

# Linc-ROR promotes endometrial cell proliferation by activating the PI3K-Akt pathway

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**Abstract. – OBJECTIVE:** To observe the expressions of Linc-ROR and proteins in the PI3K-Akt pathway in an ectopic lesion of adenomyosis.

**PATIENTS AND METHODS:** The expression of Linc-ROR in the ectopic endometrium, eutopic endometrium, and normal endometrium of adenomyosis was detected by qRT-PCR. Western blot was used to detect the protein expressions of PI3K-Akt in endometriosis and lesion endometriosis. Cell counting kit-8 (CCK-8) assay was utilized to detect cell proliferative activity. After interfering or over-expressing Linc-ROR, protein expressions of the PI3K-Akt pathway were detected by Western blot.

**RESULTS:** Linc-ROR expression in the ectopic endometrium of adenomyosis was higher than that in the eutopic endometrium and normal endometrium, and the expression level of PTEN in adenomyosis tissues was decreased, whilst expression levels of Akt, p-Akt, p-PTEN were increased. Clinical data of enrolled patients indicated that there was a relationship between Linc-ROR expression and the type and severity of dysmenorrhea of adenomyosis. However, no relationship was observed between Linc-ROR expression and age, cesarean section, uterine surgery, and menstrual cycle. Cell counting kit-8 (CCK-8) assay showed that the proliferative activity of cells was significantly decreased after knockdown of Linc-ROR in the adenomyosis cells. Western blot revealed that the expression level of PTEN increased but the expression levels of p-Akt, p-PTEN and p-PDK1 decreased. Overexpression of Linc-ROR obtained the opposite results.

**CONCLUSIONS:** Linc-ROR is highly expressed in the ectopic endometrium of adenomyosis, and it can promote the proliferative activity of endometrial cells by activating the PI3K-Akt pathway.

Key Words

Linc-ROR, PI3K-Akt, Adenomyosis, Proliferation.

## Introduction

Adenomyosis (AM) refers to diffuse or localized growth of endometrial glands and stroma in the myometrium, resulting in hypertrophy of myometrial cells<sup>1</sup>. AM is a common gynecologi-

cal disease of women in childbearing age, whose clinical manifestations are menorrhagia, secondary dysmenorrhea, and infertility, which seriously affects the life quality of AM patients<sup>2,3</sup>. The incidence rate of AM is as high as 8%-20%, the pathogenesis so far is unknown<sup>4</sup>. With the development of science and technology, the sensitivity and specificity of ultrasound and MRI in the diagnosis of AM have increased<sup>5</sup>, but there is still no effective method for the early diagnosis of AM<sup>6</sup>. AM significantly affect female health, and the lack of early diagnosis and efficient treatment greatly limits the AM prevention. Therefore, it is of great significance to discover the mechanism of the development of AM to further understand the pathophysiology of the disease, which helps clinical diagnosis and treatment of AM.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules that are longer than 200 nt (nucleotide) in length and do not possess protein-coding functions<sup>7</sup>. LncRNA participates in various biological processes, including cell proliferation, invasion, migration, and apoptosis at transcriptional or post-transcriptional levels, which is also associated with the development and progression of tumors<sup>8,9</sup>. Linc-ROR is one of the intergenic lncRNAs located on chromosome 18q21.31 with a total length of 2.6 kb. Initially, Loewer et al<sup>10</sup> found that embryonic stem cells are involved in cell reprogramming and are nominated by this. Scholars have shown that Linc-ROR is abnormally expressed in a variety of tumor tissues and cells, such as breast cancer<sup>11-13</sup>, lung cancer<sup>14</sup>, ovarian cancer<sup>15</sup>, pancreatic cancer<sup>16</sup>. It is proved to be related to the process of tumor cell proliferation, invasion, and migration<sup>17</sup>. However, there are few reports about Linc-ROR in AM.

