Follistatin-like 1 promotes endometriosis by increasing proinflammatory factors and promoting angiogenesis

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Abstract

Endometriosis (EMS) is a chronic benign inflammatory disease characterized by the growth of endometrial-like tissue in aberrant locations outside of the uterine cavity. Angiogenesis and abnormal immune responses are the fundamental requirements of endometriotic lesion survival in the peritoneal cavity. Follistatin-like 1 (FSTL1) is a secreted glycoprotein that exhibits varied expression levels in cardiovascular disease, cancer and arthritis. However, the role of FSTL1 in the development of EMS remains to be fully elucidated. Results of the present study demonstrated that the expression of FSTL1 was significantly increased in ectopic endometrial stromal cells (ESCs) and peritoneal fluid from patients with EMS, compared to the control group. Both conditions of hypoxia and estrogen treatment induced human ESCs to produce increased levels of FSTL1 and disco-interacting protein 2 homolog A (DIP2A). Furthermore, the expression levels of DIP2A, IL8 and IL1β were increased in FSTL1 overexpressed HESCs. Additionally, FSTL1 treatment increased the proliferation of HUVECs in a dose-dependent manner in vitro and markedly increased the tube formation of HUVECs. Moreover, treatment with FSTL1 facilitated M1 polarization of macrophages, increased the secretion of proinflammatory factors and inhibited the expression of scavenger receptor CD36. Results of the present study suggested that the elevated expression of FSTL1 may play a key role in accelerating the development of EMS via enhancing the secretion of proinflammatory factors and promoting angiogenesis.

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Introduction

Endometriosis (EMS) is characterized by extra-uterine endometrial-like tissues that react to an ovary-derived estrogen stimulus. EMS affects ~10% of reproductive-aged women (Giudice & Kao 2004). The main symptoms of EMS include pelvic pain, dysmenorrhea, dyspareunia, infertility and menstrual irregularities (Nap et al. 2004). Although it is a benign chronic inflammatory disease, EMS exhibits characteristics of upregulated proliferative capacity and invasiveness, defective apoptosis, angiogenesis and recurrence (Vigano et al. 2004). Results of a previous study highlighted that the retrograde menstruation hypothesis is the most widely accepted theory of pathogenesis; however, the exact mechanisms underlying the pathogenesis of this enigmatic disease remain to be fully elucidated (Bulun 2009). Accumulating evidence indicated that the formation and persistence of ectopic lesions in the peritoneal microenvironment of EMS were associated with the intrinsic abnormalities in the endometrium of affected women, the imbalance of pro- and anti-inflammatory factors, and the dysfunction of the immune system (Osuga et al. 2011, Capobianco & Rovere-Querini 2013, Lei et al. 2018, Zhou et al. 2019). Thus, an increased understanding of the pathogenesis of EMS is required for the development of precision medicine.

