The miR-25-3p/Sp1 pathway is dysregulated in ovarian endometriosis

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Abstract

Objective: The transcription factor Specificity protein 1 (Sp1) plays important roles in many critical biological functions; however, its expression and underlying functions in endometriosis remain undefined. Bioinformatics has suggested that Sp1 is potentially regulated by miR-25-3p. This study investigated Sp1 and miR-25-3p expression and their interaction during the pathogenesis of endometriosis.

Methods: Fifteen women with American Fertility Society stage III/IV ovarian endometriosis and 14 disease-free controls were included. Sp1 expression was detected by qPCR, immunohistochemistry, and western blotting. Using both bioinformatics and genetics, we identified that Sp1 was a potential target of miR-25-3p. Then, the relationship between miR-25-3p and Sp1 was investigated by knockdown and overexpression experiments.

Results: Sp1 mRNA and protein levels were increased in ectopic and eutopic endometrium compared with normal endometrium samples, with the highest expression in ectopic endometrium samples. *In vitro* experiments and luciferase reporter assays demonstrated that Sp1 was upregulated when miR-25-3p was depleted and that Sp1 was a direct target of miR-25-3p, respectively.

Conclusions: Our study revealed increased Sp1 expression in ovarian endometriosis and subsequently demonstrated that miR-25-3p directly targets Sp1. This suggests a novel miRNA/Sp1 pathway in the pathogenesis of endometriosis, which should be further explored for other potential therapeutic targets.

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Introduction

Endometriosis is an invasive, yet benign gynecological disorder that is characterized by the implantation and growth of endometrial glands and stroma outside the uterus. It is the most common cause of dysmenorrhea, dyspareunia, pelvic pain, and infertility in reproductive-aged women, and thus negatively impacts women's lives from physical, social, and mental health perspectives.[1–4] Thus, elucidating the pathogenesis and establishing novel and effective treatments for this bothersome disease would have dramatic effects on the lives of many women. The etiology and pathogenesis of endometriosis remains incompletely understood. The retrograde menstruation theory was the first widely accepted hypothesis for the occurrence of endometriosis.[5] However, it could not fully explain why only 10% to 15% of reproductive-age women get endometriosis, despite the phenomenon of retrograde menstruation occurring in 76% to 90% in this population.[6] This suggests that there are molecular factors involved in the pelvic implantation and growth of endometrial tissue that operate independently of retrograde menstruation.

Specificity protein 1 (Sp1) is an important transcription factor that regulates many critical biological functions, including cell proliferation, apoptosis, invasion, and metastasis by binding to the promotor regions of its target genes. Previous studies have demonstrated that Sp1 is aberrantly expressed and plays important roles in cancer by stimulating the growth of tumor cells.[7–9] An imbalance between proliferation and apoptosis has also been proposed to account for the susceptibility of some women for endometriosis.[10,11] However, the expression and role of Sp1 in endometriosis remains unknown.

Epigenetic mechanisms are recognized to be key players in transcriptional regulation, and microRNAs (miRNAs) are one of the most important epigenetic regulators of gene expression. miRNAs are 21 to 23 nucleotide RNA molecules that exert critical regulatory functions by perfect or partial base pairing interactions with the 3′-untranslated region (UTR) of target mRNAs.[12,13] Each miRNA can potentially bind to hundreds of mRNAs and thus post-transcriptionally regulate diverse biological processes.[12–14] The involvement of miRNAs in biological processes is dynamic and tissue/cell specific.[15] Using both bioinformatics and genetics, we identified that miR-25-3p potentially regulated Sp1. miR-25-3p is an important factor that is involved in the proliferation of many tumors.[16] As previously stated, unchecked proliferation is one of the important characteristics of the ectopic endometrium in endometriosis.[17,18] Nevertheless, the expression pattern and underlying role of miR-25-3p in endometriosis is unknown. Furthermore, whether miR-25-3p mediates endometrial growth by regulating Sp1 has not been established. In this study, we investigated the expression patterns and relationship of Sp1 and miR-25-3p in endometriosis in an effort to provide new therapeutic targets for endometriosis.

Methods

Subjects and sample collection

This study was approved by the Institutional Review Board of Xiangya Hospital, Central South University, and written informed consent for participation was obtained from all participants. All participants had regular menstrual cycles and took no steroid hormone medications for at least 3 months before sampling. All tissue samples were confirmed by the phase of the menstrual cycle and histological analysis, which showed that all samples were in the proliferative phase of the menstrual cycle.

