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Targeting EphA2 suppresses the proliferation, migration and invasion of endometriosis *via* the AMPK signaling pathway

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ABSTRACT

Endometriosis is a benign disease with similar characteristics to tumors. Recent studies have found that EphA2 has the dual effect of promoting tumor and inhibiting tumor. The objective of this study was to explore the specific regulatory mechanism of EphA2 in endometriosis. The expression level of Eph protein family in endometriosis was analyzed by bioinformatics method. At the clinical level, qPCR, Western blot and immunohistochemistry were used to verify the correlation between increased EphA2 levels and endometriosis. The effects of blocking EphA2 on cell migration, invasion, proliferation and apoptosis of primary eutopic endometriotic stromal cells were explored *in vitro*. Our study indicated that EphA2 expression was elevated in endometriosis patients, and blocking EphA2 *in vitro* inhibited cell proliferation, migration and invasion through AMPK signaling pathway. Targeting EphA2 can inhibit the progression of endometriosis through AMPK signaling pathway.

Key words: endometriosis; EphA2; AMPK signaling pathway; ALW-II-41-27.

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Introduction

Endometriosis, defined as a condition in which endometrial tissue colonizes the area outside the uterus, affects approximately 10% of women of reproductive age, severely impairs patients' quality of life, and places a heavy financial burden on the health care system.[1,2] Current perspectives suggest that endometriosis is a steroid-dependent disease, with underlying mechanisms including retrograde menstruation, chronic inflammation, metaplasia, and neuroangiogenesis theories.[3] The main treatments for endometriosis are hormone therapy and surgery, but there is currently no suppressive method that can completely cure and prevent recurrence of the disease. Therefore, further in-depth study of the disease mechanism and development of new drug targets are essential for the treatment of endometriosis.

Erythropoietin-producing hepatoma receptor A2 (EphA2) is derived from the largest receptor tyrosine kinase family – Eph/ephrin family, only expressed in proliferative epithelial cells of adults.[4,5] Lots of studies have confirmed that EphA2 has a unique dual driving effect of cancer inhibition and cancer promotion, and is significantly related to the prognosis, metastasis and survival rate of cancer patients.[6] Blocking EphA2 suppresses immune cytotoxicity in endometriosis. This suggests that EphA2 is likely to play a vital role in the pathogenesis of endometriosis and has considerable therapeutic potential.[7] The subsequent study by Chandrashekhar et al. confirmed the dysregulation of EphA2 expression in endometriosis and synthesized new EphA2-targeting drugs.[8] Normally, EphA2 transmits bidirectional signaling by pairing with its ligand EphrinA1.[9] However, EphA2 overexpression is usually accompanied by the loss of this ligand, and EphA2 activity relies on the noncanonical signaling pathway that is a ligand-independent.[10].It has been hypothesized that overexpression of EphA2 can form homodimers, which exhibit tumor-promoting activity different from the antitumor effect.[11] However, existing studies have not sufficiently illustrated the upstream and downstream interactions of EphA2 to comprehensively determine the regulatory mechanisms of EphA2 in endometriosis. Therefore, unraveling how targeted EphA2 inhibits endometriosis not only contributes a novel theoretical framework for understanding its pathogenesis, but also establishes a foundation for the development of precision treatment strategies targeting. In this study, bioinformatics tools were used to predict the expression changes of Eph/Ephrin family in endometriosis, and to further explore the role of EphA2 in endometriosis *in vitro*. Our results indicate that targeted inhibition of EphA2 can reduce the proliferation, migration and invasion of endometriosis cells, providing a new mechanism insights and a potential therapeutic target for the treatment of endometriosis.

Materials and Methods

Ethics statement and clinical samples

The clinical study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (clinical trial number: WDRY2024-K111) and conducted in accordance with ethical guidelines. The clinical trial conformed to the Declaration of Helsinki. All participants provided written informed consent.

Eutopic endometrial tissue and ovarian ectopic tissue (Endometriosis group, n=9) were collected from patients with endometriosis who were admitted to the Renmin Hospital of Wuhan University from June 2024 to October 2024 and confirmed by postoperative pathology. Non-endometriosis patients undergoing benign gynecological surgery at the same time were included, and their normal endometrial tissues were collected (Control group, n=9). Inclusion criteria: i) women of childbearing age between 18 and 45 years old; ii) the endometriosis group was pathologically diagnosed with endometriosis, and underwent ovarian cyst removal (laparoscopic or trans-abdominal) and hysteroscopic surgery; ii) the control group was diagnosed without endometriosis and adenomyosis, and received hysteroscopic surgery or total hysterectomy. Exclusion criteria: i) incomplete clinical data (lack of pathological diagnosis or unclear treatment plan); ii) patients who received estrogen and progesterone drugs or selective estrogen and progesterone receptor modulators before surgery; iii) patients with a history of present disease or previous diabetes, autoimmune disease, cancer, or serious disease (such as liver or thyroid disease); iv) patients with adenomyosis; v) a history of smoking and drinking.

Bioinformatics analysis

RNA sequencing data were obtained from the GEO database, with the GSE25826 dataset used for expression analysis, enrichment analysis, and immune infiltration analysis. Bioinformatics analyses were conducted by using R 4.3.2.

Immunohistochemistry

Fresh tissues were fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin. The slides were dehydrated in xylene, anhydrous ethanol, 95% ethanol, 70% ethanol and 50% ethanol. After a wash in distilled water, the slides were placed in a citric acid-containing antigen repair buffer (G1202; Servicebio, Wuhan, China) for antigen retrieval and heated in a microwave oven. Keep at medium heat for 8 min until boiling, then keep at medium-low heat for 7 min.[12] After washing with PBS (PH7.4) for three times, the slides were incubated with 3% H_2O_2 at room temperature and away from light for 25 min.

