

miRNA-15a-5p regulates VEGFA in endometrial mesenchymal stem cells and contributes to the pathogenesis of endometriosis

X.-J. LIU¹, X.-G. BAI², Y.-L. TENG¹, L. SONG³, N. LU⁴, R.-Q. YANG⁵

¹Department of Pathology, the First Hospital of Jilin University, Changchun City, Jilin Province, China

²Department of Neurosurgery, People's Hospital of Jilin Province, Changchun City, Jilin Province, China

³Department of Respiratory, the First Hospital of Jilin University, Changchun City, Jilin Province, China

⁴Department of Cardiovascular, the First Hospital of Jilin University, Changchun City, Jilin Province, China

⁵Department of Obstetrics and Gynecology, the Second Hospital of Jilin University, Changchun City, Jilin Province, China

Abstract. – OBJECTIVE: miRNAs have been recently reported to contribute to the etiology of endometriosis in stem cells. However, the mechanisms remain unclear.

The aim of this investigation is to explore the expression of miR-15a-5p and VEGFA in endometrial samples from patients with or without endometriosis. And then examine the regulation by miR-15a-5p on the expression of VEGFA.

PATIENTS AND METHODS: Here we collected 31 endometrial samples from patients with or without endometriosis and characterized the miRNAs expression profiles of these two groups. Then, we investigated the regulation by miR-15a-5p (miR-15a-5p) on the expression of vascular endothelial growth factor (VEGF) in endometrial mesenchymal stem cells.

RESULTS: It was demonstrated that there was dramatically down-regulation of miR-15a-5p in the patients with endometriosis, compared with control patients. Moreover, we found that the up-regulation of miR-15a-5p suppressed cell proliferation, migration and invasion of these ectopic stem cells by targeting the 3' untranslated region of VEGFA.

CONCLUSIONS: Taken together, this newly identified miR-15a-5p module provides a new avenue to the understanding of the processes of endometriosis development, especially proliferation, motility as well as angiogenesis, and may facilitate the development of potential therapeutics against endometriosis.

Key Words

Endometriosis, VEGFA, miR-15a-5p.

Introduction

Endometriosis is a chronic benign disease defined as the presence of endometriosis tissues in extra-uterine locations, and the endometriosis tis-

suces could implant to various organs such like pelvic peritoneum, ureters and bladder^{1,2}. The main symptoms of endometriosis are chronic pelvic pain, infertility, dysmenorrhea and dyspareunia, affects approximately 3%-10% of women in reproductive age^{3,4}. There are several hypotheses about the cause of endometriosis-like retrograde menstruation, coelomic metaplasia and transplantation⁵, but the pathophysiology of endometriosis is still unknown. Some papers about hypothesis-free, genome-wide have provided new sights into potent avenue leading to the development of endometriosis⁶. While it had been reported that many molecules were closely related to endometriosis, for example, killer immunoglobulin-like receptors (KIR)⁷, nuclear receptors (NRs)⁸, CA125⁹, placental protein14¹⁰ and MUC17 polymorphisms¹¹ and miRNAs¹².

Vascular endothelial growth factor (VEGF) is a member of VEGFA family, coding by a 28 kb-long gene which is located on chromosome 6p21.3¹³. It is known as a crucial regulator of angiogenesis, endothelial cells growth and migration^{14,15}. A lot of studies have reported that VEGFA plays an important role in the angiogenesis of various cancers¹⁶⁻²², endometriosis, cardiovascular disease, spermatogenesis^{23,24}, rheumatoid arthritis or macular degeneration¹³. Arsenic trioxide suppresses the expression of VEGFA by up-regulating of Ets-2 and miRNA-126²⁵. miR-93 inhibits the cells proliferation by targeting the mRNA of VEGFA²⁶. So it indicated that miRNAs play a role in modulating the expression of VEGFA and it is a potential factor to VEGFA. It also has been reported that mRNA contributes to the pathology of endometriosis²⁷⁻³⁰, it is a potential biomarker for endometriosis, but the mechanisms remains unclear.

