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Original Article

miR-9-5p promotes the invasion and migration of endometrial stromal cells in endometriosis patients through the SIRT1/NF-κB pathway

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Abstract: Objective: The present study was designed to investigate the expression of miR-9-5p and to study the effect of miR-9-5p expression on the invasion and migration of endometrial stromal cells in endometriosis patients. Methods: We recruited 17 eutopic endometrium patients, 19 ectopic endometrium patients, and 13 normal endometrium patients, and we measured their miR-9-5p and SIRT1 expressions. Western blot was used to measure the protein expressions, and cellular immunofluorescence was used to check the positions of the p65 position protein in cells. A Transwell chamber and cell scratch tests were used to test cell invasion and migration, respectively. Results: miR-9-5p was highly expressed, and SIRT1 was lowly expressed in the endometria of the endometriosis patients, and there was a negative correlation between miR-9-5p and SIRT1 mRNA in the endometriosis patients. A dual luciferase reporter gene system showed that miR-9-5p targeted the inhibition of SIRT1 expression in the endometrial stromal cells. Moreover, the up-regulation of miR-9-5p expression using the miR-9-5p-mimics significantly increased the distance of endometrial stromal cell migration and the number of cells that entered into the lower chamber of the Transwell chamber, and the down-regulation of miR-9-5p using the miR-9-5p-inhibitor significantly decreased the distance of endometrial stromal cell migration and the number of cells that entered into the lower chamber of the Transwell chamber. Moreover, the miR-9-5p-mimics significantly increased the expressions of the P-p65/p65 protein and the 65 protein in the nuclei, and the miR-9-5p-inhibitor significantly decreased the expressions of the P-p65/p65 protein and the 65 protein in the nuclei. Conclusion: miR-9-5p is highly expressed in the endometria of endometriosis patients, and miR-9-5p can promote the invasion and migration of endometrial stromal cells in vitro by targeting the SIRT1 expression via the NF-κB pathway.

Keywords: miR-9-5p, endometriosis, invasion, migration

Introduction

Endometriosis (EMs) is a common gynecological disease in women caused by the implantation of active endometrial cells outside the endometrium. Epidemiological studies have found that the incidence of EMs in women of childbearing age is as high as 10-15%, the number of patients worldwide is over 200 million, and the incidence rate has increased yearly in recent decades [1, 2]. Previous studies postulated that although the dysmenorrhea, infertility, pain of sexual intercourse, and chronic pelvic pain caused by EMs seriously affects patients' quality of life, it would not endanger their safety [3, 4]. However, recent studies suggest that although EMs is not pathologically malignant, it is still called "benign cancer" because of its biological invasion, local dissemination, uncontrolled cell proliferation, distant metastasis, recurrence, and other biological behaviors similar to malignant tumors [5, 6].

MiR-9-5p is one of the most widely studied miRNAs, and previous studies have confirmed that miR-9 is abnormally expressed in a variety of malignant solid tumor tissues. For example, it is up-regulated in glioma [7], laryngeal squamous cell carcinoma [8], and breast cancer [9], and down-regulated in colorectal cancer [10], colon cancer [11], and gastric cancer [12]. In addition, miR-9 has also been found to promote tumor cell invasion and migration [13, 14]. However, the expression of miR-9-5p and its function in endometriosis patients has been unclear. In the present study, we aimed to

investigate whether the expression levels of miR-9-5p are changed in endometriosis patients, and the effect of miR-9-5p expression on the invasion and migration of endometrial stromal cells in endometriosis patients.

Materials and methods

Tissue and ethics statement

A total of 17 eutopic endometrium patients, 19 ectopic endometrium patients, and 13 normal ectopic patients were recruited to obtain biopsies from 2018 to 2019 in Sanmen People's Hospital of Zhejiang. According to the American Fertility Association (AFS) revised standard staging score (the RAS score): 10 cases were in stage II, 6 cases were in stage III, 3 cases were in stage IV, and 17 cases of lining endometrial paraffin block as the research object, in which the proliferative period 10 For example, the secretory period is 7 cases.