Cell culture and transfection

Endometrial stromal cells (ESCs) from endometriosis or disease-free women were isolated using a previously reported protocol,[19] and then suspended in DMEM/F12 1:1 (GIBCO/BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. The media were changed at 48-hour intervals. When the cells reached 80% confluency, they were collected and plated in 24-well dishes and maintained in a humidified 5% CO_2 incubator at 37°C. 293T cells (human embryonic kidney cells) were obtained from the Laboratory for Cancer Proteomics and Translational Medicine of Xiangya Hospital, Central South University, and cultured under the same condition as ESCs. Transfection was performed after 16 to 18 hours when the cells reached 50% confluency. hsa-miR-25-3p mimic, hsa-miR-25-3p inhibitor, and hsa-mir-negative control (NC) were purchased from RiboBio (Guangzhou, China), and the construction and production of lentiviral vectors were performed by Genechem (Shanghai, China). The miR-25-3p mimic or inhibitor was transfected at a final concentration of 100 nM using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), according the protocol recommended by the manufacturer; after a 48-hour incubation, the cells were lysed directly in the wells with TRIzol reagent (Invitrogen) for mRNA isolation. The Sp1 lentiviral expression vector was transduced to establish the Sp1-overexpressing (OE) normal ESC line, whereas the Sp1-knockdown (KD) vector was transfected to establish the Sp1-knockdown eutopic ESC line; 72 hours after transfection, the cells were selected with 4 µg/mL puromycin in the culture medium for >10 days. Transfection efficiency was evaluated by quantitative PCR (qPCR). All experiments were performed in triplicate and repeated three times.

Quantitative real-time RT-PCR

Total RNA was isolated from the cyst walls of ovarian endometriomas, eutopic endometriums, and primary cultured or transfected ESCs using TRIzol reagent (Invitrogen) according the protocol recommended by the manufacturer. The quality and concentration of purified RNA were analyzed with a NanoVue Plus spectrophotometer (Healthcare Bio-ScienceAB, Uppsala, Sweden).

To analyze Sp1 mRNA levels, the extracted RNA was used for cDNA synthesis by reverse transcription. The cDNAs obtained were amplified in triplicate using the SYBR Green real-time RT-PCR kit and reactions were performed on a 7500 Fast Real-Time PCR machine according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Actin was used as endogenous control. The specific primers for amplification were as follows: *SP1*, 5′-GAGGGC AGGGTGCCAATG-3′ (forward) and 5′-TT CTGTAAGTTGGGAGCGGC-3′ (reverse); Actin, 5′-CCTGGCACCCAGCACAAT-3′ (forward) and 5′-GGGCCGGACTCGTCA TAC-3′ (reverse). A blank control was set on each optical plate for each primer, and each sample was analyzed in triplicate. In each set, data are expressed as relative mRNA expression using the $2^{-\Delta\Delta Ct}$ method and are presented as the mean ± SD normalized to the internal control (Actin). All experiments were repeated three times.

To detect miR-25-3p-3p levels, miRNA qRT-PCR was conducted with the Bulge-Loop qRT-PCR primer set (RiboBio), according to the manufacturer's protocol. Specific primers for miR-25-3p were purchased from RiboBio. miRNA expression was normalized to U6 small nuclear RNA (snRNA) levels.

Immunohistochemistry

Immunohistochemical staining was conducted on formalin-fixed paraffin-embedded

endometrial tissues that were sectioned at 4-µm thickness. Each specimen was histologically evaluated with hematoxylin and eosin staining. Sections were deparaffinized, and dehydrated through a series of xylene and ethanol washes. After incubation in 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity, the sections were blocked with 10% normal goat serum for 30 minutes to block non-specific sites. Primary antibodies, including rabbit anti-human Sp1 (Abcam, Cambridge, UK; diluted 1:500) was incubated with the slides in a humidified chamber for 24 hours at 4°C. Tissue sections were then washed with phosphate-buffered saline and incubated with biotinylated or streptavidin/peroxidase-conjugated secondary antibodies for 30 minutes according to the manufacturer's instructions (Beijing Zhongshan Biotech Company, Beijing, China). Sections were counterstained with hematoxylin, and then dehydrated and mounted. Negative control sections were incubated with PBS instead of primary antibody. All slides were analyzed by three blinded observers.