Follistatin-like 1 (FSTL1), also referred to as follistatin-related protein or TGFβ-stimulated clone 36, is a secreted extracellular glycoprotein belonging to the secreted protein acidic rich in cysteine (SPARC) protein family (Sylva et al. 2013). Like other SPARC protein family
members, FSTL1 possesses extracellular calcium-binding E-F hand motif and follistatin-like domain. However, FSTL1 has low protein sequence homology to follistatin (16%) (Ouchi et al. 2010), the activin-A binding protein. Furthermore, FSTL1 does not function through binding partners of TGFβ superfamily proteins like follistatin or FSTL3. DIP2A (disco-interacting protein 2 homolog A), a cell-surface receptor of FSTL1, combines with FSTL1 and involves in vascularization and regulation of immune response (Ouchi et al. 2010, Mattiotti et al. 2018). FSTL1 is ubiquitously expressed in mammalian cells of mesenchymal lineage, and it participates in regulation of multiple biological and pathological processes, such as embryonic development, cardiac and lung dysfunction and repair, tumorigenesis, vascularization and immune response (van Rooij 2016, Cheng et al. 2017, Liu et al. 2017b, Tania et al. 2017, Yang et al. 2017d, Zheng et al. 2017, Kudo-Saito et al. 2018, Mattiotti et al. 2018). Expression of FSTL1 is upregulated during heart failure and ischemic injury. Moreover, FSTL1 increases cardiomyocyte survival and stimulates angiogenesis to enhance cardiac regeneration and repair by phosphorylation of AKT (Oshima et al. 2008, Wei et al. 2015, van Rooij 2016). The role of FSTL1 in tumorigenesis remains controversial, as decreased expression levels of FSTL1 were associated with poor prognosis of numerous cancer types, such as ovarian and endometrial cancer, prostate malignancy, renal clear cell carcinoma and lung adenocarcinoma (Meehan et al. 2002, Chan et al. 2009, Liu et al. 2016, Chiou et al. 2017). On the other hand, upregulated expression levels of FSTL1 were observed in colon cancer, hepatocellular carcinomas, glioblastoma and esophageal squamous cell carcinoma (Reddy et al. 2008, Torres et al. 2013, Lau et al. 2017, Yang et al. 2017c). These differing expression levels of FSTL1 may be due to heterogeneity in different cancers, and multiple mechanisms underlying the processes involved in tumor development (Mattiotti et al. 2018). FSTL1 plays a key role in diverse cell type-specific functions in the regulation of cell survival, metabolism, cell differentiation and migration (Lee et al. 2017, Liu et al. 2017b). Additionally, FSTL1 is an important mediator in the pathogenesis of immune disease and exhibits a dual function in inflammatory processes. For example, in the acute phase of inflammation, FSTL1 acts as an anti-inflammatory factor. However, during long-term and chronic inflammatory disease, FSTL1 plays a proinflammatory role (Mattiotti et al. 2018). Thus, increasing evidence demonstrates that FSTL1 acts as a mediator between cells in dynamic cytokine environments during inflammatory diseases (Wang et al. 2016, Cheng et al. 2017).

As the specific role of FSTL1 in EMS as a persistent disease with chronic inflammation is yet to be fully established, the present study aimed to investigate the role of FSTL1 in EMS development. In the present pilot study, the expression levels of FSTL1 were examined in ectopic endometrial stromal cells (ESCs) and the peritoneal fluid of patients with EMS. Subsequently, the effects of FSTL1 on the biological behavior of HESCs, HUVECs and macrophages were examined. The results of the present study suggested that FSTL1 plays a key role in EMS and may act as a novel therapeutic target for the treatment of EMS.

Materials and methods

Subjects and sample collection

Participants (age, 20–45 years) were recruited from Shanghai First Maternity and Infant Hospital. Prior to sample collection, all subjects signed a written consent form. The research protocol was approved by the Medical and Life Science Ethics Committee of Tongji University. EMS peritoneal fluid (n = 18) or ectopic endometrial tissues (n = 7) were obtained from 25 patients undergoing laparoscopic excision of ovarian endometrioma. One sample of EMS peritoneal fluid contaminated with blood was excluded. The control group was selected according to previous studies (Chang et al. 2017, Yang et al. 2017b). Control endometrial tissues (n = 6) and control peritoneal fluid (n = 9) were obtained from 15 women who underwent hysterectomy for leiomyoma but did not have EMS or adenomyosis. All samples were collected during the proliferative phase of the cycle. None of the included patients had received any hormonal therapy within 6 months before the surgical procedure, and none had experienced pelvic inflammatory disease. The diagnosis of EMS was confirmed according to laparoscopic and histopathological evaluation. According to the revised American Fertility Society classification of EMS, patients with EMS were divided into two groups: Stage I–II and stage III–IV. The endometrial tissues were collected under sterile conditions and transported to the laboratory on ice in DMEM/F-12 (Hyclone; Cytiva), and subsequently used for isolating and culturing ESCs. Peritoneal fluids were centrifuged at 453 g at 4°C for 10 min, and the supernatant was stored at −80°C.