The PI3K/Akt (phosphoinositide 3-kinase/protein kinase B) signaling pathway is one of the important intracellular signal transduction pathways that help to maintain the normal physiological cell functions, such as growth, differentiation, metabolism, and cytoskeletal rearrangement<sup>17</sup>. PI3K/

Akt pathway has been widely found in many physiological functions of the body. Evidence has shown that activation of signal transduction pathway resist cell apoptosis and promote cell proliferation, and this pathway is closely linked to proliferative diseases. Tang et al<sup>19</sup> found that NES1/KLK10 can promote trastuzumab resistance in gastric cancer by activating PI3K/Akt signaling pathway. Tang et al<sup>20</sup> found that hnRNP A1 promotes corneal cell survival through the PI3K/Akt/mTOR pathway. However, few reports have been reported on the relationship between PI3K/Akt signaling pathway and AM *in vitro*.

We detected the expression of Linc-ROR in AM in tissues and cells. We also investigated the effect of Linc-ROR on the endometrial cell biology after interfering and overexpressing Linc-ROR. This study aims to elucidate the relationship between AM and the PI3K/Akt pathway, thus to provide a new theoretical basis and experimental data for the occurrence and development of AM.

## Patients and Methods

### Material Source

Tissue samples were chosen from 40 AM patients (pathologically diagnosed with AM) who underwent surgery in the Department of Obstetrics and Gynecology from Urumqi maternal and child health hospital of Xinjiang Uygur Autonomous Region. Selected women patients had a normal menstrual cycle (28 to 35 days), and did not use hormonal drugs within 3 months. Relevant preoperative examinations were performed, patients with severe coronary heart disease, hypertension and other systemic diseases, inflammatory diseases and autoimmune diseases, pelvic inflammatory disease were excluded. During the same period, the normal endometrium of 40 patients who underwent hysterectomy for uterine fibroids was selected as the control group, and submucosal fibroids, endometrial polyps, and endometrial hyperplasia were excluded. The study was approved by the Ethics Committee of our Institution and patients and their families agreed with it. The endometrium of the patient was in the stage of proliferation.

### Tissue RNA Extraction

A proper amount of tissue was weighed and put in 1.5 mL RNAase and EP tube placed on ice. Then, we cut the tissue by sterile ophthalmic scissors, 800  $\mu$ L of TRIzol (Invitrogen, Carlsbad, CA, USA) were added, the homogenizer broke the

tissue into slurry. Afterwards, we added 200  $\mu$ L of chloroform (Yeasen Biotechnology, Shanghai, China) with forced oscillation for 20 s, and incubated at room temperature for 10 min. Tissues were centrifuged at 4°C, 12000 rpm for 15 min, the supernatant was removed to a new Eppendorf (EP) tube. An equal volume of isopropanol (Yeasen, Shanghai, China) was added, the mixture was shaken and placed at room temperature for 10 min; then, it was centrifuged at 4°C, 12000 rpm for 10 min, the supernatant was discarded. 800  $\mu$ L of 75% alcohol with DEPC water was added for washing. It was centrifuged at 4°C, 12000 rpm for 5 min, the ethanol (Yeasen, Shanghai, China) was carefully aspirated, RNA was in the bottom, and then it was dried at room temperature. Finally, 30  $\mu$ L of DEPC water was added to dissolve the RNA.

### Cell Isolation and Culture

Endometrial samples obtained under aseptic conditions were isolated and cultured as soon as possible (< 2 h). Then, the endometrial glandular epithelial cells were isolated from endometrial tissues by enzymatic digestion at room temperature combined with differential centrifugation, and were placed in 37°C, 5% CO<sub>2</sub> incubator. After incubation for 1-1.5 h, suspended epithelial cell lumps were transferred to a new culture dish. Thereafter, medium was changed every 2 d. An inverted microscope was utilized to observe the cell morphology and growth every day.