All the patients or their guardians were made aware of the study and signed the informed consent. The ethics committee of Sanmen People's Hospital of Zhejiang reviewed and supervised the study.

Real-time quantitative PCR

We extracted the total RNA tissues using a RNAiso Plus (9108, Takara, Japan). After preparing the cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (RR047A, Takara, Japan), 20 μL of qPCR system was prepared and analyzed as described in the instructions of the GoTaq qPCR Master Mix (A6001, Promega, USA). The qPCR primers were: miR-9-5p forward, 5'-ACACTCCAGCTGGGTCTTTGGTTATGTAGCT-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3'; SIRT1 forward, 5'-TAGCCTTGTCAGATAAGGAAGGA-3' and reverse, 5'-ACAGCTTCACAGTCAACTTTGT-3'. The relative expression of the miRNA or mRNA was calculated by method, and β-actin/U6 was used as a loading control.

Cell transfection

miR-9-5p-NC (5'-AUUGUCCUUAUUCAUUGUCAU-3'), miR-9-5p-mimic (5'-UCUUUGGUUAUCUAGCUGUAUGA-3'), and the miR-9-5p-inhibitor (5'-ACAAACCAAUAGAUCGACAUACU-3') were synthesized by Sangon Biotech Company (Shanghai, China). And 25 nmol miRNAs were directly transfected into 10^6 cells using Lipofectamine® 2000 transfection reagent (11668019, Invitrogen, USA) at 37°C for 72 hours, and we performed the next experiment 72 hours after the transfection after verified the transfection using qPCR.

Western blot

The protein expression levels were determined using western blot as previously described, with GAPDH as a standard control protein [15]. Briefly, total protein from the tissues and cells was extracted using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, pH 7.4), and then the protein concentration was determined with a BCA kit (23225, ThermoFisher, USA). 40 μg total protein was analyzed by 10% SDS-PAGE. After being blocked by membranes with 5% skimmed milk powder at room temperature for 2 hours, the membranes were incubated overnight at 4°C with the following primary antibodies: SIRT1 antibody (8469, 1:1000, Cell Signaling Technology, USA), p65 antibody (8242, 1:1000, Cell Signaling Technology, USA), and p-p65 antibody (3033, 1:500, Cell Signaling Technology, USA).

Cell migration assay

10^6 endometrial stromal cells were seeded in 6-well cell culture plates. After 24 hours, we used tips to make scratches, and then PBS was used to wash the abandoned cells three times. Finally, serum-free DMEM medium was added. The cells were cultured at 37°C in a 5% CO_2 incubator for four days, and pictures were taken.

Cell invasion assay

We used a Transwell chamber (140652, Thermo Fisher, USA) to assess the invasion ability of the endometrial stromal cells. In brief, 3.0×10^3 endometrial stromal cells were inoculated into the upper chamber with the culture medium. Media containing 20% FBS (Gibco, Sacramento, USA) was added into the lower chamber for 24 h at 37°C. Finally, we removed the medium and washed the cells three times with PBS. Next, we added methanol to fix the cells, and we dried them and then fixed them for 30 minutes. After being stained with crystal violet for 20 minutes, the relative invasion was determined using measuring the absorbance at 595 nm.