Cells were cultured on coverslips for 48 h and then fixed with 4% paraformaldehyde. Using a histochemical pen, circles were drawn around the tissue sections or in the middle of the coverslips. The slides were blocked with 3% BSA, first by incubation overnight at 4°C with primary antibodies against EphA2 (#6997; Cell Signaling Technology, Danvers, MA, USA), CK19 (GB15198-100; Servicebio), and Vimentin (GB11192-100; Servicebio) diluted 1:500. In the negative control group, the tissue was incubated with PBS only and contained no primary antibody. Since EphA2 is overexpressed in breast cancer, MDA-MB-231 cell line with high EphA2 expression was used as a positive control[13,14]. HRP-conjugated secondary antibodies (G1213-100UL; Servicebio) were added and incubated at room temperature for 1 h at 1:200 dilution. DAB was used for color development for 1-3 min. The slides were visualized and imaged using a microscope (B53; Olympus, Tokyo, Japan).

The results of IHC were evaluated by two independent pathologists, who then assessed the staining strength based on the percentage of positive areas. Quantification was performed with ImageJ 1.8.0.

Isolation and culture of primary eutopic endometriotic stromal cell

Fresh tissues were washed with PBS and cut into approximately 1mm fragments, then digested with 1ng/mL collagenase IV (C8160; Solarbio) for 1 h. The digestion mixture was ground and filtered through a 200-mesh filter. The filtrate was centrifuged, and the supernatant was discarded. The cell pellet was resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin/streptomycin (G4003-100M; Servicebio), and cultured in a 37°C, 5% CO_2 incubator.

Cell transfection and drug treatment

Small interfering RNA targeting EphA2 (si-EphA2; GenePharma, Shanghai, China) was transfected into the primary eutopic endometriotic stromal cells by using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) and cultured at 37°C, 5% CO_2 for 48 h before subsequent experiments. The EphA2 inhibitor ALW-II-41-27(abs818923-10 mg, Absin Bioscience Inc., Shanghai, China)[15] was dissolved in sterile DMSO and stored in aliquots at −20°C. Primary cells were treated with a concentration gradient of ALW-II-41-27 for 48 h.

Quantitative real-time PCR

Total RNA was extracted from tissues and primary cells using Trizol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using a reverse transcription kit (R211-01; Vazyme, Nanjing, China) to obtain cDNA. cDNA was mixed with primers and qPCR reagents (Q712-02; Vazyme), and Quantitative real-time PCR was conducted using a fluorescence quantitative PCR analyzer (Lightcycler 4800II; Roche, Basel, Switzerland). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize RNA expression. Data were analyzed using the 2−ΔCt method.[16] Primer sequences for each gene are listed in Table 1.

Western blot

Protein samples from tissues and cells were obtained using cell lysis buffer (P0013; Beyotime Biotech Inc., Haimen, China), mixed with an appropriate volume of loading buffer (G2075-1ML; Servicebio), and denatured at 100°C for 5 min. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes, which were blocked with 5% non-fat milk at room temperature for 1 h. The membranes were then incubated overnight at 4°C with primary antibodies against EphA2 (#6997; Cell Signaling Technology), p-AMPK (TA3423S; Abmart, Shanghai, China), AMPK (WL02254; Wanleibio, Shenyang, China), Caspase3 (T40044F; Abmart), Bax (WL01637, Wableibio), Bcl-2 (T40056F; Abmart), and GAPDH (ET1601-4, HuaBio, Shanghai, China) diluted 1:1000. After washing, the membranes were incubated with 1:1000 diluted HRP-conjugated secondary antibodies (HA1001; HuaBio) at room temperature for 1 h. Detection was performed using ECL reagent (E422-01; Vazyme), and chemiluminescence was captured using a Gel Doc XR system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Cell counting kit-8 assay

Primary eutopic endometriotic stromal cells from endometriosis patients were collected and seeded into 96-well plates, with 100 µL of diluted cell suspension (1×10^4 cells/well) added to each well. At 0 h and 48 h, 10 µL of CCK8 solution (G4103-5ML; Servicebio) was added to each well and incubated at 37°C for 2 h. Absorbance was measured at 260 nm using a Envision Multimode Plate Reader (PerkinsElmer, Shelton, CT, USA).

Wound healing assay

Primary eutopic endometriotic stromal cells were cultured in six-well plates until they reached 100% confluence. Sterilized rulers and 20 µL pipette tips were used to create perpendicular cross-shaped scratches in each well. The old medium was discarded, and the wells were washed twice with PBS before adding serum-free medium. Images were captured at 0, 24, and 48 h using an inverted microscope (Olympus IX71). Cell migration distance was calculated using ImageJ 1.8.0 software to evaluate migration capability.

Transwell migration assay

A 100 µL suspension of primary eutopic endometriotic stromal cells (1×10^4 cells in serum-free medium) was seeded in the upper chamber of transwell inserts, while the lower chamber was filled with DMEM/F12 medium containing 10% FBS. After 48 h, non-invasive cells in the upper chamber were removed, and the remaining cells were stained with crystal violet solution at room temperature. Images were captured using an inverted microscope, and quantification was performed with ImageJ 1.8.0.

Transwell invasion assay

Matrigel (211222; NEST Biotechnology, Wuzi, China) was thawed at 4°C and coated on the upper chamber of transwell inserts, which were then dried at 37°C for 3 h. A 100 µL suspension of primary eutopic endometriotic stromal cells (1×10^4 cells in serum-free medium) was seeded in the upper chamber, and the lower chamber was filled with DMEM/F12 medium containing 10% FBS. After 48 h, non-invasive cells in the upper chamber were removed, and the remaining cells were stained with crystal violet solution at room temperature. Images were captured using an inverted microscope, and quantification was performed with ImageJ 1.8.0 .