In this work, we investigated the expression level of VEGFA and miR-15a-5p in eutopic and ectopic endometriosis tissues, analyzed the correlation of VEGFA and miR-15a-5p, then determined in details the function and mechanism of miR-15a-5p on the proliferation, migration and invasion of endometriosis stromal cells (ESCs). The study implied the function of miR-15a-5p in pathology of endometriosis, and clarified the reaction mechanisms.

Patients and Methods

Endometriosis tissues, cell culture and transfection

31 endometriosis patients were diagnosed by laparoscopic surgical examination, and undergone surgical excision of endometriosis tissues. The control tissues were collected from 31 premenopausal patients without endometriosis. All these patients had not received any pre-operative hormonal therapy or taken any medicine for at least three months. The resected tissues were minced and stored at -80°C before the total RNA and protein extraction. Written consent was obtained from each patient before the study. This study was approved by the Research Ethics Committee of First Hospital of Jilin University, China.

The endometriosis stromal cells (ESCs) were bought from American Type Culture Collection (ATCC) (Rockville, MD, USA), and were cultured in DMED/F12 (Gibco, Rockville, MD, USA), which was supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 20 nM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 100 $\mu\text{g}/\text{ml}$ of streptomycin and 100 U/ml of penicillin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C 5% CO_2 . ESCs were seeded into a 24-well plate and transfected with the miR-15a-5p mimics or control miRNAs by Lipofectamine[®] 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative by RT-PCR

To investigate the expression level of VEGFA mRNA and miR-15a-5p, we extracted the total RNA from endometriosis tissues or ESCs. Tissues or cells were lysed with the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's specification. mRNAs were dissolved in RNase-free water and

stored at -70°C before utilize. The quantitative analysis of RNA was performed by RT-PCR using One Step SYBR PrimeScript PLUS RT-PCT Kit (Takara, Tokyo, Japan) for each sample according to the manufacturer's manual. After the reaction, the VEGFA mRNA level and the miR-15a-5p level was calculated and presented as the relative level of VEGFA to β -actin (as control) by $\Delta\Delta\text{Ct}$ method, each sample was measured for three independent experiments.

Western Blot Analysis

Western blot analysis was performed to determine the expression of VEGFA on the protein level. Harvested the ESCs post transfecting, and lysed the cells into cell lysis buffer (Bio-Rad, Hercules, CA, USA). Centrifuged the samples at $12,000 \times g$ for 15 min at 4°C and collected the supernatant. Protein extracts were boiled in SDS/ β -mercaptoethanol and separated in a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Then, transferred the protein sample to a nitrocellulose membrane (Millipore, Bedford, MA, USA), blocked with 5% skimmed milk powder overnight at 4°C . The membrane was incubated with VEGFA-specific antibody in TBST (mouse monoclonal antibody, Abcam, Cambridge, UK, 1:500) at 37°C for 1 h, washed with TBST, next incubated with the HRP-linked secondary anti-mouse antibody (New England Biolabs, Ipswich, UK) for 30 min at 37°C . ECL kit (Life Science, Woodland Hills, CA, USA) was used to carry out the chemiluminescence reaction. The membrane was scanned by a Smart Chemi[™] lamp Analysis System (Thermo Scientific, Rockford, IL, USA) and quantified according to the band density by Quantity One software with β -actin as loading control.

Dual luciferase assay

The sequence of 3' UTR of VEGFA from Homo sapiens, Mus musculus, Rattus Norvegicus and Homo sapiens miR-15a-5p (hs-miR-15a-5p) were download from Genbank (NCBI), aligned by Megalign (DNASTAR) (GATC Biotech, Konstanz, Germany). The sequence of mutant 3' UTR of VEGFA was synthesized by Sangon Biotech (Shanghai, China). All the sequences in this study were amplified by PCR (polymerase chain reaction) with Phusion polymerase (New England Biolabs, Ipswich, MA, USA). 3' UTR of VEGFA and the mutant 3' UTR of VEGFA were cloned into the upstream