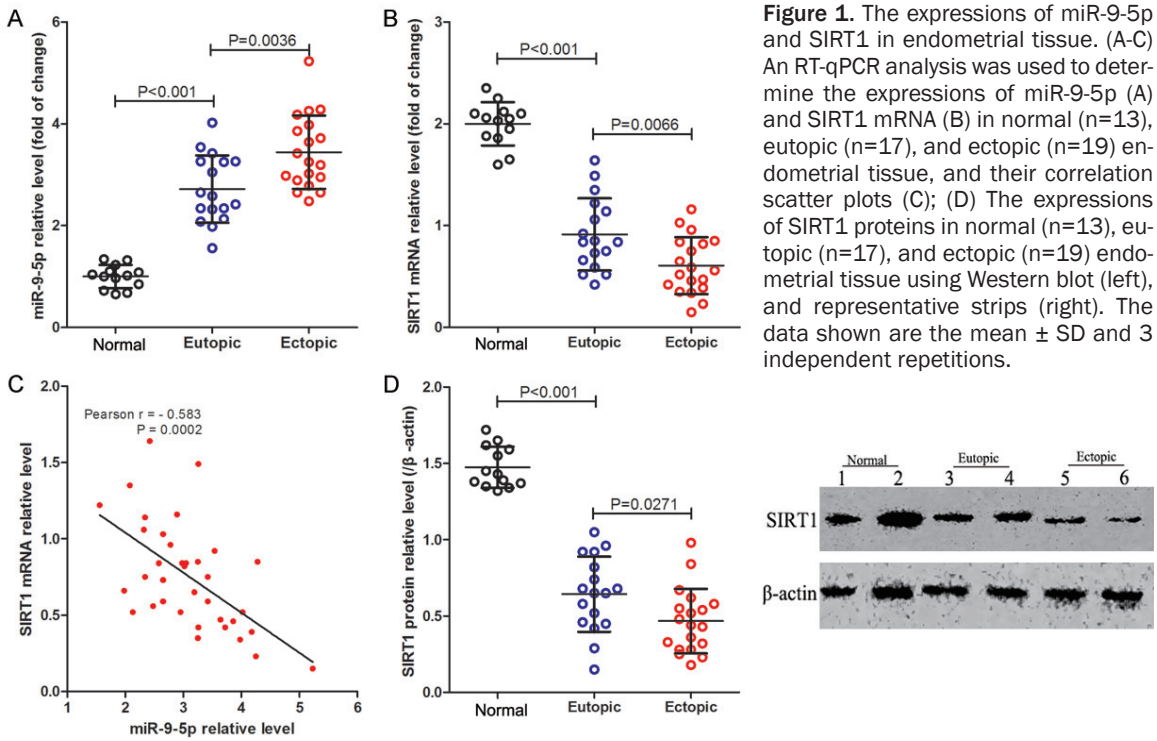

Figure 1. The expressions of miR-9-5p and SIRT1 in endometrial tissue. (A-C) An RT-qPCR analysis was used to determine the expressions of miR-9-5p (A) and SIRT1 mRNA (B) in normal (n=13), eutopic (n=17), and ectopic (n=19) endometrial tissue, and their correlation scatter plots (C); (D) The expressions of SIRT1 proteins in normal (n=13), eutopic (n=17), and ectopic (n=19) endometrial tissue using Western blot (left), and representative strips (right). The data shown are the mean ± SD and 3 independent repetitions.

Statistical analysis

We used GraphPad Prism 5 to analyze the data in this study. The Duncan's test is a valid post-hoc test following ANOVA if there are 3 groups, and Tukey's post-hoc test is a valid post-hoc test following ANOVA if there are >3 groups. The Pearson correlation coefficient was used to analyze the relationships between two groups. A *P* value less than 0.05 indicates a significant difference.

Results

miR-9-5p and SIRT1 expressions in endometriosis patients

A total of 17 eutopic endometrium patients, 19 ectopic endometrium patients, and 13 normal ectopic patients were recruited to obtain biopsies, and we measured their miR-9-5p and SIRT1 mRNA expressions using RT-qCPR. As shown in **Figure 1A**, the expressions of miR-9-5p in endometria of the endometriosis patients were significantly higher than they were in the normal endometria patients, and the miR-9-5p expressions in the ectopic endometria patients were significantly higher than they were in the ectopic endometria patients. For SIRT1 mRNA, we found that (**Figure 1B**) the SIRT1 mRNA expressions in the normal endometria patients were the highest, and in the ectopic endometria patients they were the lowest. We also analyzed the correlation between the miR-9-5p and SIRT1 mRNA expressions in the endometriosis patients and found that (**Figure 1C**) the miR-9-5p expression was negatively correlated to SIRT1 mRNA in the endometriosis patients. Moreover, we used western blot to measure the expressions of the SIRT1 protein in the endometria, and found that (**Figure 1D**) the expression of SIRT1 protein in the endometria of the endometriosis patients was significantly lower than it was in normal endometria, and the SIRT1 protein in the ectopic endometria patients was significantly higher than it was in the ectopic endometria patients.