### Real-Time Quantitative PCR

RNA was reversely transcribed into cDNA using RT kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR (quantitative Real-time polymerase chain reaction) was performed to detect the expression levels of genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal reference. The reaction system was followed by TaKaRa (Tokyo, Japan) SYBR Green I kit instructions, the total system was 20  $\mu$ L. The reaction conditions were pre-denaturation at 95°C for 30 s, 95°C for 5 s, 51°C for 20 s and 72°C for 20 s, for a total of 40 cycles; finally, it was cooled at 37°C for 20 min, and Real-time polymerase chain reaction (PCR) was used to detect the fluorescence at the end of the reaction. The primers were: GAPDH-F: 5'-CC-CACCTCCTCCACCTTTGAC-3', GAPDH-R: 5'-GGATCTCGCTCCTGGAAGATG-3'. Akt-F: AGCGACGTGGCTATTGTGAAG, Akt-R: GC-CATCATTCTTGAGGAGGAAGT. Linc-ROR-F: TATAATGAGATACCACCTTA, Linc-ROR-R: AGGAAGTGTTCATACCGTTTC.

### Cell Transfection

Cells were seeded in a 6-well plate ( $2 \times 10^5$ /well) and cultured for 24 h. After the cell density was up to 70%, cells were transfected with negative controls, si-Linc-ROR or oe-Linc-ROR according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced after 6 h.

### CCK-8 Assay

Cells were made into a cell suspension and 100  $\mu$ L of the suspension was taken to a 96-well plate, and each plate was inoculated with 5 replicate wells. Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) and serum-free medium Dulbecco's Modified Eagle Medium/Roswell Park Memorial Institute 1640 (DMEM/RPMI-1640, Gibco, Grand Island, NY, USA) were mixed in a volume ratio of 1:10; 100  $\mu$ L of mixture was added in each well and incubated at 37°C, 5% CO<sub>2</sub> incubator for 1 h. Absorbance at 450 nm wavelength was recorded with a microplate reader (Bio-Rad, Hercules, CA, USA). The value of each plate was measured.

### Western Blot

Cells were harvested, lysed in cell lysate containing protease inhibitors and centrifuged at 4°C, 12000 g for 15 min. Proteins were extracted and quantified by BCA (bicinchoninic acid) (Abcam, Cambridge, MA, USA) method and stored at -80°C. The protein was boiled for 5 min, 20  $\mu$ g/well protein was loaded. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF, Merck, Millipore, Billerica, MA, USA) membrane. Membranes were blocked with tris buffered saline-Tween (TBS-T, Beyotime, Shanghai, China) solution containing 3% bovine serum albumin (BSA) for 60 min at room temperature, and incubated with corresponding primary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 2 h at room temperature. After washing them, secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were incubated with the membrane for 1 h at room temperature, and electrochemiluminescence (ECL) method was used for luminescence. Gray values were determined using Image Quant LAS 4000 (GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA).

### Statistical Analysis

We used statistical product and service solutions (SPSS) 19.0 statistical software (IBM, Ar-

monk, NY USA), GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) for image editing. Quantitative data was analyzed by *t*-test and expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Classification data was compared using the chi-square test.  $p < 0.05$  was considered statistically significant; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