Cell isolation and culture

Normal ESCs and ectopic ESCs were isolated and cultured according to the previously reported method (Yu et al. 2016). Endometrial tissues or ovarian endometrioma tissues were minced into small pieces (1–2 mm³) and then digested by Collagenase type IV (0.1%, Sigma-Aldrich; Merck KGaA) in a 37°C shaker for 40–60 min, and subsequently filtered through 100-µm nylon mesh and 40-um nylon mesh (Falcon cell strainers; Thermo Fisher Scientific, Inc.) to remove cell debris. The ESCs were cultured in DMEM/F-12 containing 10% FBS (ScienCell Research Laboratories, Inc.), ampicillin (100 IU/mL) and streptomycin (100 IU/mL). The immortalized human endometrial stromal cell line (HESC; cat. no. CRL-4003™) was obtained from the American Type Culture Collection, and HESCs were maintained in DMEM/F-12. HUVECs were purchased from Shanghai Zhong Qiao XinZhou Biotechnology Co., Ltd. and cultured in endothelial cell medium (ECM) (Hyclone; Cytiva) supplemented with 10% FBS, 100 IU/mL
ampicillin and 100 IU/mL streptomycin. Human THP-1 cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in RPMI 1640 medium (Hyclone; Cytiva) with 10% FBS, 100 IU/mL ampicillin and 100 IU/mL streptomycin. All cells were cultured in a humidified atmosphere at 37°C with 5% CO₂. To explore the impacts of FSTL1 on macrophages, PMA (100 ng/mL, MCE) was used to induce THP-1 cells into M0 cells for 48 h. And then, M0 cells were administrated with PBS or rhFSTL1 (200 ng/mL) for 36 h.

RT-qPCR
Total RNA of NESC, EESC, FSTL1 overexpressed HESC and rhFSTL1 treated M0 cells was extracted using TRIzol® reagent (Takara Bio, Inc.) according to the manufacturer’s protocol. Total RNA was reverse transcribed into cDNA using a Prime Script RT Master Mix (Takara Bio, Inc.). Expression levels of FSTL1 mRNA were detected using TB Green Ex TaqTM II (Takara Bio, Inc.) with specific primers (sequences of primers listed in Table 1) according to the manufacturer’s protocol. qPCR was performed by Applied Biosystem StepOneTM RT-PCR System (Applied Biosystem; Thermo Fisher Scientific, Inc.). A three-step PCR was performed for 40 cycles. The samples were denatured at 95°C for 30 s, annealed at 95°C for 5 s and extended at 60°C for 30 s. Analysis of relative gene expression data using the 2−ΔΔCT method. For each sample, the relative mRNA level was normalized to the internal reference gene GAPDH. All experiments were carried out in triplicate.

ELISA
The peritoneal fluid was collected as previously mentioned and then centrifuged at 453 g at 4°C for 10 min to remove cellular debris. Peritoneal fluid contaminated with blood was excluded. The levels of FSTL1 in peritoneal fluid were measured using a human FSTL1 ELISA kit (cat. no. KOA0509; Rochland) according to the manufacturer’s protocol. The sensitivity of this assay product is 10 pg/mL with a range of 312–20,000 pg/mL. Each sample was repeated in duplicate.

Hypoxia and estradiol treatment
Hypoxia was induced in a hypoxia incubator at 2% O₂. HESC were treated in hypoxia condition for 0, 24, 48 and 72 h, respectively, after that the total protein level of FSTL1 was analyzed by Western blotting. To explore the joint effect of hypoxia and estradiol treatment on the expression of FSTL1 in HESC, HESC were administrated with different concentration (0, 1 × 10⁻⁸ and 1 × 10⁻⁷ mol/L) of 17β-estradiol (Sigma-Aldrich; Merck KGaA) and normal oxygen (21% O₂) and different concentration (0, 1 × 10⁻⁸ and 1 × 10⁻⁷ mol/L) of 17β-estradiol + hypoxia condition (2% O₂) for 48 h. The total protein level of FSTL1 and DIP2A were analyzed by Western blotting. The integrated density (IntDen) was calculated by ImageJ software. IntDen ratio of each FSTL1 and DIP2A band relative to GAPDH band was used to normalize band intensities.

Western blot analysis
HESC treated by hypoxia and estradiol were washed twice with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). A BCA Protein Assay kit (Beyotime Institute of Biotechnology) was used to determine total protein concentration. Equal amounts of proteins (20 μg/lane) were separated by SDS-PAGE on a 10% gel and subsequently transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat dry milk at room temperature for 1 h and incubated with the following primary antibodies: Rabbit anti-human disco-interacting protein 2 homolog A (DIP2A) antibody (1:1,000; cat. no. PA5-49457; Thermo Fisher Scientific, Inc.), rabbit anti-human FSTL1 antibody (1:500; cat. no. ab71548; Abcam), rabbit anti-human GAPDH antibody (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) at 4°C overnight. Following primary incubation, membranes were washed three times in TBS-Tween-20 and subsequently incubated with HRP-linked anti-rabbit IgG (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) at 4°C overnight. Protein bands were visualized using ECL reagent (Millipore, Sigma) and exposed using an imaging analysis system (Bio-Rad Laboratories, Inc.).