of firefly luciferase (Fluc) gene behind the Cytomegalovirus promoter in pmirGLO (Promega, Madison, WI, USA) with restriction endonuclease and DNA ligase (New England Biolabs, Ipswich, MA, USA), received VEGFA-Fluc and mutant-VEGFA-Fluc recombinant plasmid. The ESCs seeded in a 24-well plate were co-transfected with the miR-15a-5p mimics/miR-Ctrl and VEGFA-Fluc/mutant-VEGFA-Fluc by Lipofectamine® 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). 48 hours after transfecting, collected the cells and assayed with the Dual-Luciferase Assay kit (Promega, Madison, WI, USA) used GLOMAX (Promega, Madison, WI, USA) and recorded data, the relative luciferase activity (%) represented the expression level of VEGFA.

Cell proliferation, migration and invasion assay

ESCs were transfected with miR-15a-5p mimics or miR-Ctrl with an initial inoculation of 10^3 /ml cells, were counted the ESCs number at 12, 24, or 48 hours post transfecting, and were curved the changing trend of the cell number to illustrate the proliferative capability of the transfected cells.

The cells migration assay was performed with a typical transwell assay (Costar, 6.5 mm diameter, 8 μ m pore size) (Costar, Lowell, MA, USA). The top chamber was seeded with a number of 10^4 /ml cells in 200 μ l serum-free medium, meanwhile, 500 μ l medium with high concentration of serum was injected into the bottom and incubated at 37 °C 5% CO₂ for 12 hours. Put the filter into 4% PFA for 15 min and removed the cells on the upper surface with a cotton swab, then stained the cells on the bottom and calculated the cell number per field. The protocol of invasion assay was almost the same to migration assay, unless the membrane were treated with 24 μ g/ μ l matrigel (R&D system China, Shanghai, China) before cells seeded and cultured at 37 °C 5% CO₂ for 8 hours. The cell number was calculated in the same way.

Statistical Analysis

The statistical analysis was performed using the GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The VEGFA on mRNA level and miR-15a-5p expression level between eutopic and ectopic endometriosis tissues were analyzed by Student's *t*-test. Statistical significance was considered when *p*-value < 0.05 or less.

Results

Negative association of VEGFA level with miR-15a-5p in Ectopic endometrial tissues

To investigate the express level of VEGFA in endometriosis, we examined the mRNA level of VEGFA and miR-15a-5p level by quantitative RT-PCR in 31 eutopic endometriosis tissues and 31 ectopic endometriosis tissues. It had shown that the relative mRNA level of VEGFA was significantly up-regulated in ectopic endometriosis tissues than in eutopic endometriosis tissues (Figure 1A, *p* < 0.0001), with β -actin as an internal control. The relative level of miR-15a-5p was reduced in ectopic endometriosis tissues (Figure 1B, *p* < 0.0001), with U6 as internal control. By analyzing the correlation of relative VEGFA mRNA level with the miR-15a-5p level in ectopic endometrial tissues, we found an inverse correlation between them. The high mRNA level of VEGFA accompanied a low expression level of miR-15a-5p (Pearson correlation, $R^2 = 0.2961$, *p* = 0.0016), as shown in Figure 1C, miR-15a-5p had a potential in modulating the expression level of VEGFA.

Transfection with miR-15a-5p mimics inhibits the expression of VEGFA in both mRNA and protein levels in human endometrial stromal cells (ESCs)

To further determined whether the expression of VEGFA was down-regulated by miR-15a-5p, we transfected a miR-15a-5p mimics into endometriosis stromal cells (ESCs); then, we examined the relative level of miR-15a-5p and the expression of VEGFA in both mRNA and protein levels after 48 hours' transfection. The level of miR-15a-5p was significantly increased after the transfection with 30 nm or 60 nm miR-15a-5p mimics (Figure 2A, *p* < 0.0001), compared with the control miRNA (miR Ctrl). However, Figure 2B demonstrated that the mRNA level of VEGFA was reduced post the transfection with 30 nm or 60 nm miR-15a-5p mimics (*p* < 0.01). Western blot analysis was used to examine the expression of VEGFA at the protein level, with β -actin as an internal control (Figure 2C). The expression level of VEGFA was decreased in ESCs transfected with miR-15a-5p mimics rather than miR Ctrl. The band intensity of VEGFA was down-regulated in ESCs transfected with miR-15a-5p mimics (Figure 2D, *p*