Colony formation assay

After harvesting primary eutopic endometriotic stromal cells from endometriosis patients, 500 cells were seeded into six-well plates containing 2 mL of complete medium. The cells were cultured at 37°C, 5% CO_2 for 2 weeks to form colonies. After 2 weeks, the medium was discarded, and the cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet solution for 15 min. The plates were rinsed with running water, and images were captured for colony counting.

Apoptosis assay by flow cytometry

Primary endometrial stromal cells from endometriosis patients were collected and apoptosis was detected using a flow cytometry kit (E-CK-A211; Elabscience, Wuhan, China). Cells were resuspended in binding buffer and stained with 5 µL Annexin V-FITC and propidium iodide (PI) dyes, then incubated in the dark at room temperature for 15 min. Apoptosis was assessed using a flow cytometer (CytoFlex; Beckman Coulter, Brea, CA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0, and data are presented as mean ± SD from at least three independent experiments. Comparisons between two groups were made using the *t*-test, and one-way ANOVA with Tukey's multiple comparison test was used for multiple group comparisons. A *p*-value <0.05 was considered statistically significant.

Table 1. Primer sequences for genes in PCR.

Name of primer	Sequences
EphA2-F	AGAGGCTGAGCGTATCTTCAT
EphA2-R	GGTCCGACTCGGCATAGTAGA
EphA4-F	TTCGCCCTATTTTCGTGTCTC
EphA4-R	TGGTAGGTTCGGATTGGTGTAT
EFNA1-F	TCAGGCCCATGACAATCCAC
EFNA1-R	GTGACCGATGCTATGTAGAACC
EphB4-F	CGCACCTACGAAGTGTGTGA
EphB4-R	GTCCGCATCGCTCTCATAGTA

Results

Bioinformatics analysis of EphA2 in endometriosis

We conducted bioinformatics analysis using the GSE25826 dataset from the GEO database. The results showed an upregulation of EphA2 in endometriosis, though the increase was not obvious (Figure 1A). This dataset included ectopic endometrial tissue, eutopic endometrial tissue, and normal endometrial tissue. EphA2 expression levels were changed among these groups (Figure 1 B,C). Further analysis of EphA2 expression and immune cell infiltration revealed that EphA2 expression was associated with macrophages, activated dendritic cells, CD8+ T cells, and B cells ($p<0.01$, Figure 1E), indicating that EphA2 plays a role in immune regulation in endometriosis. Pathway enrichment analysis showed that EphA2 is mainly involved in pathways related to inflammatory response, metabolic regulation, and oxidative phosphorylation (Figure 1D).

EphA2 expression is upregulated in endometriosis tissues

To validate the bioinformatics analysis results, we examined the expression levels of EphA2 in ectopic endometrial tissues, eutopic endometrium, and normal endometrium. RT-qPCR results indicated that the mRNA expression of EphA2 ($p=0.0054$) and EphA4 ($p=0.0016$) was increased in both ectopic and eutopic endometrium (Figure 2A), while the expression of EphB4 showed no significant difference ($p=0.2463$, Figure 2A). EphrinA1 mRNA expression was elevated in ectopic endometrium but lower in eutopic endometrium ($p=0.0036$, Figure 2A). Consistent with the mRNA trends, the protein expression level of EphA2 also showed an increasing trend in ectopic endometrium ($p=0.0043$, Figure 2B). Immunohistochemical results respectively indicated that the EphA2-positive percentage of eutopic ($p=0.0006$) and ectopic endometrium ($p=0.0002$) in endometriosis patients was significantly higher than that in normal endometrium tissue, further confirming the abnormal upregulation of EphA2 in endometriosis (Figure 2C). These results suggest that the occurrence of endometriosis is associated with elevated levels of EphA2.

Knockdown or inhibition of EphA2 induces changes in the AMPK pathway in endometriosis cells

Primary eutopic endometriotic stromal cells were extracted from eutopic endometrium of endometriosis patients for related biological experiments. Immunohistochemistry indicated that the primary cells expressed Vimentin but not CK19, confirming that the extracted cells were endometriotic stromal cells (Figure 3C). First, si-EphA2 was used to knock down EphA2 expression in primary cells (Figure 3D). Western blot results of si-EphA2 cells showed that after knocking down EPHA2, the expression of phosphorylated AMPK (p-AMPK) was significantly downregulated ($p<0.0001$), while total AMPK remained unchanged ($p=0.0776$), indicating that EphA2 can activate AMPK and regulate downstream signaling pathways (Figure 3A). However, the level of the apoptosis protein Caspase3 did not change ($p=0.9739$, Figure 3A). ALW-II-41-27, a targeted inhibitor of EphA2, was used to further explore the related mechanisms. The results showed that EphA2 and p-AMPK were similarly downregulated in ALW-II-41-27-treated cells ($p=0.0034$), while the levels of apoptosis proteins Caspase3, Bcl2, and Bax did not change ($p=0.5357$, Figure 3B).