Table 1 The sequences of primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
</thead>
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<td>FSTL1</td>
<td>TGGCAGTAAAGGGCAAGACGCT</td>
<td>GATACTGAACACATGGTTGCT</td>
</tr>
<tr>
<td>DIP2A</td>
<td>TGCTTCGCTTGCATCCT</td>
<td>GAAGGCGGATTGGGAGGGCT</td>
</tr>
<tr>
<td>BECN1</td>
<td>GGGTCTGCGCTATTAGGGCTG</td>
<td>TTCCTCTGTGTCCTCAAATC</td>
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<tr>
<td>MAP1LC3B</td>
<td>CAGCTATACATAGGACATC</td>
<td>GTCCTGACCACCAAGGAGAGG</td>
</tr>
<tr>
<td>SQSTM1</td>
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<td>AGCGGGCCGCTTACAGAAG</td>
</tr>
<tr>
<td>COX2</td>
<td>GCCATGCGGTGAGCTTACATC</td>
<td>CCGT AGACCGAGAGAGAG</td>
</tr>
<tr>
<td>TFA</td>
<td>TCCCGAGACCGCTTCTCCTA</td>
<td>ACGTGGAGGTTGCTCACCAAG</td>
</tr>
<tr>
<td>I1B</td>
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<td>AGGGAGCGTTCGCTTGGAGG</td>
</tr>
<tr>
<td>IL8</td>
<td>CACTGCCGCAACACGAAAT</td>
<td>TCACTGACCCGCTTCAAAACCTC</td>
</tr>
<tr>
<td>IL6</td>
<td>AGTCTCAGTACGTCTCCGT</td>
<td>CTACTGGTGCCGAGGCC</td>
</tr>
<tr>
<td>CD86</td>
<td>AGAGCTGTCCATCAGGCTT</td>
<td>CCGCCGTTGTGCTAGTTCCA</td>
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<td>CD206</td>
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<td>CD14</td>
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<td>TLR4</td>
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<tr>
<td>CD36</td>
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<td>AGGGTACCGGAAACCAACTCA</td>
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<tr>
<td>GAPDH</td>
<td>TGGTGAAGGCTGCGTCTGAAC</td>
<td>GCTCTTGGAGATGTTGAG</td>
</tr>
</tbody>
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https://rep.bioscientifica.com
FSTL1 overexpression plasmid transfection

FSTL1 overexpression plasmids (24906-3, Lot 133C76F) and control plasmids (CON083, Lot P17060100) were purchased from Shanghai GeneChem Co., Ltd. Plasmids were transfected into HESCs using Attractene Transfection Reagent (Qiagen GmbH) according to the manufacturer’s protocol. The overexpression efficiency was confirmed using RT-q PCR.

Matrigel invasion assay

The invasion of ESCs was evaluated using a Matrigel invasion assay according to a previously reported method (Li et al. 2012, Shao et al. 2016). Cell inserts (pore size, 8 μm pore size; diameter, 6.5 mm; Corning, Inc.) were coated with 60 μL of eight-fold diluted Matrigel (BD Biosciences, CA, USA). A total of 2 × 10^4 normal primary ESCs in 200 μL DMEM/F12 medium supplemented with 10% FBS were seeded into the upper chamber. PBS or rhFSTL1 (10 ng/mL) was also placed in the upper chamber. A total of 800 μL DMEM/F12 medium supplemented with 10% FBS was plated in the lower chamber. Following 48 h of incubation at 37°C, the Matrigel and uninvaded cells were removed from the upper surface of the inserts using a cotton bud. The invaded cells were fixed with 100% methanol at room temperature for 10 min and subsequently stained with crystal violet at room temperature for 20 min. Images of invaded cells were captured using an Olympus BX51+DP70 light microscope (Olympus Corporation).

