Original Article
MiR-424-5p regulates proliferation and apoptosis by targeting FGFR1 in endometriosis cells

Wenwu Yang, Li Hong, Xuexian Xu, Qin Wang, Jinling Huang, Li Jiang

Department of Gynaecology and Obstetrics, Renmin Hospital of Wuhan University, Wuhan 430060, P. R. China

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Abstract: Objective: Endometriosis is a chronic disease that influences approximately 5-15% of women of reproductive age. Some studies have confirmed that numerous microRNAs (miRNAs) are unconventionally expressed in the ectopic endometrium. However, the accurate pathological mechanism of the effect of miRNAs on endometriosis is not completely clear. The aim of this study was to investigate the functional role of miR-424-5p in endometriosis. Methods: The levels of the miR-424-5p were detected by qRT-PCR. The protein levels of fibroblast growth factor receptor1 (FGFR1) and signal transducer and activator of transcription-3 (STAT3) were measured by western blot. Online software and luciferase reporter assay system were used to predict and verify the targets of miR-425-5p. Cell proliferation and apoptosis were examined in vitro by MTT and apoptosis assay. Results: The expression level of miR-424-5p was significantly downregulated, and the level of FGFR1 was significantly upregulated in the tissues from patients with endometriosis compared with normal endometrium. MiR-424-5p directly targeted FGFR1 and effectively inhibited FGFR1 expression in endometriosis cell line CRL-7566. In addition, miR-424-5p significantly suppressed proliferation and promoted apoptosis of CRL-7566 cells, whereas FGFR1 overexpression reversed the effect of miR-424-5p on CRL-7566 cells. Moreover, miR-424-5p reduced pSTAT3 expression, while FGFR1 overexpression attenuated the inhibitory effect of miR-424-5p on pSTAT3 expression in CRL-7566 cells. Conclusion: miR-424-5p is down-regulated in the ectopic endometrium and regulates endometriosis cell proliferation and apoptosis through STAT3 signaling by targeting FGFR1.

Keywords: MiR-424-5p, endometriosis, FGFR1, STAT3

Introduction
Endometriosis is a common and intricate gynecological disorder with the existence and development of endometrial tissue and stromal cells outside the uterus, which causes dysmenorrhea, dyspareunia, severe chronic pelvic pain and infertility [1, 2]. The progress of ectopic endometrial tissues suggests inherent cellular mechanisms contributing to unrestricted growth, invasion and angiogenesis of endometriotic cells [3]. A report showed that many factors may cause endometriosis, such as coelomic metaplasia, retrograde menstruation, and lymphatic and vascular spread [4]. The development of new ways for the diagnosis and therapy of endometriosis is limited owing to knowing little about the etiopathogenesis and natural progress of endometriosis. Though sustained effort has been made in exploring endometriosis, its causative mechanisms have yet not been fully clear [5]. Most women suffer from retrograde menstruation, the most primary risk factors for endometriosis, while merely 10-15% of women are subjected to endometriosis [6], implying that other elements take part in the progress of endometriosis.

MicroRNAs (miRNAs) are a type of endogenous 18- to 22-nucleotide (nt)-long small non-coding RNAs that modulate gene expression post-translationally [7]. It is well understood that miRNAs are capable of regulating expression of approximately 30% of human genes [8]. MicroRNAs play a vital role in biological and pathological process and are currently used as diagnostic and prognostic markers in many diseases [9-11]. For instance, a finding revealed that abnormal expression of miRNA is concerned with pathogenesis of endometriosis [12]. Shi et al. showed that downregulation of miR-183 increased the invasive capacity and
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repressed the endometrial stromal cell apoptosis in endometriosis [13]. The result presented by Zhao et al. indicated that upregulation of miR-20a may be involved in endometriosis by inhibiting NTN4 [14]. Recently, a report with miRNA expression profile in endometriosis showed that down-regulation of miR-424-5p was observed in patient with endometriosis [15]. However, the functional role of miR-424-5p in endometriosis has been unclear.