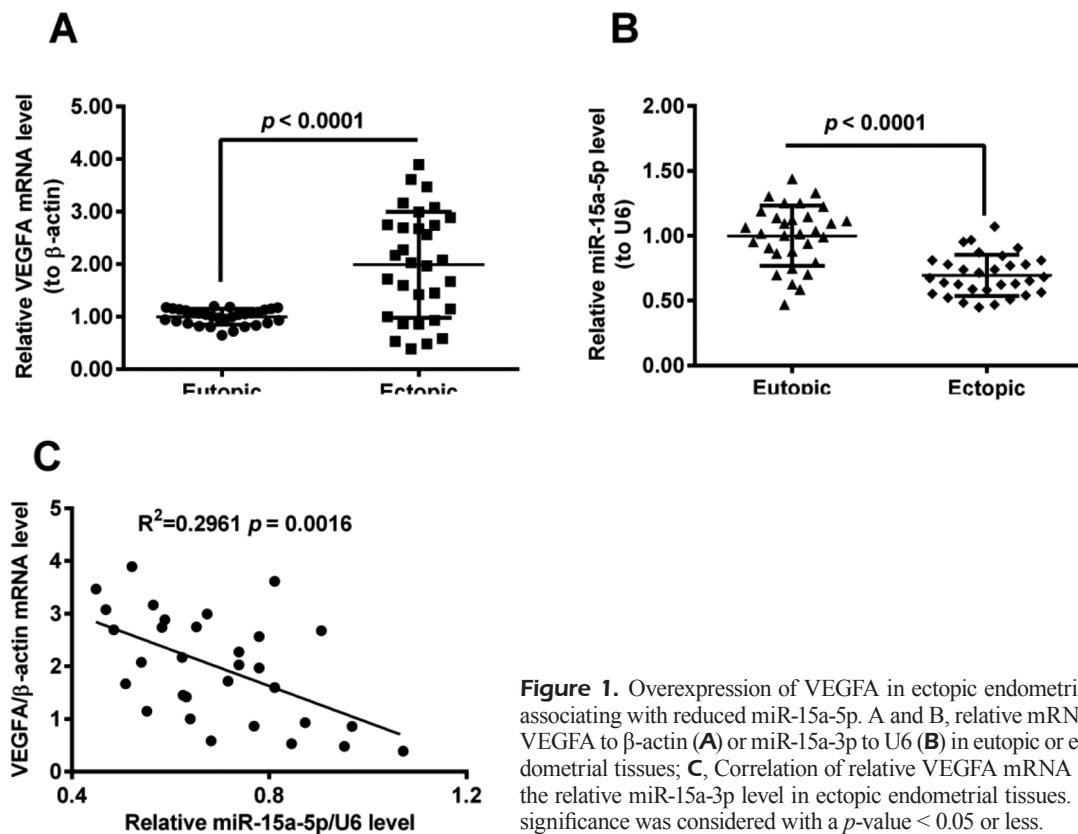


Figure 1. Overexpression of VEGFA in ectopic endometrial tissues, associating with reduced miR-15a-5p. A and B, relative mRNA level of VEGFA to β -actin (A) or miR-15a-3p to U6 (B) in eutopic or ectopic endometrial tissues; C, Correlation of relative VEGFA mRNA level with the relative miR-15a-3p level in ectopic endometrial tissues. Statistical significance was considered with a p -value < 0.05 or less.