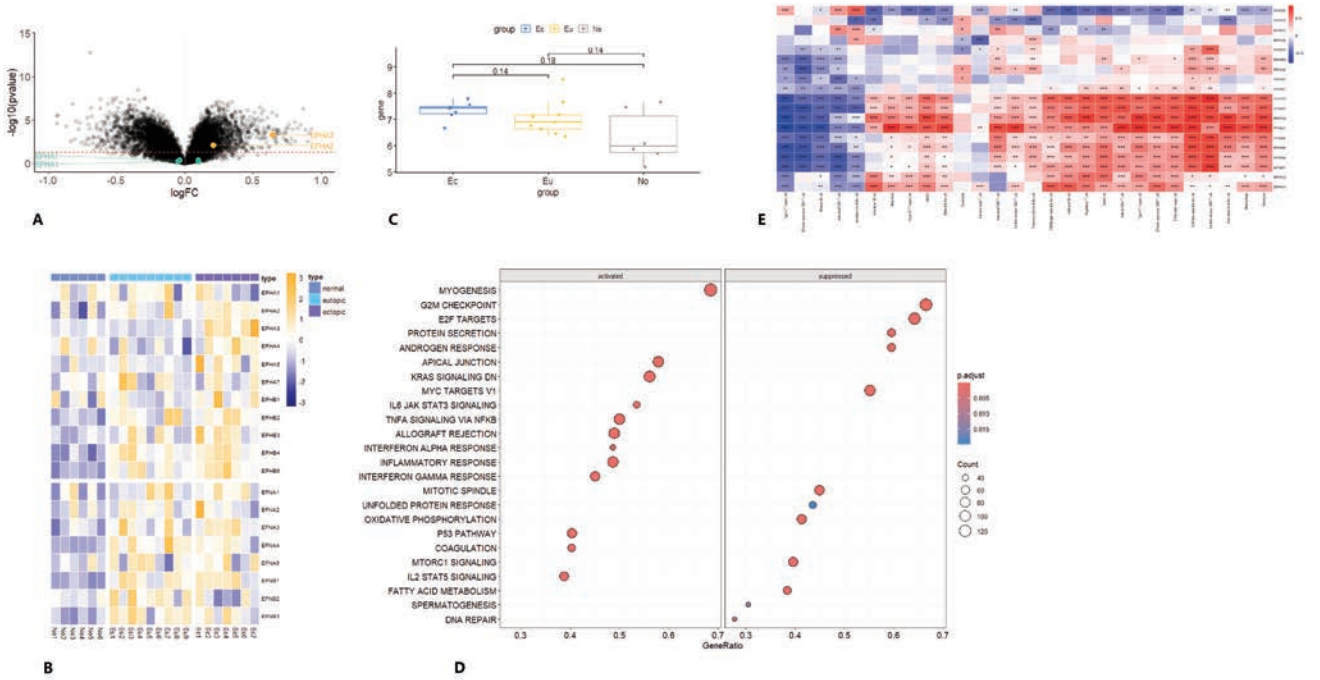

Figure 1. Bioinformatics analysis of EphA2 in endometriosis. **A**) Volcanic map of endometriosis protein expression. **B**) Heat maps of Eph/ephrin family mRNA expression in endometriosis. **C**) EphA2 mRNA expression difference in normal, ectopic, and eutopic endometrium. **D**) Immunoinfiltration correlation analysis of EphA2 in endometriosis. **E**) Pathway enrichment analysis of EphA2 in endometriosis. The values represent mean±SD of three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns, no statistical significance.

This indicates that EphA2 is associated with the AMPK pathway in endometriosis, and this pathway likely does not involve the regulation of apoptosis in endometriosis cells.

Knockdown or inhibition of EphA2 reduces cell migration and invasion

We used wound healing assays to observe changes in cell migration ability after si-EphA2 (p=0.0108) or ALW-II-41-27 (p=0.0021) treatment. The results showed that the migration ability of both treatment groups was reduced compared to the NC group (Figure 4 A,B). In transwell migration assays, we again confirmed that the migration ability of cells treated with si-EphA2 (p=0.002) and ALW-II-41-27 (p=0.001) was reduced (Figure 5A). In the Transwell invasion assay, the ability of NC group cells to penetrate the Matrigel was significantly higher than that of si-EphA2 (p<0.0001) and ALW-II-41-27 (p=0.004) treated cells (Figure 5B).