Fibroblast growth factor receptor (FGFR) family is a portion of growth factor receptor tyrosine kinases (RTK) capable to trigger some cellular events including cell proliferation, inhibition of apoptosis, angiogenesis, and cell migration [16]. As a member of FGFR family, FGFR1 was reported to promote an epithelial-to-mesenchymal transition (EMT) of primitive streak-localized epiblast cells into mesoderm cells [16]. Turner et al suggested that the abnormal FGFR1 expression was observed in the progression of various tumor cell types, including prostate cancer, colorectal cancer and non-small lung cancer [16]. Shiang et al. demonstrated that the upregulation of FGFR1 increased cell proliferative capability, whereas its downregulation induced cell apoptosis in breast cancer [17]. Recently, a research indicated that the level of FGFR1 was significantly increased in ectopic endometrium in comparison with either its eutopic counterpart or endometrium from normal patients [18]. Although upregulated FGFR1 has been observed in endometriosis, its elaborate molecular mechanism remains to be further explored.

In this study, we detected the expression level of miR-424-5p using qRT-PCR in normal endometrium and in paired ectopic and eutopic endometrium tissues from women. And then the effect of miR-424-5p on CRL-7566 cells proliferation and apoptosis was investigated. Additionally, a potential target and signal pathway by which miR-424-5p exerted its effect on endometriosis cell was identified to better elucidate the functional role of miR-424-5p in endometriosis.

Materials and methods

Tissue acquisition

Ectopic and eutopic endometrial tissues were obtained from 26 patients diagnosed as endometriosis with an average age of 30 ± 5.1 years, and normal endometrial tissues were from 26 healthy control subjects at Renmin Hospital of Wuhan University, Wuhan, China. All patients participating in the experiment signed an informed consent form in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) and the study received approval from the Ethics Committee of Renmin Hospital of Wuhan University. All patients participating in this study had normal menstrual cycles and none of the patients had received hormonal treatment prior to surgery. Samples from the proliferative phase of the menstrual cycle were maintained in liquid nitrogen for further biochemical assays.

Cell culture and transfection

We purchased endometriosis cell line CRL-7566 from the American Type Culture Collection (ATCC; Manassas, VA, USA). CRL-7566 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) with the addition of 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA) with the addition of 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA) in a 5% CO₂ atmosphere at 37°C.

The miR-424-5p mimics, miR-424-5p inhibitor and negative control (NC), mutant and wild-type FGFR1 3’UTR, the FGFR1 overexpression plasmid and the siRNA plasmid were transfected into the cells by means of Lipofectamine 2000 reagent (Invitrogen) at 20 nM according to the manufacturer’s instructions and incubated at 37°C for 24 h. We collected all samples for qRT-PCR, Western blot analysis, MTT assay or flow cytometry analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer’s instructions. The reverse-transcription reactions were carried out using a TaqMan™ microRNA assay kit (Applied Biosystems, Foster City, CA, USA) and a Prime Script™ RT reagent kit (Takara, Shiga, Japan) following the instructions from the respective manufacturers. Real-time PCR was performed using the StepOnePlus™ real-time PCR system (Applied Biosystems, Courtaboeuf, France) and Platinum
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SYBR Green qPCR SuperMix UDG (Invitrogen). The expression levels of miRNA were normalized to the endogenous U6 small nuclear RNA (U6-snRNA) and calculated by the $2^{-\Delta\Delta\text{Ct}}$ method.

Luciferase assay

The wild-type FGFR1-3'UTR (WT) and mutant FGFR1-3'UTR (MUT) harboring the possible binding site of miR-424-5p were constructed and cloned into pmirGLO dual luciferase miRNA reporter vectors (Promega, Madison, WI, USA). The reporter vectors and miR-424-5p mimics or NC were co-transfected into CRL-7566 cells by Lipofectamine 2000 (Invitrogen). After 48 h of incubation, cells were lysed to measure Renilla luciferase activities using the dual-luciferase assay system (Promega).

Western blot analysis

Protein samples were isolated from cells by a total protein extraction kit (Kaiji Biological, Inc.). The protein concentration was detected by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). For western blot analysis, 50 µg of proteins were separated in 10% SDS-PAGE, and then transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). The membranes were blocked in Tris-Buffered Saline and 0.1% Tween 20 (TBST) buffer with 5% BSA for 2 h at room temperature and then were incubated with primary antibodies overnight at 4°C. After rinsing twice in TBST, the membranes were subsequently incubated with secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) labeled with HRP for 1 h at 37°C and detected by ECL. Finally, the interest proteins were detected by ECL (Pierce, Rockford, IL, USA) and the signal intensity was determined by Image J software (NIH, USA). The primary antibodies used in this study are anti-FGFR1 (1:10,000; CST, Inc., Danvers, MA, USA), anti-STAT3 (1:10,000; CST), anti-pSTAT3 (1:10,000; CST) and anti-β-actin (1:10,000; CST).