miR-9-5p targeted the inhibition of the SIRT1 expressions

We analyzed the sequence of miR-9-5p and SIRT1 and found that they have a mutual sequence (**Figure 2A**). As shown in **Figure 2B**, we transferred miR-9-5p-NC, the miR-9-5p-mimics, and the miR-9-5p-inhibitor into endometrial stromal cells and used an RT-qPCR analysis to determine the expression of miR-9-5p and found that the miR-9-5p-mimics successfully increased miR-9-5p expression and the miR-9-5p-inhibitor successfully decreased the miR-9-5p expressions. And the results of the dual

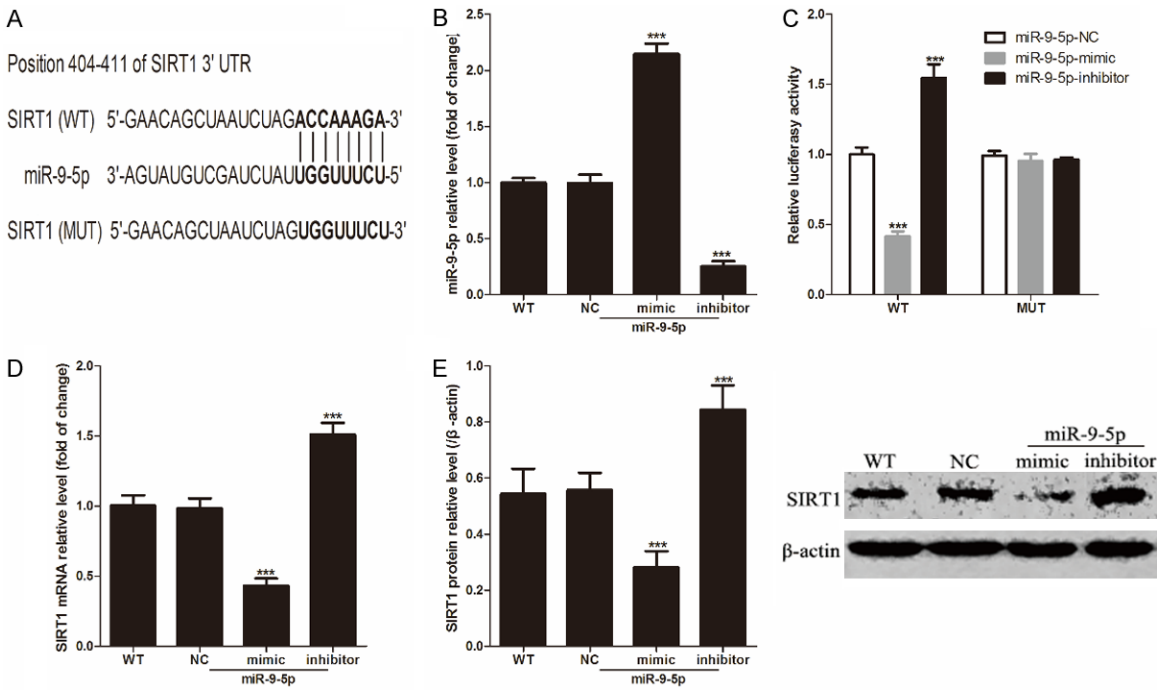

Figure 2. SIRT1 is a target gene of miR-9-5p in endometrial stromal cells. (A) The website predicted that SIRT1 targets miR-134-5p; (B) The expression of miR-9-5p in endometrial stromal cells after miR-9-5p-NC, the miR-9-5p-mimics and miR-9-5p-inhibitor were transferred to the endometrial stromal cells using RT-qPCR; (C) miR-9-5p negatively targets SIRT1 expression in endometrial stromal cells which was determined using a dual luciferase reporter gene system; (D, E) The expression of SIRT1 mRNA (D)/protein (E) in endometrial stromal cells after miR-9-5p-NC, the miR-9-5p-mimics, and the miR-9-5p-inhibitor were transferred to endometrial stromal cells using RT-qPCR. The data are shown as the means ± SD and 3 independent repetitions; *** was P<0.001 vs. the miR-9-5p-NC group.

luciferase reporter gene system suggested that (**Figure 2C**) the miR-9-5p-mimic decreased relative to the luciferase activity, and the miR-9-5p-inhibitor increased the relative luciferase activity, but it did not work in the MUT group. In addition, we also measured the expressions of SIRT1 and found that (**Figure 2D** and **2E**) the miR-9-5p-mimics significantly decreased the SIRT1 expression, and the miR-9-5p-inhibitor significantly increased the SIRT1 expression.