Tubular formation assay

HUVECs were pretreated with PBS or rhFSTL1 (200 ng/mL) at 37°C for 24 h. Matrigel was thawed at 4°C, and 50 μL Matrigel was evenly spread in each well of a 96-well plate. Following solidification in 5% CO_2 at 37°C for 1 h, the pretreated HUVECs were digested and the density was adjusted to 8.0 × 10^3 cells/mL. A total of 100 μL cell suspension was added to each well, and the plate was subsequently cultured in 5% CO_2 at 37°C for 9 h. In order to trace tubular formation, Calcein-AM (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to stain living HUVECs at 37°C for 30 min. We took general pictures for each well (96-well plate) at 100× magnification. And for quantification of tubular formation effect, we took pictures from five randomly selected 400x fields. Capillary-like tubular formation was quantified by counting the number of junctions, nodes, branches and total length using ImageJ software (Version 1.43, National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay

The viability of HUVECs was measured using a CCK-8 assay (Dojindo Laboratories, Inc., Tokyo, Japan) according to the manufacturer’s protocol. Cells were seeded in 96-well plates at a density of 1 × 10^4/well and treated with different concentrations (0, 10^{-4} and 10^{-2}mol/L) of 17β-estradiol and cultured at varying durations (0, 24, 48, 72 h) of hypoxic conditions (2% O_2). Western blotting was performed to detect the expression levels of FSTL1. Results of the present study demonstrated that conditions of hypoxia for 24 h and 48 h significantly increased the expression of FSTL1 compared with normal oxygen (21% O_2) (Fig. 2A). However, 72 h of hypoxia treatment exhibited a suppression effect (Fig. 2A). Moreover, treatment with high concentrations (10^{-2}mol/L) of 17β-estradiol increased the expression levels of FSTL1 and DIP2A, a functional receptor specific to FSTL1 was detected at a wavelength of 450 nm using a DigiScan Microplate Reader (ASYS Hitech GmbH). Each group was tested in duplicate using six wells. Each experiment was repeated three times.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software Inc.). Data are presented as the mean ± S.E.M. from three independent experiments. Differences between two groups were analyzed using paired t-tests, unpaired t-tests or Mann–Whitney test. Comparisons among ≥3 groups were analyzed using one-way ANOVA followed with Bonferroni’s multiple comparisons test. P < 0.05 was considered to indicate a statistically significant difference.

Results

FSTL1 is highly expressed in ectopic ESCs and peritoneal fluid of patients with EMS

To investigate the expression levels of FSTL1 in normal ESCs and ectopic ESCs, RT-qPCR analysis was performed. The results demonstrated that the relative mRNA expression of FSTL1 was significantly upregulated in ectopic ESCs, compared with normal ESCs (Fig. 1A). Moreover, protein expression levels of FSTL1 were also increased in the peritoneal fluid of patients with EMS, compared with the control group (Fig. 1B). We further analyzed the relationship between FSTL1 expression level and the clinical severity of EMS. The expression of FSTL1 increased as the severity of EMS worsened (Fig. 1C). FSTL1 in peritoneal fluid from ESM(III–IV) group was dramatically increased compared with FSTL1 in the control group (P = 0.0185) (Fig. 1C). However, there was no statistical difference between the control group and ESM(I–II) group. Also, ESM(I–II) group and ESM(III–IV) group did not show statistical difference even though the level of FSTL1 increased significantly (Fig. 1C).

Hypoxia and estrogen increase the expression of FSTL1 in ESCs

To explore if hypoxia and estrogen exhibit an effect on the expression of FSTL1, HESCs were treated with different concentrations (0, 10^{-4} and 10^{-2} mol/L) of 17β-estradiol and cultured at varying durations (0, 24, 48, 72 h) of hypoxic conditions (2% O_2). Western blotting was performed to detect the expression levels of FSTL1. Results of the present study demonstrated that conditions of hypoxia for 24 h and 48 h significantly increased the expression of FSTL1 compared with normal oxygen (21% O_2) (Fig. 2A). However, 72 h of hypoxia treatment exhibited a suppression effect (Fig. 2A). Moreover, treatment with high concentrations (10^{-2}mol/L) of 17β-estradiol increased the expression levels of FSTL1 and DIP2A, a functional receptor specific to FSTL1.
**FSTL1 promotes the secretion of inflammatory cytokines and the invasion of ESCs**