Figure 1. Expression levels of the miR-424-5p and FGFR1 in endometrial tissues were detected by RT-qPCR and miR-424-5p directly targeted FGFR1. A: MiR-424-5p mRNA level was significantly down-regulated in eutopic and ectopic endometrium tissues (n=26). B: FGFR1 mRNA level was increased significantly in eutopic and ectopic endometrium tissues (n=26). C: The sketch map of the miR-424-5p binding site in FGFR1 3'UTR. D: The luciferase reporter assay showed the luciferase activity of WT-FGFR1 and MUT-FGFR1 in CRL-7566 cells at 48 h after transfection. **P<0.01, ***P<0.001.
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MTT assay

The cells were seeded at a density of 3.0×10^3 cells/well into 96-well plates (Corning Costar, Corning, NY, USA) containing DMEM with 10% FBS. After transfection, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) solution (5 mg/ml in ddH2O) were added to each well at indicated times (day 0, 24, 48, and 72) followed by incubation for 4 h at 37°C. Thereafter, the culture medium was removed followed by the addition of 100 µl of dimethyl sulfoxide (DMSO) to each well, and the plates were incubated for 15 min at 37°C to dissolve intracellular formazan crystals. Finally, absorbance was recorded at 490 nm using a Tecan Infinite F 200 microplate reader (Crailsheim, Germany).

Flow cytometry

Cultured cells were collected and washed with phosphate-buffered saline (PBS). Cells (1×10^6) from each sample were resuspended in binding buffer and stained with the Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. Apoptotic cells were determined and quantified by flow cytometry (BD FACS Aria; BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Data were presented as means ± SD, and statistical significance were analyzed using student’s t test or analysis of variance (ANOVA). Experiments were independently repeated at least three times. Statistically significant p values were labeled as follows: *P<0.05; **P<0.01, ***P<0.001.

Results

MiR-424-5p was downregulated and FGFR1 was upregulated in the ectopic endometrium

The expressions of miR-424-5p and FGFR1 in the normal, eutopic and ectopic endometrium tissues from women were detected by qRT-PCR. The data indicated that the level of miR-424-5p was significantly decreased in eutopic and ectopic endometrium compared with normal endometrium, and even the level of miR-424-5p in ectopic endometrium was significantly lower than that in the eutopic endometrium (Figure 1A). In addition, the expression level of FGFR1 was significantly increased in eutopic and ectopic endometrium compared with normal endometrium, and the expression level of FGFR1 was upregulated significantly in the ectopic group in comparison with the eutopic group (Figure 1B).

MiR-424-5p inhibited FGFR1 expression by targeting FGFR1-3’UTR in CRL-7566 cells

To define whether miR-424-5p targets directly FGFR1, we firstly seek for target genes of miR-424-5p by using miRBase (http://www.mirbase.org/) and TargetScan5.1 (http://www.targetscan.org/) miRNA databases. The bioinformatics predicted that FGFR1 contained a con-
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Next, we established a firefly luciferase reporter harboring 3’UTR of wild FGFR1 (WT-FGFR1) or 3’UTR of the mutant FGFR1 (MUT-FGFR1). The reporter assay indicated that miR-424-5p was capable of significantly suppressing luciferase expression of the WT-FGFR1 reporter, but not the mutant reporter (Figure 1D).

To confirm the effect of miR-424-5p on FGFR1, the level of miR-424-5p and FGFR1 were determined by qRT-PCR and western blot in CRL-7566 cells transfected with miR-424-5p mimics, inhibitor, or NC. The results revealed that miR-424-5p expression was efficiently up-and down-regulated by miR-424-5p mimics and miR-424-5p inhibitor, respectively (Figure 2A). As shown in Figure 2B, miR-424-5p mimics were capable of significantly inhibiting FGFR1 expression, whereas the miR-424-5p inhibitor significantly promoted its expression. All these data indicated that miR-424-5p directly targeted and inhibited FGFR1 expression in CRL-7566 cells.