miR-9-5p promotes the metastasis of endometrial stromal cells

First, a cell scratch test was used to assess the migration of the endometrial stromal cells and a Transwell chamber was used to evaluate the invasion of the endometrial stromal cells after regulating the expression of miR-9-5p. As shown in **Figure 3**, the miR-9-5p-mimics significantly increased the distance of the endometrial stromal cell migration and the number of cells that enter into the lower chamber of the Transwell chamber, and the miR-9-5p-inhibitor significantly decreased the distance of the endometrial stromal cell migration and the number of cells that enter into the lower chamber of the Transwell chamber.

miR-9-5p activates the NF-κB pathway in endometrial stromal cells

Resveratrol i a specific activator of SIRT1, and EX572 is a specific inhibitor of SIRT1, so we used them to regulate the expression of SIRT1 in the endometrial stromal cells. As shown in **Figure 4A** and **4B**, resveratrol significantly decreased the expression of the P-p65/p65 protein and EX527 significantly increased the expression of the P-p65/p65 protein. Similarly, the miR-9-5p-mimics played the same function as EX527, which significantly increased the expression of the P-p65/p65 protein (**Figure 4C** and **4D**). The miR-9-5p-inhibitor played the same function as resveratrol, which significantly decreased the expression of the P-p65/p65 protein (**Figure 4C** and **4D**).

Discussion

In this study, we first found that miR-9-5p is highly expressed in the endometria of endome-