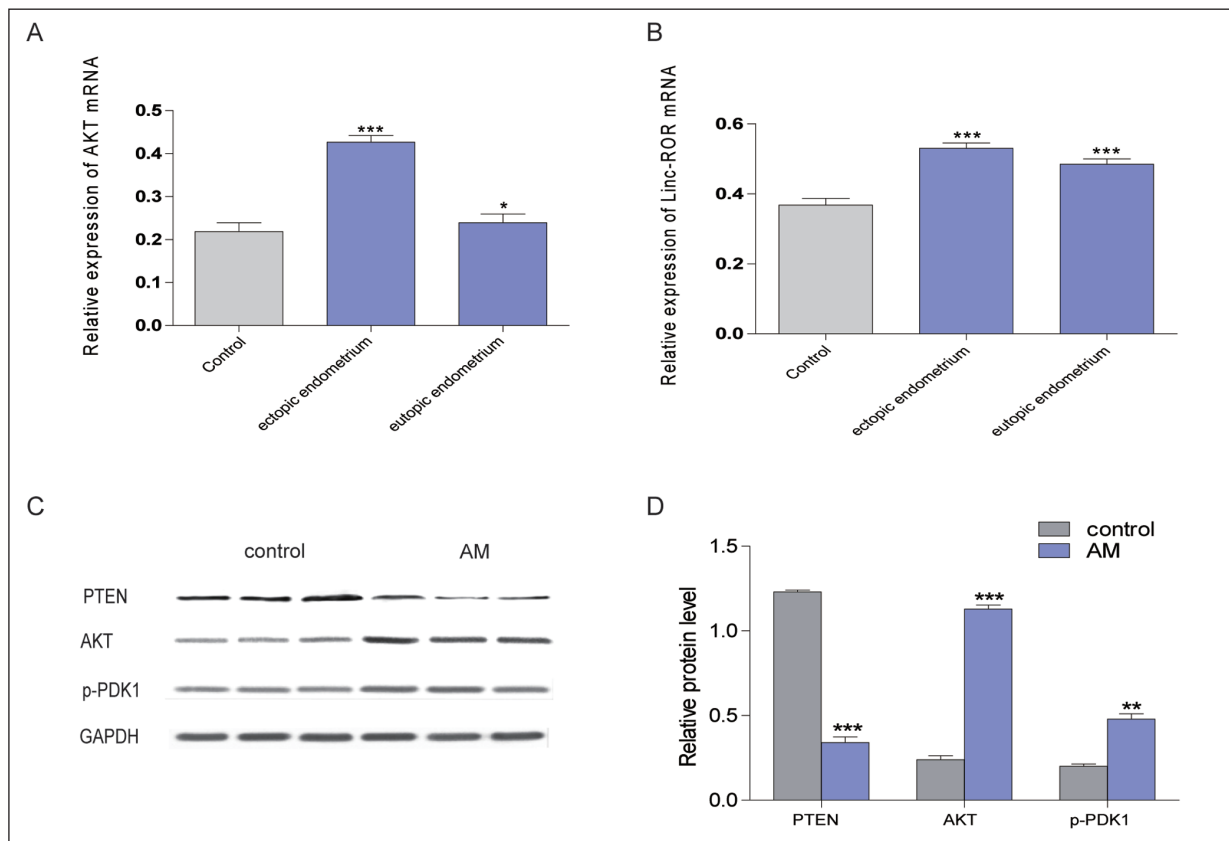
## Results

### Linc-ROR Was Highly Expressed in Endometrial Adenomyosis

40 cases of adenomyosis and 40 cases of normal endometrial tissue were performed qRT-PCR. The results showed that in AM patients, Linc-ROR and Akt expressions were found in endometriosis, ectopic endometrium, eutopic endometrium and normal endometrial tissue samples. The expression of Linc-ROR in the ectopic endometrium of AM patients was significantly higher than that of the eutopic endometrium and normal endometrium. Expression of Linc-ROR in the eutopic endometrium was higher than that of the normal endometrium (Figure 1B). The mRNA expression of Akt in the ectopic endometrium and eutopic endometrium of AM patients was significantly higher than that of the normal endometrium. The mRNA expression of Akt in the ectopic endometrium was higher than that in the eutopic endometrium (Figure 1A). Clinical data analysis demonstrated that the expression of Linc-ROR in AM patients was related to the AM type and the severity of dysmenorrhea, but not with age, history of cesarean section, history of uterine surgery, and menstrual cycle (Table I).