Upregulation of FGFR1 restored the inhibitory effect of miR-424-5p on FGFR1 expression

To further explore the link between miR-424-5p and FGFR1, we co-transfected CRL-7566 cells

Figure 3. The expression correlations between miR-424-5p and FGFR1 in CRL-7566 cells. Cells were co-transfected with miR-424-5p mimics and FGFR1 overexpression plasmid or miR-424-5p inhibitor and si-FGFR1 for 24 h. A: FGFR1 upregulation did not change the increase in miR-424-5p mRNA expression in miR-424-5p mimic-transfected CRL-7566 cells. B: FGFR1 siRNA did not change the reduction in miR-424-5p mRNA expression in CRL-7566 cells transfected with miR-424-5p inhibitor. C: FGFR1 overexpression reversed the reduction in FGFR1 protein expression in miR-424-5p mimic-transfected CRL-7566 cells. D: FGFR1 knockdown reversed the increase in FGFR1 protein expression in miR-424-5p inhibitor-transfected CRL-7566 cells. *P<0.05, **P<0.01, ***P<0.001.
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Figure 4. MiR-424-5p regulated cell proliferation and apoptosis by targeting FGFR1 and modulated pSTAT3 expression. A: MTT assay was carried out to evaluate cell proliferation. miR-424-5p mimics inhibited significantly CRL-7566 cells proliferation, whereas FGFR1 overexpression reversed the inhibitory effect of miR-424-5p mimics on cell proliferation. B: MiR-424-5p inhibitor promoted significantly CRL-7566 cells proliferation, whereas FGFR1 siRNA attenuated the positive effect of miR-424-5p inhibitor on cell proliferation. C: Flow cytometric analysis was used to detect the cell apoptosis. miR-424-5p mimics increased significantly the apoptotic rate of CRL-7566 cells, whereas FGFR1 overexpression relieved the inductive effect of miR-424-5p mimics on apoptosis. D: MiR-424-5p inhibitor reduced significantly the apoptotic rate of CRL-7566 cells, whereas si-FGFR1 knockdown overturned the inhibitory role of miR-424-5p inhibitor in apoptosis. E: Protein levels of total STAT3 and pSTAT3 was detected by western blot. pSTAT3 protein level was reduced significantly in CRL-7566 cells transfected with miR-424-5p mimics, while FGFR1 overexpression reversed inhibitory effect of miR-424-5p mimics on pSTAT3 expression. F: PSTAT3 protein level was increased significantly in CRL-7566 cells transfected with miR-424-5p inhibitor, whereas si-FGFR1 reversed the positive effect of miR-424-5p inhibitor on pSTAT3 expression. *P<0.05, **P<0.01.
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fected with miR-424-5p mimics (Figure 3A). We simultaneously transfected CRL-7566 cells with miR-424-5p inhibitor and FGFR1 siRNA plasmid, and found that the FGFR1 knockdown did not change the reduction in miR-424-5p expression in cells transfected with miR-424-5p inhibitor (Figure 3B).

In addition, western blot was used to detect the protein level of FGFR1. The results showed that miR-424-5p mimics significantly reduced the FGFR1 level, while FGFR1 overexpression attenuated the inhibitory effect of miR-424-5p mimics on FGFR1 (Figure 3C). In addition, miR-424-5p inhibitor significantly increased the FGFR1 level, and si-FGFR1 reversed the positive influence of miR-424-5p inhibitor on FGFR1 (Figure 3D).

FGFR1 reversed the effect of miR-424-5p on cell proliferation and apoptosis

To investigate the functional role of miR-424-5p in regulating cell proliferation and apoptosis in CRL-7566 cells, MTT assay and flow cytometry assay were performed. MTT assay indicated that the miR-424-5p mimics markedly inhibited CRL-7566 cells proliferation, whereas FGFR1 overexpression reversed the inhibitory effect of miR-424-5p mimics on cell proliferation (Figure 4A). However, growth of CRL-7566 cells transfected with miR-424-5p inhibitor was promoted, while si-FGFR1 overturned the positive effect of the miR-424-5p inhibitor on cell proliferation (Figure 4B). Apoptosis analysis displayed that miR-424-5p mimics significantly increased the percentage of apoptotic cells in comparison with the control group, whereas FGFR1 overexpression attenuated the effect of miR-424-5p mimics on apoptosis (Figure 4C). Additionally, miR-424-5p inhibitor obviously suppressed apoptosis in CRL-7566 cells, and si-FGFR1 reversed the role of miR-424-5p inhibitor in apoptotic (Figure 4D). The data indicated that miR-424-5p regulated endometriosis cell proliferation and apoptosis by targeting FGFR1.