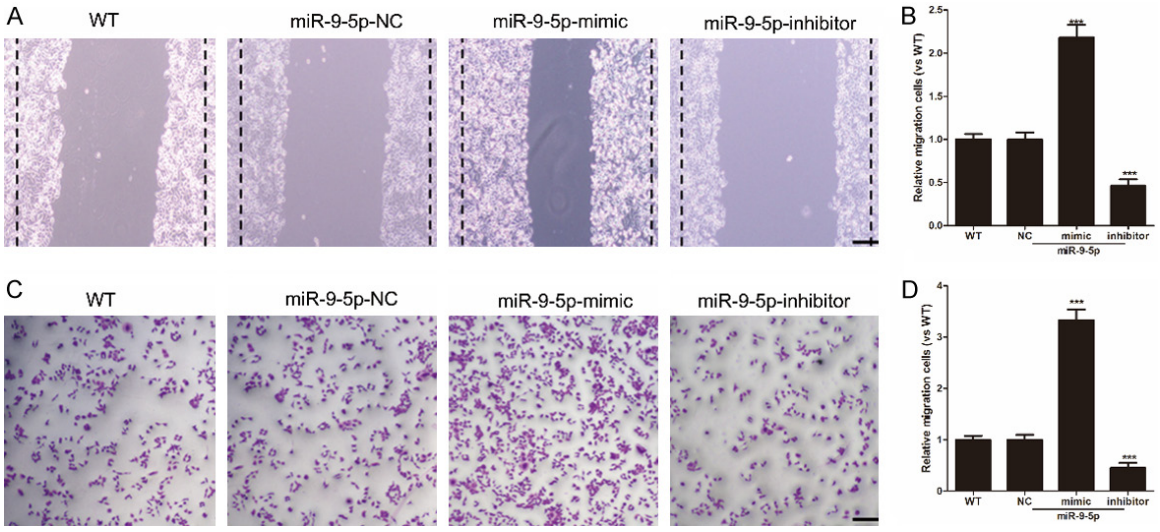

Figure 3. The effect of miR-9-5p expression on the invasion and migration of the endometrial stromal cells. (A, B) Cell scratch tests were used to evaluate the migration of the endometrial stromal cells (A) and a statistical analysis of the scratch distance (B). (C, D) A Transwell chamber was used to assess the number of cells in the migration of the endometrial stromal cells (C) and a statistical analysis of the cells that enter into the lower chamber of the Transwell chamber (D). The data are shown as the means ± SD and 3 independent repetitions; *** was P<0.001 vs. the miR-9-5p-NC group.

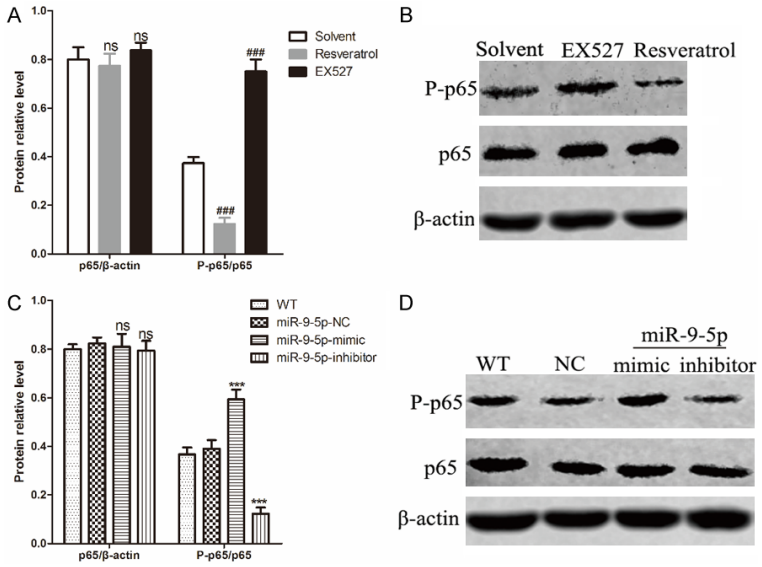

Figure 4. The effect of the miR-9-5p expression on the NF-κB pathway in endometrial stromal cells. A, B. The expressions of the p-p65 and p65 protein after treatment with resveratrol and EX527 using Western blot; C, D. The expression of the p-p65 and p65 protein after treating with resveratrol and EX527 using Western blot after miR-9-5p-NC, the miR-9-5p-mimics, and the miR-9-5p-inhibitor were transferred to the endometrial stromal cells. The data are shown as the mean ± SD and three independent repetitions; ns was no significant vs. the solvent group or the miR-9-5p-NC group, ### was P<0.001 vs. the solvent group and *** was P<0.001 vs. the miR-9-5p-NC group.

triosis patients, and more importantly that the miR-9-5p expression in ectopic endometria is significantly higher than it is in ectopic endometria. In the study of malignant tumors, miR-9-5p has been implicated in the regulation of the invasion and migration of malignant tumor cells, for example, when Liu et al. found that MicroRNA-9 promotes the proliferation, migration, and invasion of breast cancer cells by down-regulating FOXO1 [18] and when Park et al. found that microRNA-9 suppressed cell migration and invasion through the downregulation of TM4SF1 in colorectal cancer [19]. Therefore, we decided to explore the effect of miR-9-5p, a high expression of endometrium in patients with EMs, on the invasion and migration of endometrial-associated cells. There are a large number of endometrial stromal cells and glandular epithelial cells in the human endometrium, and Zeitvogel et al. found that glandular epithelial cells and stromal cells of endometriotic tissue have a similar invasiveness and ability to