### Linc-ROR Promotes Cell Proliferation

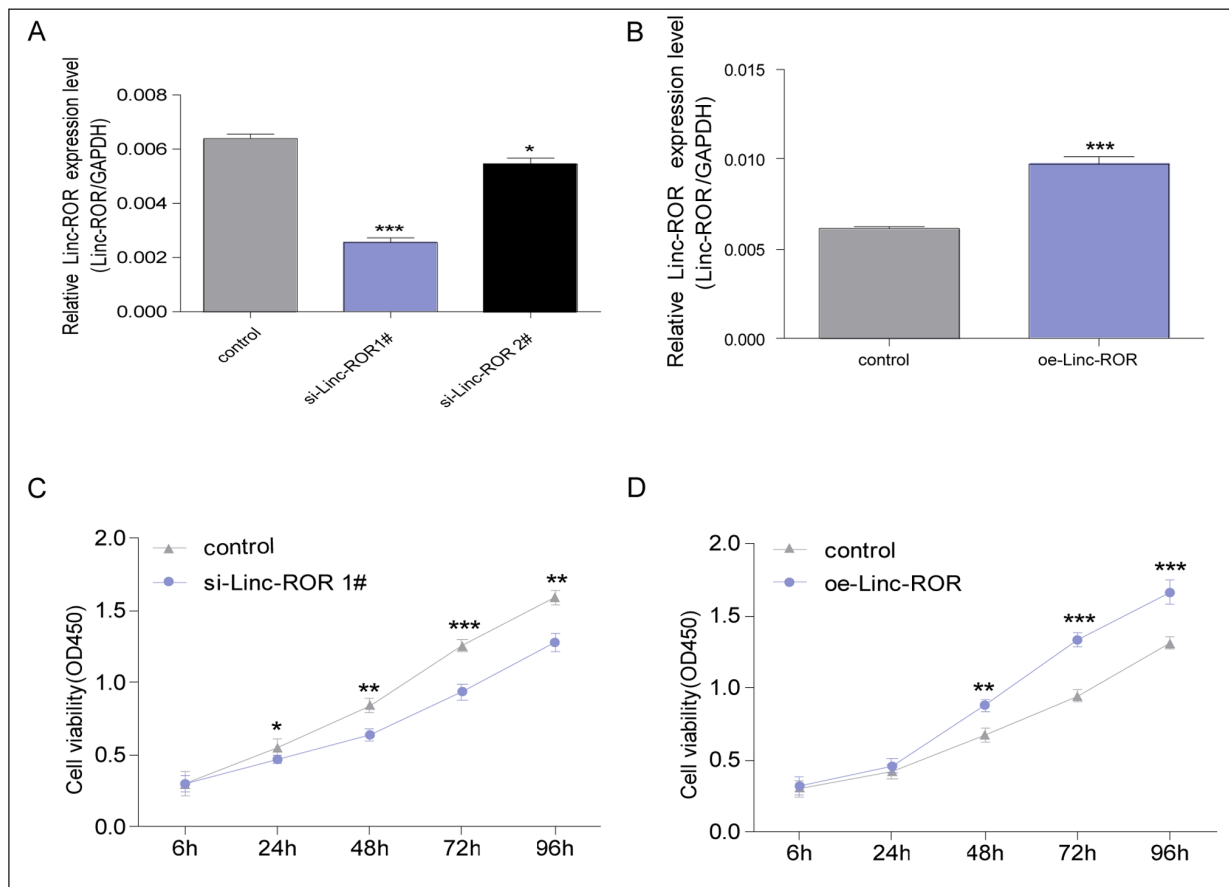
The si-Linc-ROR treatment was performed on epithelial cells isolated from the adenomyosis endometrium. The transfection results were shown in Figure 2A. The interference effect of si-Linc-ROR 1 # was the strongest. The efficiency of oe-Linc-ROR after overexpression Linc-ROR was shown in Figure 2B. CCK-8 pointed out that viability of endometrial epithelial cells decreased after interference with Linc-ROR (Figure 2C). The viability of endometrial cells increased after overexpression of Linc-ROR (Figure 2D). These results suggested that Linc-ROR can promote the proliferation of endometrial epithelial cells.



**Figure 1.** Linc-ROR was highly expressed in the endometrium of adenomyosis. **A**, The mRNA expression of Akt in ectopic and eutopic endometrium in AM patients was significantly higher than that in normal endometrium. The mRNA expression of Akt in ectopic endometrium was higher than that in eutopic endometrium. **B**, The expression of Linc-ROR in ectopic endometrium of AM patients was significantly higher than that in eutopic endometrium and normal endometrium, while the expression of Linc-ROR in eutopic endometrium was higher than that in normal endometrium. **C-D**, The expression of PTEN decreased and the expressions of Akt, p-Akt and p-PTEN increased in ectopic endometrium of AM patients.