Upregulation of FGFR1 reversed the effect of miR-424-5p on pSTAT3

To further investigate the molecular mechanism of miR-424-5p in regulating CRL-7566 cell growth, we determined the levels of total STAT3 and phosphorylated STAT3 (pSTAT3) in CRL-7566 cells by western blot. The data showed that miR-424-5p mimics reduced the level of pSTAT3 compared with NC group, whereas FGFR1 overexpression reversed the role of miR-424-5p mimics in inhibiting the expression of pSTAT3 (Figure 4E). Consistent with this, miR-424-5p inhibitor increased the expression of pSTAT3, and FGFR1 knockdown reversed the effect of miR-424-5p inhibitor on promoting the expression of pSTAT3 (Figure 4F). These data suggested that miR-424-5p modulated STAT3 signaling by targeting FGFR1.

Discussion

It is reported that 5-15% of reproductive age women worldwide suffered from endometriosis, a common, benign gynecological disease [5]. Previously, Ohlsson et al. confirmed that expression levels of 22 miRNAs were different in paired ectopic vs. eutopic endometrium, and differential expressions of miRNAs potentially regulated the expression of 2340 genes [19]. In addition, target genes were demonstrated differentially expressed between endometrial ectopic and eutopic endometrium in an mRNA microarray study [20]. Recently, the results represented by Braza-Boils confirmed that 156 mature miRNAs were differentially expressed in eutopic endometrium tissue compared with control endometrial tissue, and 79 miRNAs were up-regulated and 77 miRNAs were down-regulated [15]. Moreover, miR-424-5p was found to be down-regulated significantly in patient endometrial tissue than that in healthy (control) endometrium [15, 19]. Consist with this, we found that the level of miR-424-5p was decreased significantly in the eutopic and ectopic endometrium tissues compared with normal endometrium tissue. Previous reports showed that miR-424-5p targeted vascular endothelial growth factor (VEGF-A) and played a vital role in down-regulating the angiogenic activity of VEGF-A [21]. Thus, we speculated that miR-424-5p might play an important role in endometriosis.

To investigate the functional role of miR-424-5p in endometrium, we predicted that miR-424-5p directly targeted FGFR1. Zhao et al. screened some molecules involved in endometriosis pathogenesis using a bioinformatics-based method and confirmed that FGFR1 was overexpressed significantly in eutopic and ectopic endometrium of endometriosis compared with its eutopic counterpart [18]. WEI et al.
revealed that overexpression of FGFR1 influenced osteosarcoma MG63 cell cycle by regulating CDK1 expression and significantly contributed to cell proliferation [22]. Similarly, our result found that FGFR1 expression was upregulated significantly in patient endometrial tissue than that in normal tissue. Moreover, FGFR1 overexpression promoted CRL-7566 cell proliferation and inhibited apoptosis. We speculated that the inhibitory role of miR-424-5p in FGFR1 expression played a vital role in endometriosis.

STAT3 exists in the cytoplasm until activated by phosphorylation. Activated STAT3 transfers to the nucleus accompanied by the formation of homodimers or heterodimers and then binds to promoter regions for target gene expression [23]. It has been reported that STAT3 signaling takes part in oncogenesis by stimulating cell proliferation, promoting angiogenesis, mediating immune evasion and conferring resistance to apoptosis [24]. Recently, Kim et al. found that expression level of pSTAT3 was upregulated significantly in the eutopic endometrium comparison with normal endometrium [25]. The results indicated that abnormal activation of STAT3 signaling might play a vital role in the pathogenesis of endometriosis. In our study, we observed that pSTAT3 expression was modulated by miR-424-5p and FGFR1 in CRL-7566 cells. MiR-424-5p suppressed pSTAT3 expression, whereas FGFR1 could reverse the inhibitory role of miR-424-5p in regulating pSTAT3 expression in CRL-7566 cells.

In summary, our results revealed that miR-424-5p inhibited proliferation and promoted apoptosis in CRL-7566 cells by negatively regulating FGFR1 expression. Moreover, miR-424-5p suppressed the activation of STAT3 signaling by modulating FGFR1 expression. Thus, miR-424-5p functions as a negative regulator in endometriosis and may develop into a new therapeutic target for the treatment of endometriosis. Still, the role of miRNAs in endometriosis remains largely unknown. Given this, the elucidation of miRNA mechanisms involved in endometriosis and potential pathogenesis of endometriosis should be further investigated.

Disclosure of conflict of interest
None.

Address correspondence to: Li Hong, Department of Gynaecology and Obstetrics, Renmin Hospital of Wuhan University, No. 238 Jiefang Road, Wuhan 430060, P. R. China. Tel: +86-027-88041911; E-mail: cmdrnt@163.com

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