To further investigate the function of FSTL1 in ESCs, HESCs were transfected with FSTL1 overexpression plasmids, and the efficiency of overexpression was verified using RT-qPCR analysis. Results of the present study demonstrated that the relative mRNA expression of FSTL1 in the overexpression group was increased by 214-fold, compared with the control group (Fig. 3A). Moreover, results of the RT-qPCR analysis demonstrated that overexpression of FSTL1 in HESCs promoted the expression of DIP2A (Fig. 3B). Additionally, inflammatory cytokines IL8 and IL1B were markedly upregulated in HESCs, compared with the control group (Fig. 3C). However, no significant difference was observed for the levels of autophagy gene (BECN1, MAP1LC3B and SQSTM1) in FSTL1 overexpressed HESCs, compared with the negative control group (Fig. 3D). Meanwhile, rhFSTL1 (10 ng/mL) treatment increased the expression of IL8 and IL1B in HESCs dramatically compared with control group (Supplementary Fig. 1). However, there was no statistical difference in the expression of DIP2A (Supplementary Fig. 1C). Furthermore, the role of FSTL1 in the regulation of HESCs biological behavior was investigated, and results of the present study demonstrated that treatment with rhFSTL1 (10 ng/mL) significantly increased the invasiveness of ESCs, compared with the non-treated group (Fig. 3E and F).

**FSTL1 stimulates endothelial cell survival and the formation of vascular-like structures**

To further explore the effects of FLST1 on angiogenesis, HUVECs were treated with PBS or rhFSTL1 (200 ng/mL) for 24 h, and tubular formation assays were conducted. Results of the present study suggested that FSTL1 treatment stimulated the formation of network structures by HUVECs (Fig. 4A). According to the statistical analysis, the number of junctions, nodes, branches and total length were markedly increased in the rhFSTL1 treated group, compared with the non-treated group (Fig. 4B). Furthermore, rhFSTL1 treatment increased the viability of HUVECs in a concentration-dependent manner (Fig. 4C).
These findings suggested that FSTL1 in endometriotic milieu may stimulate angiogenesis and promote EMS.

**FSTL1 regulates proinflammatory cytokine secretion and the polarization of macrophages**

To investigate the potential effects of FSTL1 on the endometriotic immune microenvironment, the role of FSTL1 in the regulation of macrophage differentiation and function was investigated, as macrophages are the most abundant immune cells in endometriotic milieu (Halme et al. 1987, Kubicka et al. 1996, Berbic et al. 2009). Results of the present study demonstrated that the expression of inflammatory cytokines, such as COX2, IL6, IL8, TNFA and IL1B, in M0 cells were significantly upregulated by rhFSTL1, compared with the control group (Fig. 5A). Moreover, the expression of DIP2A was also increased in rhFSTL1 treated group (Fig. 5A). Notably, the expression of CD86 in M0 cells was markedly increased, and the expression of CD206 was reduced in the FSTL1 treated group, compared with the control group (Fig. 5B). These findings indicated that

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**Figure 3** FSTL1 promotes the secretion of inflammatory cytokines and the invasion of ESCs. (A) Transfection efficiency of FSTL1 overexpression plasmids was confirmed using RT-qPCR (unpaired t-tests). (B, C and D) mRNA levels of DIP2A, IL8, IL1B, BECN1, MAP1LC3B and SQSTM1 were detected using RT-qPCR (unpaired t-tests or Mann–Whitney test). (E) HESCs were treated with PBS or rhFSTL1 (10 ng/mL) and the invasion of HESCs was detected. Five fields of vision were randomly selected and transmembrane cells were counted. (F) Statistical analysis of invaded HESCs (unpaired t-test). Data are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. NS, not significant; NC, negative control; OE, overexpression; FSTL1, Follistatin-like 1; ESCs, endometrial stromal cells; RT-qPCR, RT-quantitative PCR; DIP2A, disco-interacting protein 2 homolog A; SQSTM1, sequestosome-1; HESCs, human endometrial stromal cells.
FSTL1 polarized macrophages toward an M1 phenotype. However, no significant difference was observed in the expression levels of TLR4 and CD14 (Fig. 5C). Notably, CD36, a phagocytic biomarker of macrophages, was decreased in rhFSTL1 treated group, compared with the control group (Fig. 5D), indicating that FSTL1 suppresses the phagocytosis of macrophages.