**Table I.** The relationship between the Linc-ROR expression and clinicopathological features of AM patients (N = 40).

| Clinicopathologic features  | Linc-ROR expression |             | p-value |
|-----------------------------|---------------------|-------------|---------|
|                             | Low (n=20)          | High (n=20) |         |
| Age (year, $\bar{x}\pm s$ ) | 38 $\pm$ 6          | 35 $\pm$ 4  | 0.4391  |
| Uterine surgery history     |                     |             |         |
| Yes                         | 10                  | 8           | 0.5250  |
| No                          | 10                  | 12          |         |
| Cesarean section            |                     |             |         |
| Yes                         | 9                   | 7           | 0.5186  |
| No                          | 11                  | 13          |         |
| Pathologic subtype          |                     |             | 0.0110* |
| Focal type                  | 13                  | 5           |         |
| Diffuse type                | 7                   | 15          |         |
| Dysmenorrhea score          |                     |             | 0.0098* |
| <3 points                   | 12                  | 4           |         |
| $\geq$ 4 points             | 8                   | 16          |         |
| Menstrual cycle             |                     |             | 0.3421  |
| Regular                     | 8                   | 11          |         |
| Irregular                   | 12                  | 9           |         |



**Figure 2.** Lin-ROR promoted proliferation of endometrial cells. **A**, Interference effect of si-Linc-ROR. **B**, Overexpression effect of oe-Linc-ROR. **C**, Down-regulation of Linc-ROR inhibited the proliferation of endometrial cells. **D**, Upregulation of Linc-ROR promoted proliferation of endometrial cells.

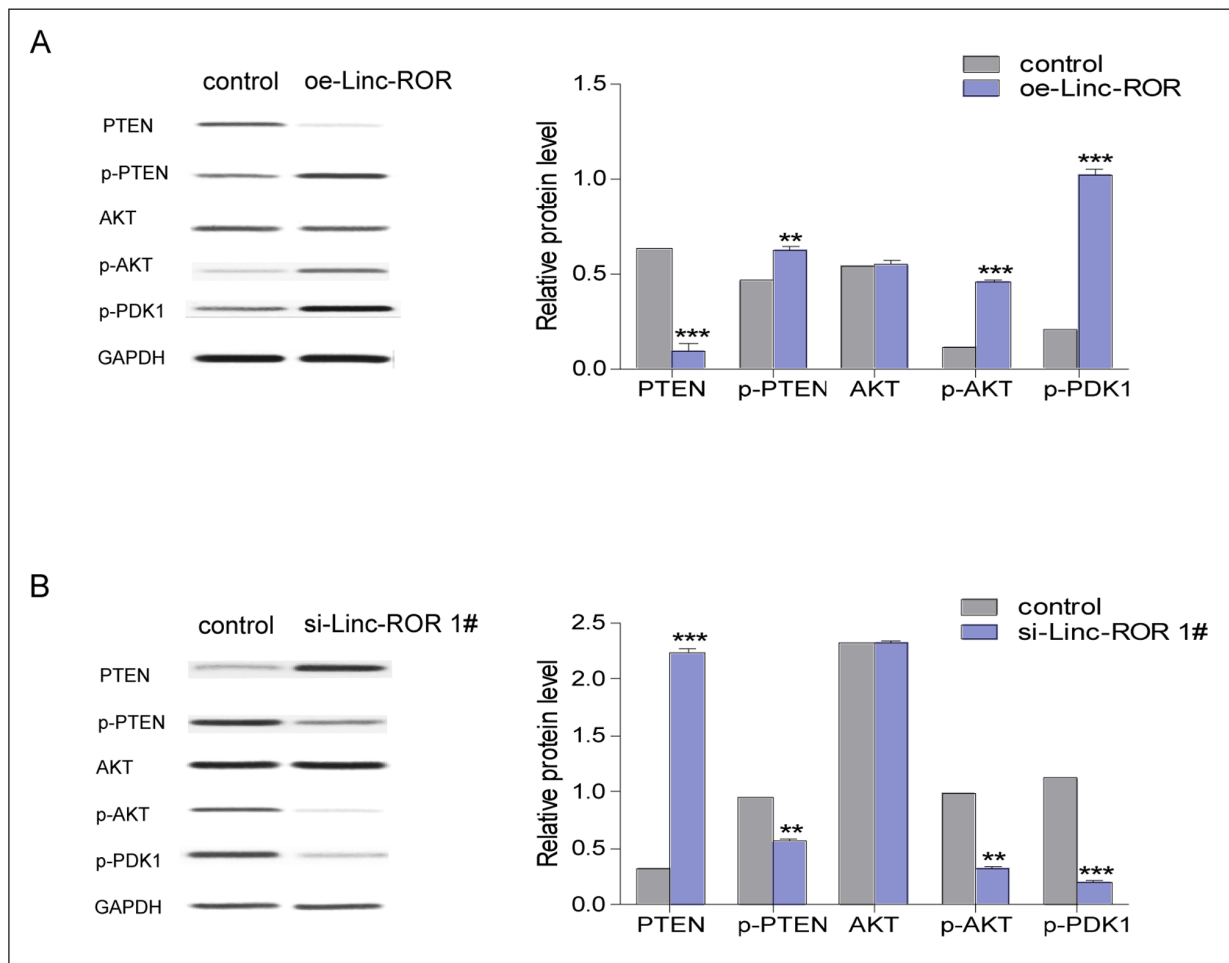
### Related Protein Expression Levels of P13K/Akt Signaling Pathway After Interfering Linc-ROR