Western blot analysis

Tissue samples were lysed with RIPA buffer (Dingguo, Inc., Beijing, China) for protein extraction. Protein concentration was determined by the BCA assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (10 µg) of total protein was loaded onto SDS–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes, which were prepared by formaldehyde activation for 5 minutes and being lysed in Tris-buffered saline for 10 minutes. Subsequently, the membranes were incubated in blocking buffer (5% non-fat milk) for 1 hour, then immunoblotted overnight at 4°C with primary antibodies against SP1 (Abcam) or GAPDH (Bioleaf, Shanghai, China). After washing the membranes

three times for 15 minutes with TBST [10 mM Tris–HCl (pH 7.4), 0.5 M NaCl, Tween 20 (0.1% v/v)], the membranes were incubated for 90 minutes with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000; Invitrogen), followed by washing the membranes three times for 10 minutes with TBST. Immunoreactive bands were detected by the enhanced chemiluminescence system (Pierce, Rockford, IL, USA) and quantifications were estimated using Image Lab 4.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

In situ hybridization (ISH) for miR-25-3p

The expression pattern of miR-25-3p in ectopic, eutopic, and normal endometrium samples was detected using a miRCURY locked nucleic acid (LNA) miRNA detection probe (Exiqon, Vedbaek, Denmark), following the manufacturer's instructions. A scrambled miRNA probe was used as negative control.

Luciferase reporter assay

A 708-bp fragment of the *SP1* 3′UTR was amplified and cloned into the pmiR-RB-REPORT vector (Promega, Madison, WI, USA), which contained the luciferase reporter system. Site-directed mutagenesis was made at the seed sequence of the miR-25-3p-binding site in the *SP1* 3′UTR. 293T cells were plated into 24-well plates in triplicate at 1.5×10^4 cells/100 µL, and then co-transfected with 100 ng/mL luciferase reporter plasmid containing the *SP1* 3′UTR and 50 nM of miR-25-3p or miRNA-NC (Guangzhou RiboBio). After 48 hours, the cells were harvested, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. All transfections and assays were conducted in triplicate.

Statistical analysis

Results are expressed as mean ± SD, and all statistical analyses were performed with SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). The Student's *t* test was used for comparisons between two groups, and 1-way ANOVA with a post-hoc test was used for multiple comparisons. A 2-tailed *P* value <0.05 was considered statistically significant.

Results

Patient characteristics

In total, 29 subjects aged 21 to 40 years were enrolled, of which 15 were laparoscopically and histologically diagnosed with ovarian endometriosis, and 14 were controls who had an operation *via* laparoscopy or laparotomy because of subserosal or broad ligament myoma and were confirmed to not have endometriosis during the operation. Normal endometrium samples were obtained from the 14 disease-free women; cyst walls of ovarian endometriomas and eutopic endometrium samples were obtained from 15 women with American Fertility Society stage III/IV endometriosis. The mean body mass index of the endometriosis and control groups were comparable (20.2 ± 2.1 vs. $20.9 \pm 1.8 \, kg/m^2$, respectively).

Increased Sp1 expression in ectopic and eutopic endometrium

We used qPCR to investigate *SP1* mRNA levels in endometriosis and found that it was
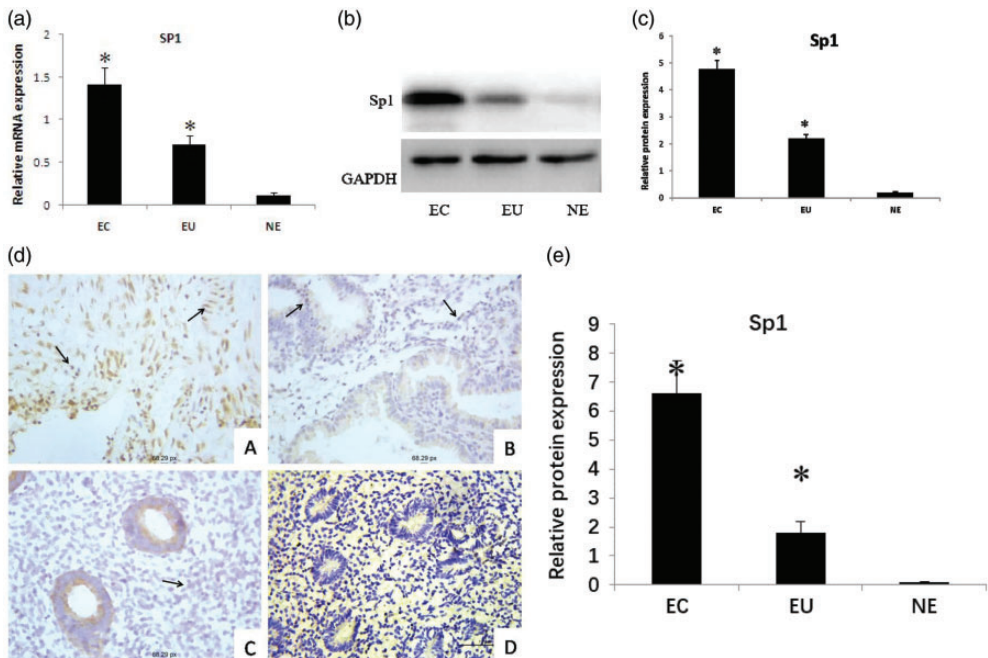
Figure 1. Sp1 expression in ectopic, eutopic, and normal endometrium. a. *SP1* mRNA was expressed at significantly higher levels in ectopic endometrium (EC) and eutopic endometrium (EU) compared with normal endometrium (NE). b & c. Sp1 protein levels were detected by western blotting, which showed significantly higher Sp1 protein levels in EC and EU than in NE. d & e. Immunohistochemistry for Sp1 was performed on sections of EC (A), EU (B), and NE (C). Black arrows indicate positive staining; *$P<0.05$ (Student's *t* test).