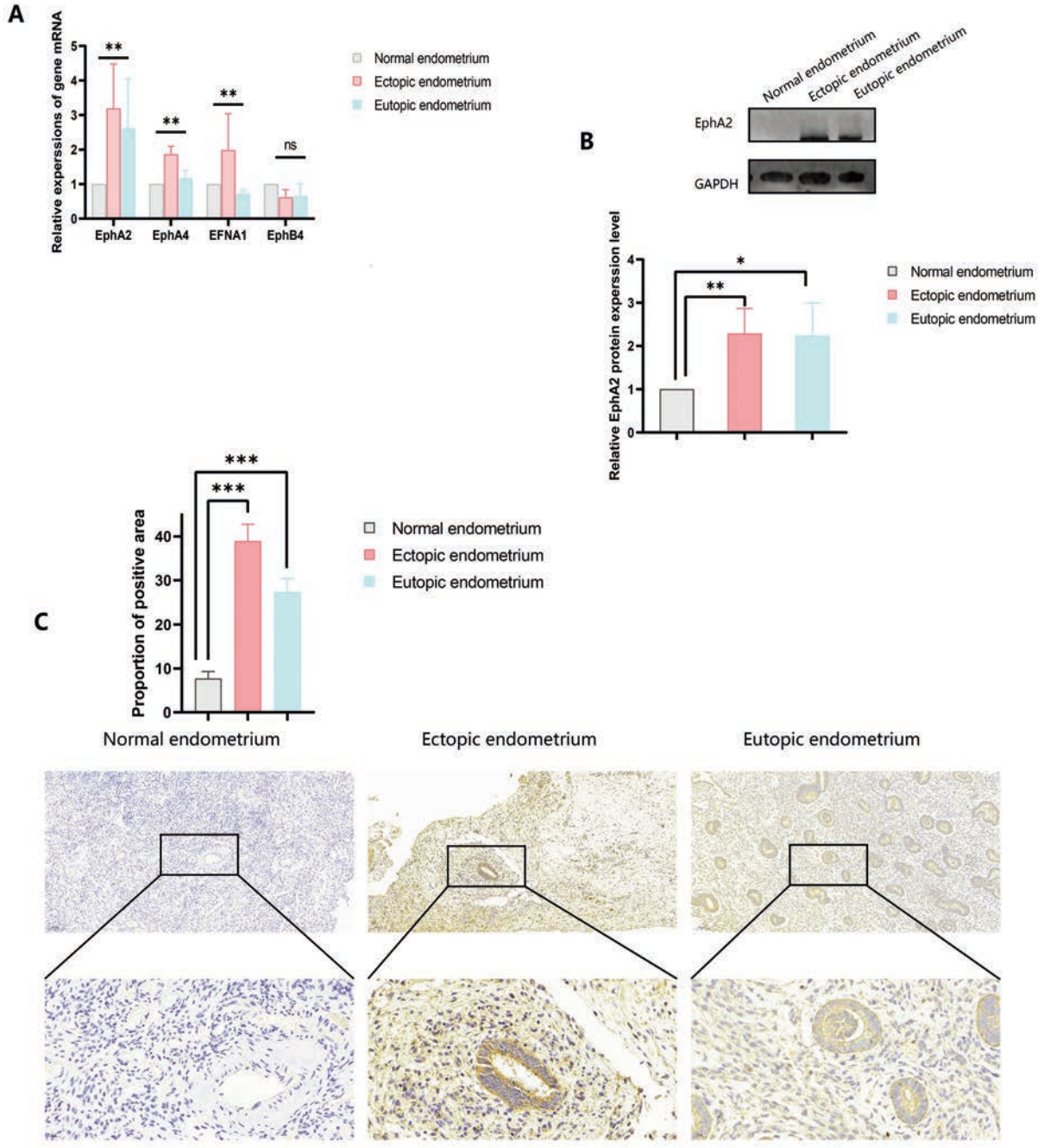

Figure 2. Changes of Eph mRNA and protein expression levels in ectopic, eutopic, and normal endometrium tissues. **A**) EphA2 mRNA expression level was increased in endometriosis tissue. **B**) EphA2 protein expression level was higher in endometriosis than in normal endometrial tissues were determined by Western blot. **C**) Immunohistochemical analysis showing that EphA2 protein was overexpressed in endometriosis. Scale bar: 20 μm (400× magnification). The values represent mean±SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, no statistical significance.

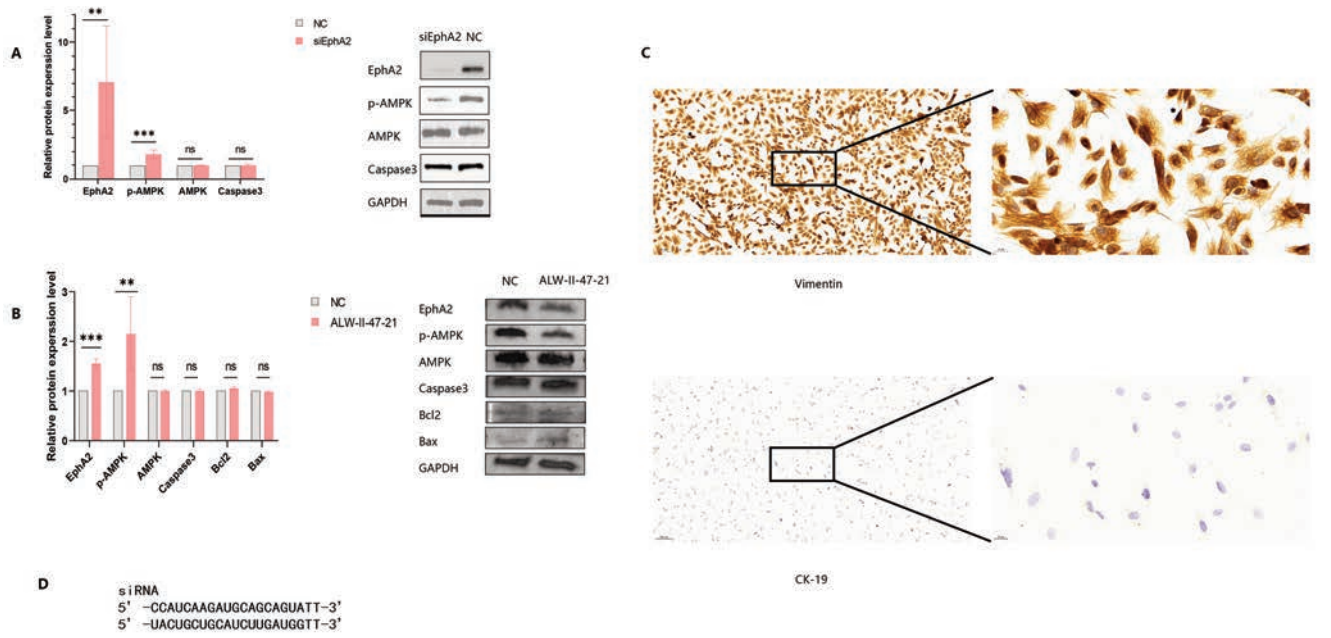

Figure 3. Protein expression levels of primary eutopic endometriotic stromal cells after transfection with si-EphA2 or treatment with ALW-II-41-27. **A**) EphA2 protein expression in primary cells after si-EphA2 transfection. **B**) EphA2 protein expression in primary cells treated with ALW-II-41-27. **C**) Immunohistochemistry identification of primary cells; scale bar: 20 μm (400× magnification). **D**) si-EphA2 sequence. The values represent mean ± SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, no statistical significance.