< 0.01). Moreover, the relative level of VEGFA was much lower in ESCs transfected with 60 nM miR-15a-5p mimics than those transfected with 30 nM miR-15a-5p mimics. Therefore, miR-15a-5p suppressed the expression of VEGFA in both mRNA and protein levels in ESCs.

miR-15a-5p targets the 3' UTR of VEGFA gene

It has been reported that miR-93 could repressed MMP3 or VEGFA expression by targeting 3' UTR of MMP3 mRNA or VEGFA mRNA directly²⁶. So, we suspected that miR-15a-5p inhibited the expression of VEGFA by targeting the 3' UTR of *VEGFA*. To verify this hypothesis, we aligned the consensus target sequence with 3' UTR of *VEGFA* from Homo sapiens, *Mus musculus* and *Rattus Norvegicus* with Homo sapiens miR-15a-5p (hs-miR-15a-5p), the sequence was listed in Figure 3A. To further determine the function of this consensus sequence, a luciferase reporter was linked with the 3' UTR of *VEGFA*, and a reverse sequence of 3' UTR of *VEGFA* was utilized as control (Figure 3B). miR-15a-5p mimics and

miR-Ctrl were transfected into ESCs which already had been transfected with the 3' UTR of *VEGFA* linked a luciferase reporter. The results showed that the relative luciferase activity was significantly decreased post transfecting 30 or 60 nM miR-15a-5p mimics (Figure 3C, $p < 0.001$). However, there was no significant difference in the relative luciferase activity between miR-15a-5p mimics and miR-Ctrl in the ESCs, which were transfected with the reporter with the mutant 3' UTR of *VEGFA* (Figure 3D). All these data demonstrates that miR-15a-5p targets the 3' UTR of *VEGFA* gene and inhibits the VEGFA expression effectively.

Transfection with miR-15a-5p mimics inhibits the growth and migration of ESC cells

miR-15a-5p could modulate the level of VEGFA, which is a regulator of cell growth and migration, so miR-15a-5p may also influence growth and migration of ESCs. Then, we curved the ESCs number at 0, 12, 24 and 48 hours after transfecting with miR-Ctrl and miR-15a-5p mimics; Figure 4A showed that the proliferation

