.

Funding

This work was supported in part by the National Natural Science Foundation of China (No. 81904233), Guangdong Provincial Hospital of Traditional Chinese Medicine Zhaoyang Talent Project (ZY2022KY04), The renowned medical studio of national famous Chinese medicine Huang Jianling ([2022]75), The renowned medical studio of national famous Chinese medicine Li Liyun (1199ws02), Liang Xuefang Guangdong Famous Traditional Chinese Medicine Inheritance Studio, Project number: ([2023]108), and You Zhaoling Academic Experience Inheritance Studio.

Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

The studies involving humans were approved by the Ethics Committee of The Second Affiliated Hospital of GZUCM, number: BF2020-123–01. The studies were conducted following the 1964 Declaration of Helsinki and its later amendments and in accordance with local legislation and institutional requirements. Written informed consent was obtained for the human samples used in this study. Patient information is confidential and cannot be disclosed. The sample can only be used for scientific research, not for commercial and profit purposes. Written informed consent was obtained from all patients for participation in this study.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-92526-3.

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