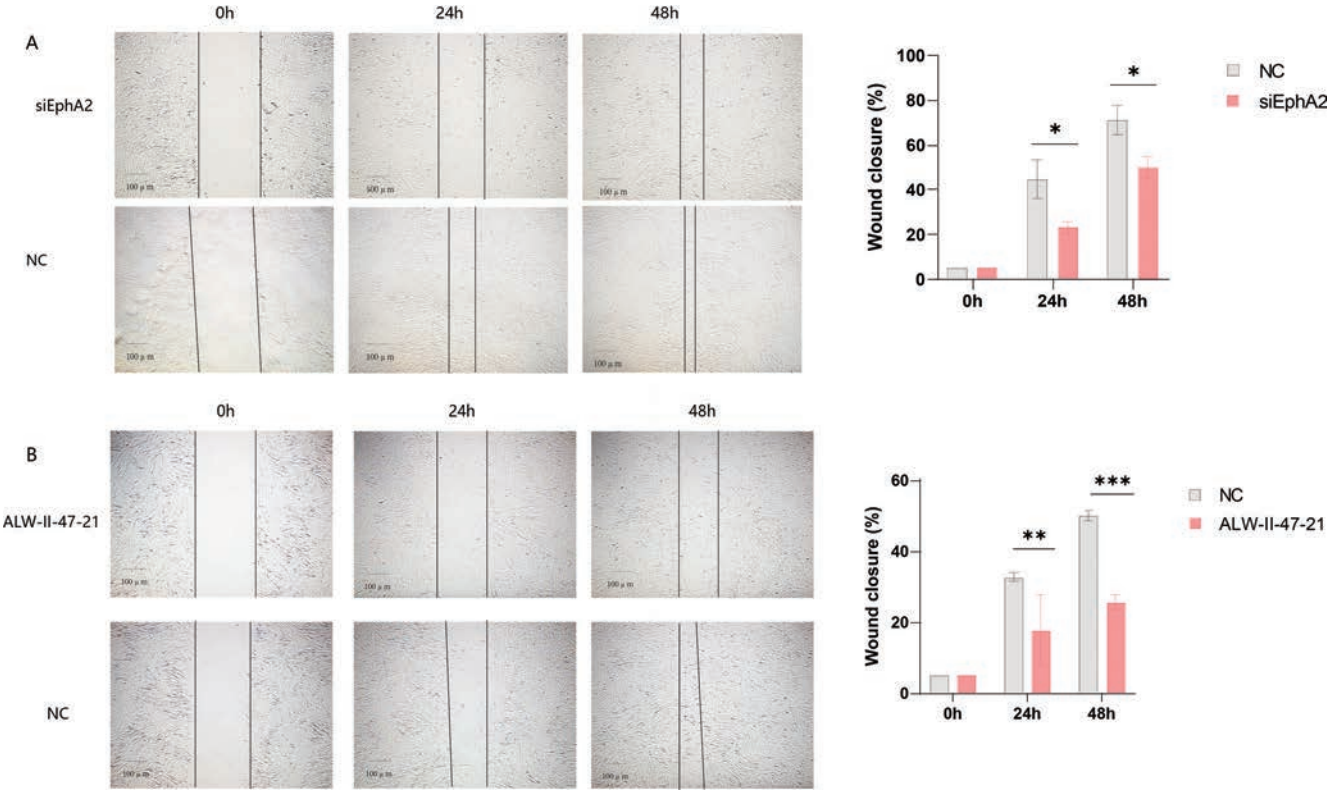

Figure 4. After transfection or si-EphA2 and treatment with ALW-II-41-27, the migration ability of primary eutopic endometriotic stromal cells were decreased. **A**) Wound healing assay of primary cells after si-EphA2 transfection. **B**) Wound healing assay of primary cells after treatment with ALW-II-41-27; representative images are shown at 0, 24 and 48 h; scale bar: 100 μm (100× magnification). The values represent mean±SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, no statistical significance.

This demonstrates that knocking down or inhibiting EphA2 can reduce the migration and invasion abilities of primary eutopic endometriotic stromal cells from endometriosis patients.

Inhibition of EphA2 suppresses cell proliferation but does not affect apoptosis

CCK8 and colony formation assays were used to assess cell proliferation ability. In the colony formation assay, the number of colonies formed in the ALW-II-41-27 treatment group was significantly lower than in the NC group (p=0.0223, Figure 6A). The CCK8 assay indicated that cell viability decreased with increasing drug concentration (p=0.0004, Figure 6B). Flow cytometry was used to detect changes in apoptosis after cell treatment, and the analysis showed no statistically significant difference in apoptosis rates between the drug-treated and control groups (p=0.3251, Figure 6C). This suggests that ALW-II-41-27 can inhibit cell proliferation without affecting cell apoptosis.

Discussion

Although hormone therapies have shown promising results in treating endometriosis over the past decade, their side effects and limitations due to non-curability have prompted researchers to explore the development of non-hormone-targeted therapeutic strategies.[17] Among emerging targets, EphA2, as the key role in the regulation of tumor microenvironment, has attracted much attention, though its pathogenic mechanism and therapeutic potential are still unclear.[18-20] Our study revealed that EphA2 was upregulated in endometriosis and contributed to immune imbalance. AMPK phosphorylation was one of the key signal transduction events mediating the cellular biological behavior regulated by EphA2. We also identified the ability of the EphA2 kinase inhibitor ALW-II-41-27 to suppress cellular proliferation and malignant behaviors in primary cell model, highlighting its translative potential in endometriosis.