significantly increased in both ectopic and eutopic endometrium compared with normal endometrium ($P < 0.05$; Figure 1a). We further measured Sp1 protein levels by western blot, and the results showed higher Sp1 protein expression in disease samples (Figure 1b and c). The spatial distribution of Sp1 was determined by immunohistochemistry, which showed that Sp1 was strongly enriched in the nucleus and cytoplasm of epithelial and stromal cells of ectopic and eutopic endometrium, whereas it was weakly expressed in the nucleus and cytoplasm of normal endometrium (Figure 1d and e). These results suggest that Sp1 is upregulated in endometriosis.
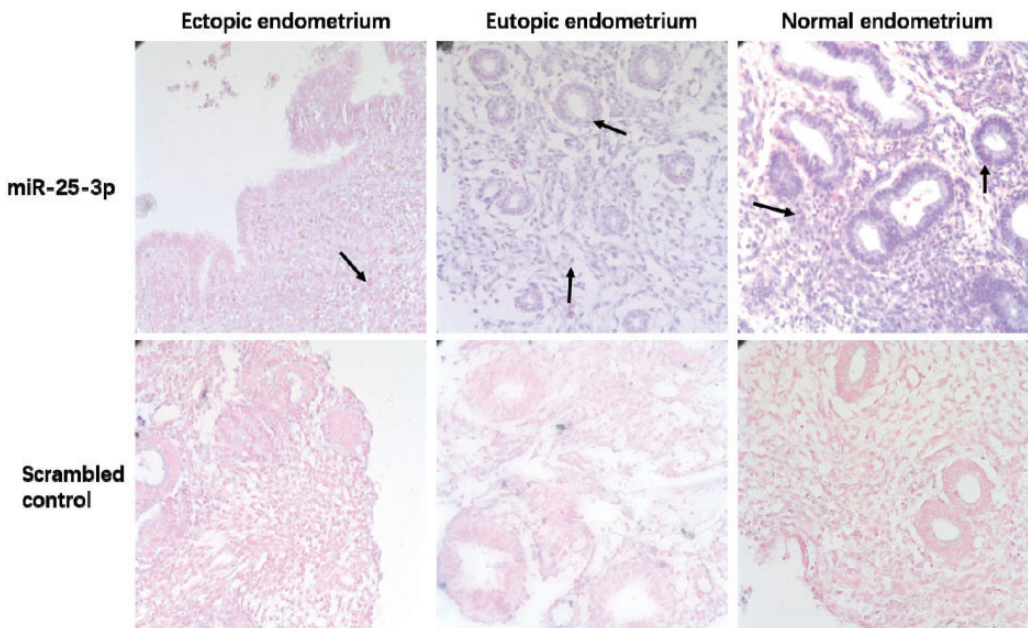
Decreased miR-25-3p expression in eutopic and ectopic endometrium

The levels of miR-25-3p expression were determined in paired eutopic and ectopic endometrium from endometriosis, and

normal endometrium samples. The results demonstrated a significant decrease in miR-25-3p in ectopic and eutopic endometrium compared with normal endometrium ($P < 0.05$; Figure 2).