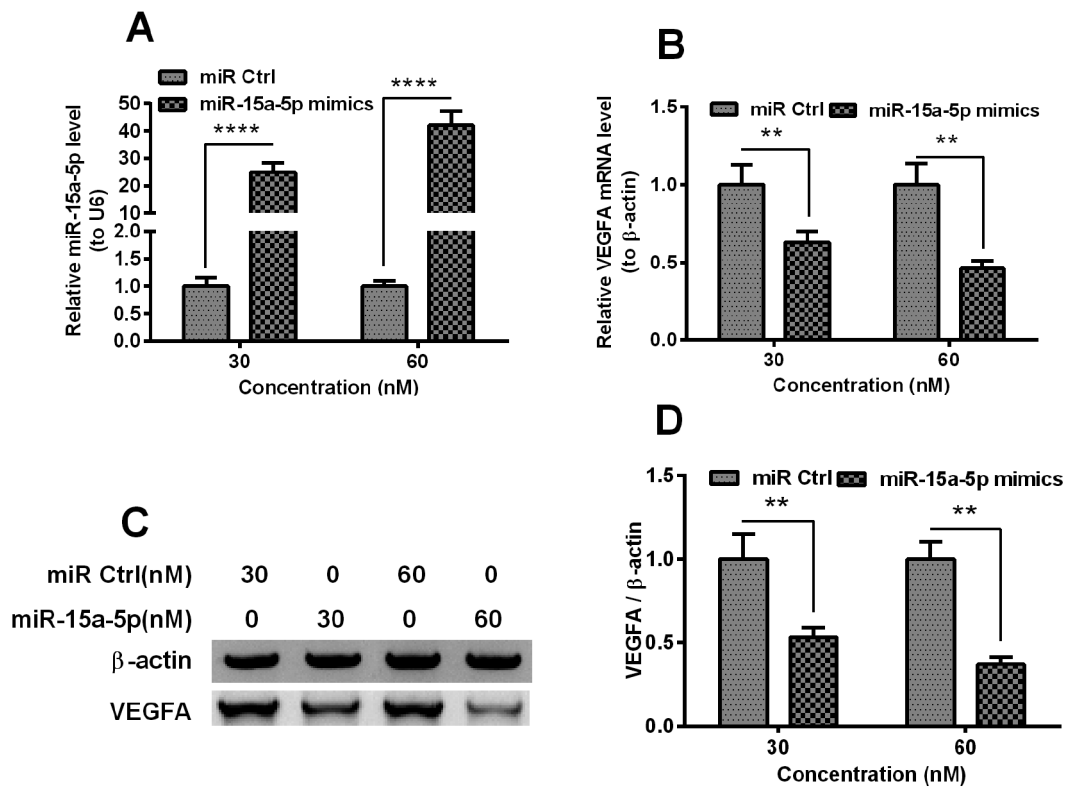


Figure 2. Transfection with miR-15a-5p mimics inhibits the expression of VEGFA in both mRNA and protein levels in human endometrial stromal cells (ESCs). **A**, miR-15a-5p level in ESCs cells, which were transfected with 30 or 60 nM miR-15a-5p mimics or control miRNA (miR-Ctrl) for 12 h; **B**, mRNA level of VEGFA in the miR-15a-5p mimics- or miR-Ctrl-transfected ESCs cells (30 or 60 nM for 12 h); **C** and **D**, Western blot analysis of VEGFA in the miR-15a-5p mimics- or miR-Ctrl-transfected ESCs cells (30 or 60 nM for 24 h), with β -actin as control. Each experiment was repeated in triplicate independently. (**) $p < 0.01$, (****) $p < 0.0001$.

ability of ESCs was significantly reduced post the transfection with miR-15a-5p mimics ($p < 0.01$ respectively for 24 or 48 hours). To further explore the ability of miR-15a-5p on cell migration and invasion, typical transwell assay was performed; and results indicated that the number of migratory cells was significantly reduced after the transfection with miR-15a-5p mimics ($p < 0.01$ respectively, Figure 4B). Meanwhile, the invaded cells were also reduced post the transfection with miR-15a-5p mimics, compared with the control group ($p < 0.05$ or $p < 0.01$, Figure 4C). Therefore, miR-15a-5p mimics inhibits the growth, migration and invasion of ESC cells.

Discussion

VEGFA is a dimeric glycoprotein that plays an important role in vasculogenesis, the overexpression of VEGFA often occurred in cancer²⁰. In this study, we compared the expression

level of VEGFA in eutopic and ectopic endometriosis tissues. The immunofluorescence staining images implied that the VEGFA was overexpressed in ectopic endometriosis tissues. It had reported that miRNAs could regulate the expression of VEGFA²⁰. In the present work, we firstly found that VEGFA mRNA level was significantly increased while the level of miR-15a-5p was markedly decreased in ectopic endometriosis tissues. The correlation analysis showed that the VEGFA mRNA level was inversely correlated with miR-15a-5p. Moreover, the *in vitro* experiments demonstrated that miR-15a-5p markedly downregulated the expression of VEGFA on both mRNA and protein levels. All these data indicated a potential role of miR-15a-5p in inhibiting expression of VEGFA.

It had reported that miR-93 repressed MMP3 or VEGFA by targeting the 3' UTR of the MMP3 mRNA or VEGFA mRNA²⁶. Accordingly, we generated sequences of VEGFA 3' UTR