This work found that Linc-ROR can promote the proliferative activity of endometrial cells. To further study its mechanism of promoting cell proliferation, we performed Western blot in three adenomyosis ectopic endometrial tissues and three normal tissues. The results illustrated that the expression level of PTEN (phosphatase and tensin homolog deleted on chromosome ten) decreased, whilst expression levels of Akt, p-Akt, p-PTEN increased (Figure 1C-D). To investigate the possible mechanism of Linc-ROR in regulating the proliferation of endometrial cells, Western blot was used to detect the protein expressions in the P13K/Akt signaling pathway before and after interfering Linc-ROR expression. The results revealed that after Linc-ROR knockdown, PTEN expression was up-regulated, while the expressions of p-Akt,

p-PTEN, p-PDK1 (phosphoinositide-dependent kinase) were down-regulated (Figure 3B). This suggested that Linc-ROR promotes endometrial cell proliferation by activating the P13K-Akt pathway.

### Protein Expression Levels of P13K/Akt Signaling Pathway After Upregulation of Linc-ROR Expression

In endometrial cells, we further confirmed that the expression level of PTEN decreased and the expression of p-Akt, p-PTEN, and p-PDK1 were up-regulated after Linc-ROR overexpression (Figure 3A). The experimental results suggested that Linc-ROR overexpression down-regulated PTEN, increased phosphorylation of PTEN and activated Akt, thereby promoting downstream signaling events regulated by Akt, even though p-Akt and p-PDK1 were up-regulated. Thus, overexpression of Linc-ROR expression can promote Akt activation and promote cell proliferation.



**Figure 3.** Related protein expression levels in PI3K/Akt signaling pathway detected by Western blot. **A**, Expression of tumor suppressor gene PTEN decreased and the expressions of Akt, p-Akt, p-PTEN increased after overexpression of Linc-ROR. **B**, Expression of tumor suppressor gene PTEN was up-regulated, while the expressions of Akt, p-Akt and p-PTEN were decreased after down-regulating Linc-ROR.

## Discussion

AM is characterized by a benign infiltration and growth in the myometrium to the endometrium, with the hypertrophy and hyperplasia of surrounding myometrial cells. With the advanced molecular biology and diagnostic techniques recently, great progress has been made in exploring the etiology and pathogenesis of AM in the myometrial growth of the basal endometrium, steroid hormones effect, genetic factors, immune factors, and gene changes.