**Discussion**

EMS is an estrogen-dependent, chronic inflammatory disease that typically affects women of reproductive age. The natural state of the endometrium, immune system and peritoneal microenvironment are altered in women with EMS (Bulun 2009, de Ziegler et al. 2010, Capobianco & Rovere-Querini 2013, Donnez et al. 2016, Izumi et al. 2018). According to the results of previous studies, FSTL1 modulates immune response and exhibits both pro- and anti-inflammatory effects (Chaly et al. 2014). Additionally, results of previous studies have revealed that FSTL1 is crucial in the pathogenesis of rheumatoid arthritis, obesity and other chronic inflammatory diseases (Wilson et al. 2010, Chaly et al. 2012, Fan et al. 2013). However, the role of FSTL1 in ESM is yet to be fully elucidated.

Results of the present study demonstrated an increased expression of FSTL1 in ectopic ESCs and the peritoneal fluid of patients with EMS. These findings suggested an association between FSTL1 and the mechanisms underlying EMS. Hypoxia is essential for the microenvironment of the ectopic lesion implants (Kim et al. 2015, Dai et al. 2019), thus, the effects of hypoxia on FSTL1 expression were explored in HESCs. Results of the present study demonstrated that the protein expression levels of FLST1 in HESCs were increased under conditions of hypoxia, and protein expression levels were highest following 48 h of incubation under hypoxic conditions. Consistent with the results of the present study, results of previous studies demonstrated that FSTL1 protein expression was markedly upregulated in primary human trophoblasts cells under hypoxic conditions (Mouillet et al. 2015). Moreover, the present study revealed that treatment with estrogen promoted the synthesis of FSTL1 in HESCs. In addition, the expression of DIP2A was also increased under conditions of hypoxia and following treatment.
with estrogen. Consistent with the results of the present study, results of previous studies demonstrated that the expression of FSTL1 was upregulated by estrogen and tamoxifen in osteoblastic cells, highlighting that FSTL1 may be an estrogen-regulated gene (Ohashi et al. 1997).

Communication among cells of the endometrial tissue, blood vessels and the immune system is required during the development of endometriotic lesions. In order to examine the role of FSTL1 secreted by ectopic ESCs in the development of EMS, FSTL1 overexpression plasmids and rhFSTL1 were used to mimic the expression of FSTL1 in ectopic milieu. Results of the present study demonstrated that transfection with FSTL1 overexpression plasmids in HESCs led to the upregulation of DIP2A and proinflammatory cytokines IL8 and IL1B. Moreover, treatment with rhFSTL1 in vitro markedly increased the levels of pro-inflammatory cytokines secreted by macrophages, including COX2, IL6, IL8, TNFA, IL1B, DIP2A, CD86, CD206, TLR4, CD14 and CD36 was analyzed using RT-quantitative PCR (unpaired t-tests or Mann–Whitney test). Data are expressed as the mean ± s.e.m. **P < 0.01, ***P < 0.001; NS, no statistical difference; FSTL1, Follistatin-like I; PMA, phorbol 12-myristate 13-acetate.
in this study, the level of FSTL1 in peritoneal fluid was approximately 2–15 ng/mL, so we treated the HESCs with 10 ng/mL rhFSTL1 in vitro. And, rhFSTL1 promoted the invasion of HESCs significantly. Also, the expression of IL8 and IL1B was increased markedly in rhFSTL1 treated group. However, when we used the same concentration of rhFSTL1 (10 ng/mL) to treat HUVECs or macrophages, there was no statistical function change. So, we increased the concentration of rhFSTL1 according to previous research (Liu et al. 2017c, Li et al. 2020, Xi et al. 2021). In a study by Yi Liu et al., the serum level of FSTL1 in the control group was 5.301 ± 0.779 ng/mL, which is similar to our results. And Yi Liu et al. used 100–250 ng/mL concentration of rhFSTL1 to perform in vitro experiments (Liu et al. 2017c). The specific reason still needs to be explored. According to the results of a previous study, the levels of autophagy were reduced in ectopic tissues, compared with normal endometrium (Yang et al. 2017a). Moreover, FSTL1 promoted airway remodeling by activating autophagy in asthma (Liu et al. 2017a). Nevertheless, results of the present study demonstrated that FSTL1 did not change the autophagy level of ESCs; however, further studies are required for verification.