EphA2 overexpression is known to promote immune escape and thus shape the immunosuppressive microenvironment in diseases like cancer.[21] The immune microenvironment of endometriosis contains both innate immune cell populations and adaptive cell populations, which evolves from a pro-inflammatory environment in the early stage to immune tolerance in the advanced stage of endometriosis.[22] Our RNA sequencing analysis revealed significant EphA2 upregulation in endometriosis lesions and associated with infiltrating macrophages, CD8+ T cells, dendritic cells and B cells positively. For instance, EphA2 drives M2 macrophage polarization,[23] impairing phagocytosis, triggering chronic inflammatory pathways that cause neuroangiogenesis and fibrosis and constituting the core phenotype of the immune niche of endometriosis.[24,25] CD8+ T cells and B cells constitute the adaptive immune system of endometriosis, and their abnormal activation is related with infertility caused by endometriosis.[22,26] EphA2 may enhance the migration and cytotoxic activity of CD8+ T cells,[27,28] while also

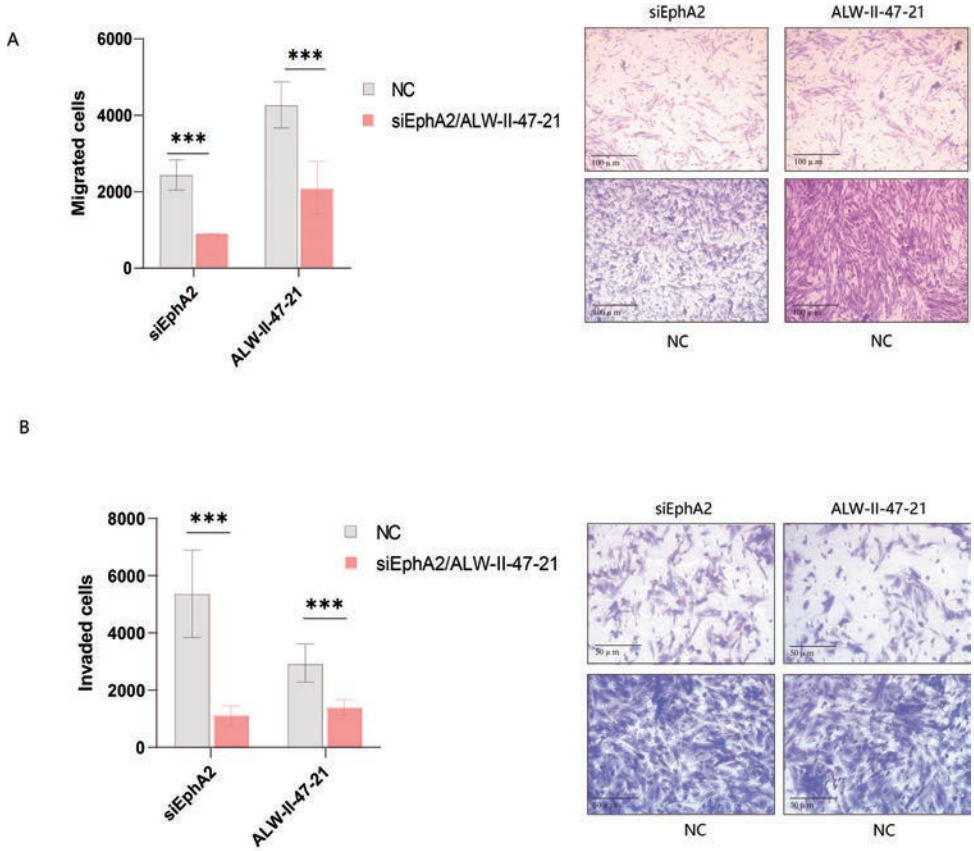

Figure 5. After transfection with si-EphA2 or treatment with ALW-II-41-27, the migration and invasion ability of primary eutopic endometriotic stromal cells were reduced. **A**) Transwell migration experiment after transfection with si-EphA2 or treatment with ALW-II-41-27. **B**) Transwell invasion experiment after transfection with si-EphA2 or treatment with ALW-II-41-27; scale bar: 100 μm (100× magnification). The values represent mean±SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, no statistical significance.

stimulating B cells to produce autoantibodies and exacerbating chronic inflammation.[29] Dendritic cells expressing EphA2 further infiltrate lesions, promoting angiogenesis and transforming into macrophages with pathogenic phenotypes.[30-32] Although these findings establish EphA2's association with immune dysfunction, the precise mechanisms require further investigation, particularly whether EphA2 directly reprograms immune cells toward a pathogenic phenotype, which could position EphA2 inhibitors as potential immunomodulators for endometriosis.

The therapeutic potential of targeting EphA2 is exemplified by ALW-II-41-27 , a type II kinase inhibitor that binds EphA2's ATP binding site of the "DFG-out" conformation.[33] Previously mainly studied in inhibiting cancer, ALW-II-41-27 has recently been discovered potential therapeutic effects in benign diseases.[34,35] And in our primary cell, it is observed that genetic and pharmacological targeting EphA2 significantly suppressed proliferation, migration, and invasion, consistent with Matzuk.[8] Interestingly, EphA2 inhibition did not show significant inhibitory or pro-apoptotic effects in endometriosis. This phenomenon may explain why endometriosis is still defined as a benign disease despite its ability to spread and invade similar to cancer.[36]

In addition to cellular behavior, the therapeutic effect of ALW-II-41-27 may own broader therapeutic effect, such as suppressing pro-inflammatory events caused by macrophage in osteoarthritis and atherosclerosis.[37,38] Besides, The inhibition of EphA2 can also disrupt neovascularization by regulating "vascular secretion" signals and endothelial cell migration.[39,40] These multiple effects suggest that targeting EphA2 has clinical application prospects for various pathogenic characteristics of endometriosis, though further validation is needed.