With the further research on lncRNA, we found that lncRNA is involved in the occurrence and development of various diseases. It affects the treatment and prognosis of patients, and regulates the proliferation, migration, and apoptosis of cells. However, the biological function and mechanism

of many lncRNAs are still not very clear and need further study. Linc-ROR is an intergenic lncRNA, a new lncRNA discovered by Loewer et al<sup>10</sup> in 2010. This study demonstrated that Linc-ROR can induce pluripotent stem cells (iPSCs) and regulate the transcriptional factors Oct4, Sox2, and Nanog, maintain the differentiation potential of embryonic stem cells. Recent studies have shown that Linc-ROR is abnormally highly expressed in many tumors such as breast cancer<sup>11-13</sup>, lung cancer<sup>14</sup>, ovarian cancer<sup>15</sup>, and pancreatic cancer<sup>16</sup>, and highly expressed Linc-ROR can enhance the growth, migration, and invasion of tumor cells, promote the process of tumor cell epithelial-mesenchymal transition and affect the characteristics of tumor stem cells. Hou et al<sup>21</sup> found that Linc-ROR can act as a molecular sponge of microRNA-205 (miR-205) to promote the epithelial-mesenchymal transition

of breast cancer cells. It significantly enhances the invasion and metastasis of breast cancer cells. Another study reported that, in pancreatic cancer and endometrial cancer, Linc-ROR regulates the expression of target genes Oct4, Sox2, and Nanog, promote stem cell differentiation, thereby promoting tumor cell proliferation, invasion, metastasis through interfering miR-145 function<sup>22-24</sup>. Therefore, Linc-ROR may play an important role in the process of tumor cell epithelial-mesenchymal transition and promote the invasion and metastasis of the tumor. Few studies have been carried out, however, in exploring the effect of Linc-ROR on the development and progression of AM. We applied qRT-PCR to detect the expression of Linc-ROR in eutopic, ectopic, and normal endometrium of AM and found that the expression level of Linc-ROR in ectopic endometrium was significantly higher than that in eutopic and normal endometrial tissue. CCK-8 assay further suggested that Linc-ROR can promote the proliferation of endocardium cells. So, which signal pathway dose Linc-ROR regulate the proliferation of AM?

The PI3K-Akt signaling pathway is closely linked to cell proliferation, differentiation, apoptosis and other cellular responses. The PI3K protein has the activity of kinase and protein kinases. Akt is an important downstream kinase in the PI3K pathway and has a filamentous/threonine kinase activity. It acts directly upon phosphorylation of the Akt protein when activated by the PI3K protein, so that downstream signaling pathway factors, such as NF-KB inhibitor protein, NF-KB, Bad, caspase-9, caspase-3, and other phosphorylation reactions are activated, and then to achieve its various biological effects, such as promoting cell cycle progression, inhibiting cells proliferation and inducing apoptosis<sup>25</sup>. Abnormally activated PI3K/AKT signaling pathway is closely related to the development and progression of diseases, thus affecting the treatment and prognosis<sup>19,26,27</sup>. The PI3K-Akt signaling pathway has become a new therapeutic target. Park et al<sup>28</sup> indicated that retinol can induce apoptosis of colon cancer cells by decreasing the activity of PI3K protein, and the study also found that the protein expression of the PI3K is positively correlated with cell apoptosis. Tang et al<sup>29</sup> and other studies found that metformin inhibits apoptosis, oxidative stress, and neuroinflammation; moreover, it improve sepsis-induced brain injury through PI3K/Akt signaling pathway. In this investigation, we found that after down-regulation of Linc-RoR, the protein expression of PTEN was up-regulated

and the expressions of p-Akt, p-PTEN, p-PDK1, and p-PDK1 were downregulated.

In summary, Linc-RoR is highly expressed in ectopic endometrial adenomyosis, which can promote cell proliferation by activating PI3K/Akt signaling pathway.

## Conclusions

We found that Linc-RoR is highly expressed in preeclampsia and promotes the proliferation of endometrial cells by activating the PI3K/AKT pathway. This provides a theoretical basis for the clinical application of Linc-RoR targeted molecular therapy in the treatment of AM.

## Conflict of Interest

The authors declared no conflict of interest.

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