grow tumors [20]. Moreover, SAMPSON [21] believed that during normal menstruation, active endometrial debris, growth factors and cytokines can flow back into the pelvic cavity through the fallopian tubes, causing intimal cells to invade and grow in the surrounding tissues, causing EMs, which is the ectopic implant theory of EMs. Therefore, we isolated endometrial stromal cells to study the biological function of miR-9-5p.

As a microRNA, miR-9-5p does not encode a protein, so it must function by regulating the expression of a target gene to exert biological functions. In the present study, we found SIRT1 and miR-9-5p have complementary sequences, and we confirmed that miR-9-5p targets the inhibition of SIRT1 expression using a dual fluorescent gene reporter system in endometrial stromal cells in vitro. And we also found that SIRT1 is lowly expressed in the endometria of endometriosis patients and has a negative correlation to miR-9-5p expression in the endometria of endometriosis patients. These finding indicate that miR-9-5p might function by regulating the expression of the SIRT1 gene in endometriosis patients. Fortunately, previous studies have found that miR-9 and SIRT1 are age-related genes [22], and miR-9 targets the expression of SIRT1 in peripheral blood mononuclear cells [23]. Furthermore, SIRT also has been found to play an important role in the regulation of cancer cell metastasis [24, 25]. Yang, et al. [24] found that SIRT1 inhibits the invasion and migration of ovarian carcinoma cells by inhibiting the epithelial-mesenchymal transition. Zhou P, et al. [25] found that evodiamine inhibits migration and invasion by Sirt1-mediated post-translational modulations in colorectal cancer. Therefore, these data suggest that miR-9-5p might regulate the invasion and migration of endometrial stromal cells by targeting SIRT1.

To further investigate the effect of miR-9-5p expression on the invasion and migration of endometrial stromal cells, we performed cell scratch tests and Transwell chamber invasion tests and found that miR-9-5p not only significantly increases the distance of endometrial stromal cell migration, but it also significantly increases the number of cells that enter into the lower chamber of the Transwell chamber. Moreover, we also found that the miR-9-5p-mimics play the same function as EX527 (a specific inhibitor of SIRT1), and the miR-9-5p-inhibitor plays the same function as resveratrol (a specific activator of SIRT1), namely that miR-9-5p significantly increases the expression of the P-p65/p65 protein and the 65 protein in the nucleus.

P65 is a key protein in the NF-κB pathway, and its degree of phosphorylation/acetylation is positively correlated with the degree of activation of the NF-κB pathway [26]. Previous studies found that NF-κB is abnormally expressed in the endometrial tissues of patients with endometriosis and might be involved in the development of endometriosis [27, 28]. And Xin found that nobiletin alleviates endometriosis by downregulating NF-κB activity in an endometriosis mouse model [29]. More important was that NF-κB was found to play an important role in the regulation of the metastasis of endometrial stromal cells in endometriosis, such as Dai et al. found that MiR-199a attenuates endometrial stromal cell invasiveness through the suppression of the IKK/NF-κB pathway and reduces interleukin-8 expression [30]. And SIRT1 is a histone deacetylase that is widely expressed in human cells, and it carries out important biological functions by deacetylating multiple transcription factors, including the inhibition of the NF-κB signaling pathway activation by inhibiting NF-κB phosphorylation or acetylation [31]. In this study, we found that miR-9 negatively regulates SIRT1 expression and positively regulate p65 expression, meaning that miR-9 positively regulates the NF-κB pathway.

In conclusion, miR-9-5p, a highly expressed microRNA in endometrial of endometriosis patients, might promote the invasion and migration of endometrial stromal cells in endometriosis patients by targeting SIRT1 via the NF-κB pathway.

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Disclosure of conflict of interest

None.

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