miR-25-3p regulated Sp1 expression in primary endometrial cells

The impact of miR-25-3p levels on Sp1 expression was determined by transfecting ESCs with miR-25-3p mimic or inhibitor. Transfecting normal ESCs from endometriosis-free women with miR-25-3p inhibitor led to a >90% decrease in miR-25-3p levels and a corresponding 20-fold increased *SP1* mRNA levels ($P < 0.05$; Figure 3a and b). Transfecting eutopic ESCs from endometriosis patients with miR-25-3p mimic increased miR-25-3p by >90%, and repressed *SP1*

Figure 2. miR-25-3p expression in ectopic, eutopic, and normal endometrium. The nuclei of normal endometrial stromal cells and glandular epithelial cells were strongly positive (blue) for miR-25-3p. Staining intensities were reduced in eutopic endometrium and were lowest in ectopic endometrium. The scrambled miRNA probe was used as the negative control. Black arrows indicate positive staining.
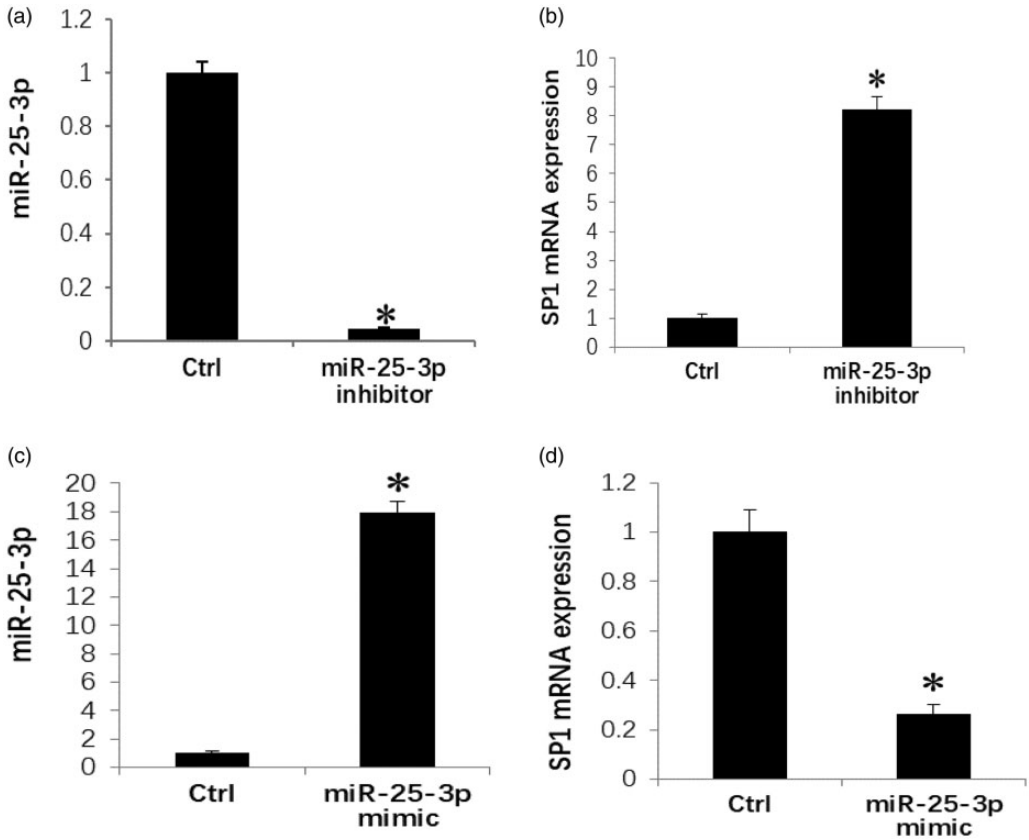
Figure 3. miR-25-3p repressed Sp1 expression in endometrial cells. Transfecting the miR-25-3p inhibitor into normal ESCs or the miR-25-3p mimic into eutopic ESCs showed a >90% transfection efficiency (a and c). *SP1* mRNA levels were significantly increased after transfecting the miR-25-3p inhibitor (b) and were decreased after treatment with the miR-25-3p mimic (d). *$P<0.05$ (Student's t test).

mRNA expression by 6.5-fold ($P < 0.05$; Figure 3c and d).

Sp1 is a direct target of miR-25-3p

We further explored whether *SP1* was a downstream target of miR-25-3p. We found that *SP1* contained potential binding sites for miR-25-3p using TargetScan 7.1 (www.targetscan.org) (Figure 4a). Co-transfecting ESCs with the pmiR-RB-REPORT™ Vector that contained most of the SP1 3′UTR (wild-type) and miR-25-3p showed significantly inhibited luciferase activity compared with controls ($P < 0.05$), whereas there

was no effect when the mutant (Mut) vector was co-transfected (Figure 4b). These data suggest that Sp1 is a direct target of miR-25-3p.