The biological function of EphA2 is mediated by both ligand-dependent and -independent pathways.[41-43] The different expression changes between EphA2 and ephrinA1 indicate that EphA2 may be involved in endometriosis through a non-ligand-dependent pathway. We further found that down-regulation of EphA2 significantly reduced the phosphorylation of AMPK, suggesting that the EphA2-AMPK axis plays a key regulatory role in endometriosis. While AMPK activation has been shown to inhibit endometriosis progression in other studies,[44,45] our data align with its tumor-promoting role in certain cancers.[46-48] These contradictions confirm the complex function of AMPK in different cell types or disease stages. Enrichment analysis also revealed that EphA2 may affect endometriosis progression through regulation of multiple signaling networks such as inflammation, metabolism and oxidative phosphorylation. These findings provide theoretical basis for developing multi-target combination therapy strategies.

Although ALW-II-41-27 is currently a widely used preclinical drug, its cross-reactivity with other kinases has caused serious off-target effects.[33] Alternative approaches repurposing the non-selective kinase inhibitor dasatinib[49] or developing monoclonal antibodies, though the latter efficacy is limited.[50] Neutral liposomal small interfering RNA drug EPHARNA, antibody-drug conjugate MEDI-547 and hSD5-vedotin have shown good therapeutic effects in preclinical trials.[51-53] These provide hope for more targeted interventions, which may be able to balance the targeting and effectiveness of the treatment.

There are still limitations in this study. Limited by experimental modulation, we did not further evaluate the role of targeting EphA2 in animal models of endometriosis. Whether EphA2 is involved in other characteristic phenotypes of endometriosis, such

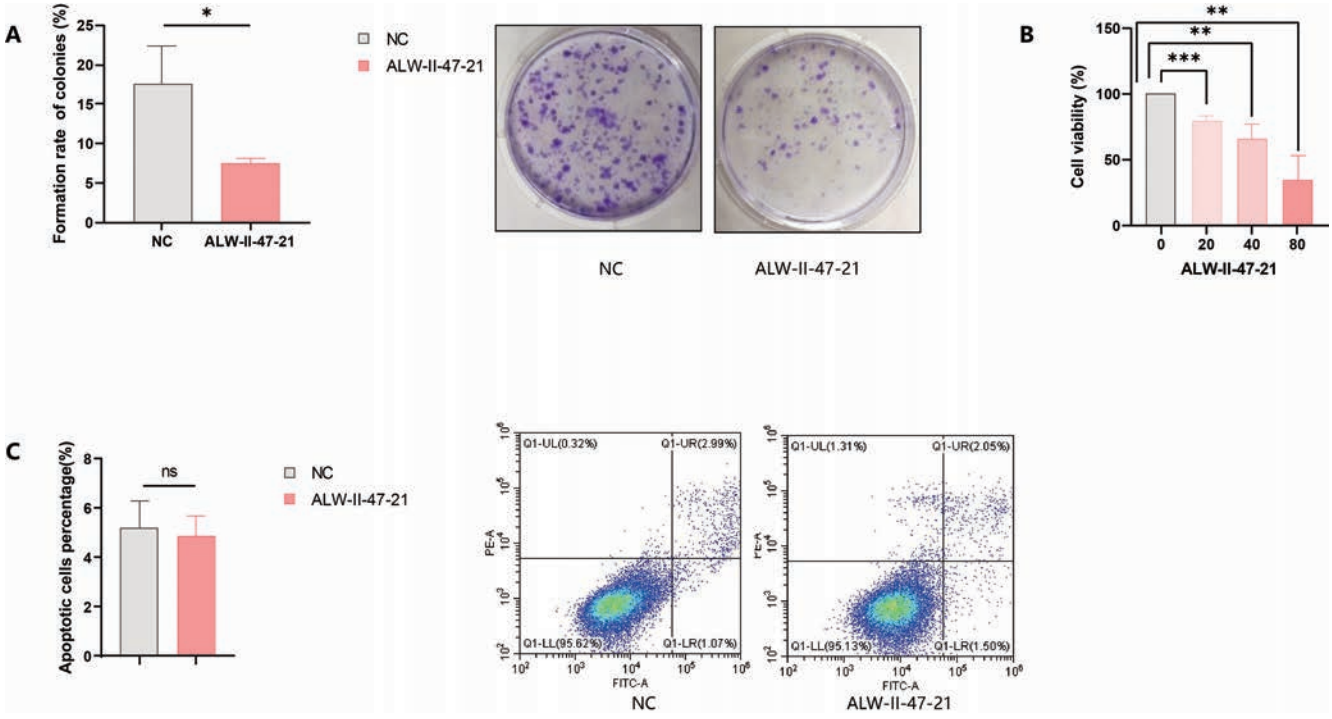

Figure 6. Changes in proliferation and apoptosis of primary eutopic endometriotic stomal cells treated with ALW-II-41-27. **A**) Colony formation assay showing that the proliferation of primary cells was inhibited by ALW-II-41-27. **B**) CCK8 assay showing that ALW-II-41-27 downregulated the proliferation of primary cells. **C**) Flow cytometry apoptosis assay showing that ALW-II-41-27 had no significant effect on apoptosis of primary cells. The values represent mean±SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, no statistical significance.

as neuroangiogenesis,[54] and a possible non-ligand-dependent pathway between EphA2 and AMPK remains to be determined. Therefore, future work should address these gaps while screening for more selective EphA2 inhibitors or synergistic combination therapies.

This study systematically elucidates the molecular mechanism by which EphA2 promotes endometriosis progression through AMPK pathway and confirms that both pharmacological and gene inhibition of EphA2 can effectively block the malignant behavior of endometrial cells. These findings not only provide a new perspective for understanding the pathogenesis of EMs, but more importantly, lay an experimental foundation for the development of non-hormone targeted therapies, especially for refractory endometriosis or pain patients who have not responded to traditional treatments. Combined with recent clinical advances in EphA2 inhibitors in the field of endometriosis, our study supports EphA2 as a potential target for endometriosis precision therapy, promising to fill the gaps in current treatment options in the future.

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