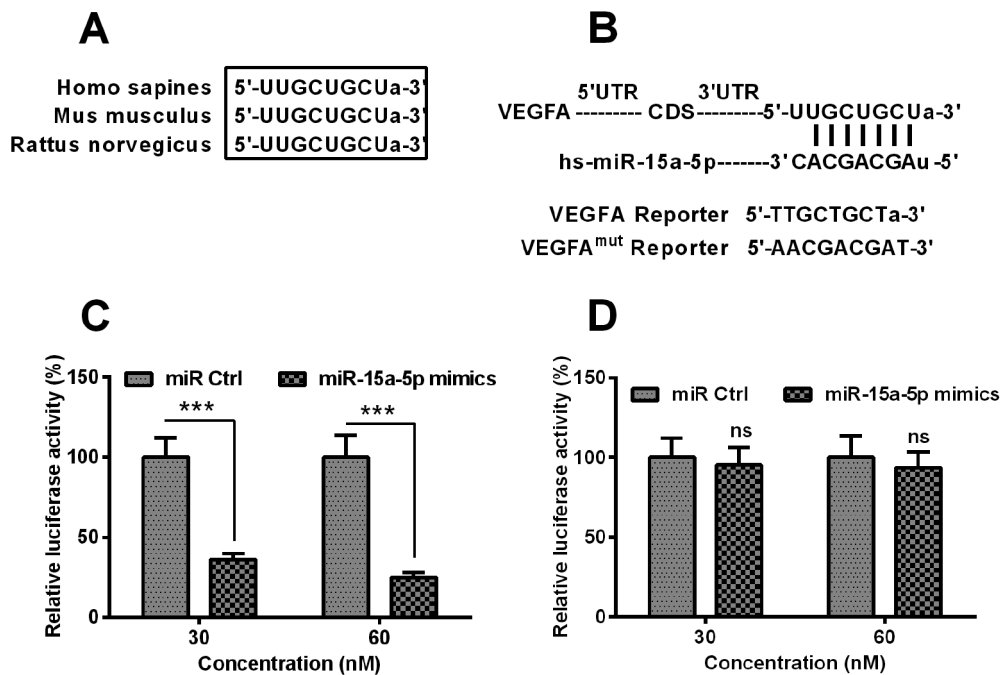


Figure 3. miR-15a-5p targets the 3' UTR of VEGFA gene and inhibits the VEGFA expression. **A**, Alignment of the consensus target sequence with VEGFA 3' UTR from *Homo sapiens*, *Mus musculus* and *Rattus Norvegicus* with Homo sapiens miR-15a-5p (hs-miR-15a-5p); **B**, Sketch of a luciferase reporter with the VEGFA 3' UTR. The VEGFA 3' UTR or the mutant VEGFA 3' UTR was inserted behind the Cytomegalovirus promoter; **C** and **D**, Influence of miR-15a-5p mimics or miR-Ctrl transfection on the relative luciferase activity in the ESCs which were transfected with the reporter with the 3' UTR of VEGFA (**C**) or with the mutant 3' UTR of VEGFA (**D**) with renilla luciferase as internal control; Each data was averaged for triple independent results. (ns) no significance, (***) $p < 0.001$.

from *Homo sapiens*, *Mus musculus* and *Rattus Norvegicus*, and aligned the consensus target sequence with Homo sapiens miR-15a-5p (hs-miR-15a-5p). We found the consensus sequence, 5'-UUUGCUGCUA-3'. And we confirmed the specific binding between miR-15a-5p and the 3' UTR of VEGFA gene, via this consensus sequence. The dual luciferase assay with the reporter with 3' UTR of VEGFA showed a significant downregulation by miR-15a-5p than miR-Ctrl, whereas there was no difference between the two miRNA mimics in the luciferase reporting assay with the reporter plasmid with mutant 3' UTR of VEGFA. Therefore, miR-15a-5p targets the 3' UTR of VEGFA mRNA and suppresses the VEGFA expression.

Endometriosis is a disease presence as tissues outside the uterus, which means the ESCs transferred to other sites, such as pelvic peritoneum³ and urinary tract². miRNAs are a regulator of gene expression in various biochemical processes. Du et al³¹ revealed that miR-195 inhibits the proliferation, migration and invasion of cervical

cancer. miR-15a-5p mimics were utilized to verify the role of miR-15a-5p on ESCs proliferation and migration. The growth rate was slow down after cells transfected with miR-15a-5p mimics, and the migration cells or invasion cells was reduced in ESCs which were transfected with miR-15a-5p compared to the control. Therefore, miR-15a-5p suppressed the ability of cell proliferation, migration and invasion.

Conclusions

VEGFA was overexpressed in ectopic endometriosis tissues, and expression level of VEGFA was negatively related with miR-15a-5p which inhibits the expression of VEGFA by targeting VEGFA mRNA 3' UTR directly. These results imply that miR-15a-5p might regulate VEGFA in endometrial mesenchymal stem cells and might contribute to the pathogenesis of endometriosis. This study may provide a potential biomarker for endometriosis therapeutics.

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Conflict of Interests

The authors report no declaration of interests. The authors alone are responsible for the content and writing of the paper.

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