Discussion

Endometriosis affects up to 10% to 15% of reproductive-aged women. While extensive research has been performed in this area, an understanding of the pathogenesis of endometriosis is distinctly lacking. Previous evidence suggests that the attachment, proliferation, and invasion of endometrial cells from retrograde menstruation into
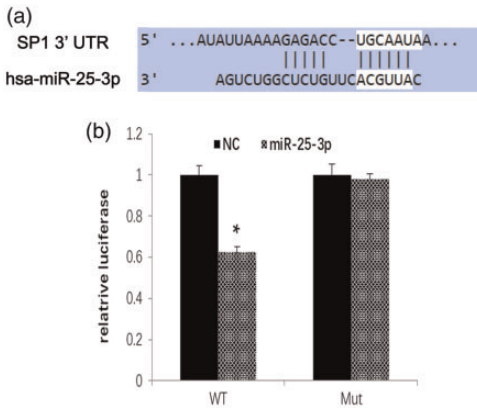
Figure 4. Sp1 is a target of miR-25-3p. a. SP1 contained potential binding sites for miR-25-3p. b. 293T cells were co-transfected with miR-25-3p or control (NC) mimics and wild-type (WT) or mutant (Mut) SP1 3′-UTR in the luciferase reporter vector. Dual luciferase activity was measured 48 hours later. The presented data are the summary of four independent experiments; *P<0.05 compared with control.

pelvic tissues results in endometriotic lesions,[10,11,20] which opened the door for us to investigate factors that regulate these processes in endometriosis.

Dysregulated expression of the transcriptional regulatory factor Sp1 has been found in various human cancers.[21,22] By interacting with several nuclear proteins, Sp1 is involved in many biological processes, including cell proliferation, apoptosis, differentiation, and angiogenesis.[23,24] Our study demonstrated that Sp1 expression was significantly elevated in paired ectopic and eutopic endometrium compared with normal endometrium, with the expression highest in ectopic endometrium. These data indicated a potential role for Sp1 in endometriosis that facilitates endometrial tissues proliferating in the pelvis following retrograde menstruation.

Dysregulated miRNA expression has been identified in ectopic endometrium, which suggests it has vital roles in endometriosis.[25–27] However, few studies have investigated miR-25-3p expression or its role in endometriosis. In this study, we found that miR-25-3p expression was lower in paired ectopic and eutopic endometrium than in normal endometrium, and was lowest in ectopic endometrium. This was in contrast to Sp1, which had high levels in ectopic endometrium, intermediate levels in eutopic endometrium, and low levels in normal endometrium. Thus, miR-25-3p and Sp1 showed opposing expression patterns in endometriosis, which suggests a negative post-transcriptional relationship between these two transcripts during the development and progression of endometriosis. To investigate the potential molecular mechanism through which miR-25-3p regulates Sp1, we used bioinformatics analysis, and over-expression and depletion experiments. Sp1 expression was decreased *in vitro* when miR-25-3p was overexpressed and increased when miR-25-3p was depleted. Thus, Sp1 is dynamically controlled by miR-25-3p expression. Luciferase assays verified direct base pairing between miR-25-3p and the *SP1* 3′UTR. These experiments demonstrated that Sp1 promotes the proliferation of ectopic endometrium and is directly regulated by miR-25-3p, which indicates that Sp1 is a potential therapeutic target for endometriosis.

This was the first study to reveal high Sp1 expression and low miR-25-3p expression in paired ectopic and eutopic endometrium compared with normal endometrium. Further experiments showed that Sp1 was directly regulated by miR-25-3p, which highlights a novel miRNA-based Sp1 regulatory pathway in endometriosis. However, this study was not without limitations. The total number of cases was relatively small, and the potential biological function of Sp1 and miRNAs in endometriosis remains unclear. Additional investigations will be needed to further study the biological processes in endometriosis that are controlled

by Sp1, which will gain new insight for exploring novel therapies.

Authors' contributions

Licong Shen designed and performed the experiments, analyzed the data, and wrote the manuscript. Xiaxia Hong and Yang Liu collected samples and performed the qPCR, immunohistochemistry, and western blot assays. Wenjun Zhou contributed to cell culture and transfections. Yi Zhang designed the experiments, analyzed data, and revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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