Angiogenesis is crucial during the formation of tridimensional cysts as they transport oxygen and nutrients to endometrial tissues. However, novel vessels are not sufficient during the formation of ectopic lesions. Results of the present study demonstrated that treatment with rhFSTL1 promoted endothelial cell survival and the formation of vascular-like structures. These findings are consistent with those of a previous study, highlighting that treatment with FSTL1 increased revascularization in the ischemic limbs of mice. Furthermore, transduction of an adenoviral vector expressing FSTL1 into HUVECs stimulated the migration and differentiation into network structures by activating the phosphorylation of AKT-endothelial nitric oxide synthase signaling under ischemic conditions (Ouchi et al. 2008).

In the peritoneal microenvironment, macrophages comprise a major population of immune cells. This study highlighted that FSTL1 stimulates M0 cells to synthesize and secrete various inflammatory cytokines, such as COX2, IL6, IL8, TNFA and IL1B, which may facilitate the adhesion, invasion or proliferation of ESCs and the progression of EMS (Wu et al. 2002, Khan et al. 2005, Richter et al. 2005, Sikora et al. 2017). Recent studies revealed that the number of activated macrophages increased in peritoneal fluid of patients with EMS. And both M1 macrophages (classically activated phenotype) and M2 macrophages (alternatively activated phenotype) have been identified in the microenvironment of peritoneal fluid from patients with EMS (Beste et al. 2014). M1 macrophages, which produce pro-inflammatory effect, promote tissue damage and worsen inflammatory disease progression (Berbic et al. 2009). However, M2 macrophages are involved in tissue repair and remodeling, and suppress inflammatory responses (Miller et al. 2020). Our present study demonstrated that FSTL1 played a role in modulating macrophage polarization and phagocytosis. Treatment with rhFSTL1 facilitated the M1 proinflammatory phenotype, and suppressed the anti-inflammatory M2 polarization of macrophages by upregulating the expression of CD86 and inhibiting the expression of CD206. Consistent with the results of the present study, results of a previous study demonstrated that FSTL1 increased the migrative property of macrophages and favored the M1 phenotype compared with the M2 phenotype (Li et al. 2020). Macrophages play an important role in the development of EMS by removing cell debris, antigen presentation and so on. However, the phagocytosis ability of peritoneal macrophages declined in line with the severity of EMS (Dmowski et al. 1998). The reason why the phagocytosis ability of peritoneal macrophages from patients with EMS decreased remains unclear. Previous studies reported that downregulation of CD36, a class B scavenger receptor of macrophages (Chuang et al. 2009, 2010), results in the reduced phagocytic ability of peritoneal macrophages of patients with EMS (Chuang et al. 2009, 2010). In our present study, we demonstrated that CD36 was inhibited by FSTL1 in vitro, suggesting...
that FSTL1 may promote the development of EMS via suppressing the phagocytic ability of macrophages in ectopic milieu.

The overall understanding of the associations between FSTL1 and the development of EMS remains limited. Therefore, future in-depth exploration of the interactive modes and mechanisms underlying FSTL1 in EMS are required to further verify the results of the present study.

Collectively, FSTL1 contributes to the development of endometriosis by stimulating proinflammatory cytokines and angiogenesis (Fig. 6). In the endometriotic milieu, FSTL1 expression levels were increased by ectopic ESCs under conditions of hypoxia and treatment with estrogens. The elevated FSTL1 levels may increase the invasiveness of ESCs and promote the synthesis of IL8 and IL1B. On the other hand, elevated levels of FSTL1 may play a key role in the communication between ESCs, macrophages and endothelial cells. Moreover, FSTL1 promoted the survival of endothelial cells and neovascularization. FSTL1 also facilitated M1 polarization of macrophages, promoted the secretion of proinflammatory factors and inhibited the expression of scavenger receptor CD36. Collectively, these findings revealed the role of FSTL1 in the modulation of EMS via an increase in proinflammatory factors and the promotion of angiogenesis, which may provide novel insights into the pathogenesis of EMS. These findings may also aid in the development of therapeutic interventions against EMS.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-21-0094.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Sha-Ting Lei performed all experiments, analyzed the data and wrote the manuscript. Ming-Qing Li provided experimental guidance and edited the manuscript. Yan-Ling Cao, Shu-Hui Hou and Hai-Yan Peng collected the samples and performed the experiments. Dong Zhao and Jing Sun conceived the study and supervised the experiments. All authors read and approved the final manuscript.

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