Silencing of cofilin-1 gene attenuates biological behaviours of stromal cells derived from eutopic endometria of women with endometriosis

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BACKGROUND: Eutopic endometria with endometriosis (EMs) differ dramatically from normal endometria, physiologically and biochemically, yet the pathogenesis of EMs remains unclear. Cofilin-1 (CFL1), a critical modulator of the actin cytoskeleton, is associated with tumour progression, cell motility, cell adhesion, cell invasion and angiogenesis. Although eutopic endometria with EMs exhibit many malignant-like behaviours and a higher expression of CFL1 than normal endometria, the effects of CFL1 on the pathogenesis of EMs are unknown. The aim of this study was to explore the role of CFL1 expression in proliferation, apoptosis, adhesion, invasion, angiogenesis and ultrastructure of endometrial cells.

METHODS: We isolated and cultured stromal cells derived from the eutopic endometria of 30 patients with advanced ovarian EMs (ESCs, Stromal Cells of eutopic endometria in Endometriosis patients) and 30 control patients without EMs (NSCs, Stromal Cells of eutopic endometria in Non-endometriosis patients), and evaluated their proliferation, apoptosis, adhesion, invasion and expression of markers of adhesion, invasion and angiogenesis in vitro. In addition, these functions were examined after short hairpin RNA (shRNA) was used to silence the CFL1 gene in ESCs, and pEGFP-N1-CFL recombinant plasmid was transiently transfected into NSCs to up-regulate CFL1 expression.

RESULTS: Under basal conditions, CFL1 mRNA and protein were overexpressed in ESCs. Proliferation, adhesion, invasion and markers of adhesion, invasion and angiogenesis were enhanced in ESCs compared with NSCs; in contrast, the apoptosis rate was lower in ESCs than in NSCs. Silencing the CFL1 gene in ESCs markedly attenuated proliferation, adhesion, invasion and expression of the markers, but enhanced apoptosis. Conversely, up-regulation of CFL1 in NSCs increased proliferation, adhesion, invasion and expression of the markers but reduced apoptosis.

CONCLUSIONS: The overexpression of CFL1 in ESCs is associated with enhanced proliferation, adhesion, invasion and angiogenesis and reduced apoptosis in EMs. These malignant-like behaviours of ESCs in EMs can be attenuated by inducing CFL1 gene silencing with shRNA interference.

Key words: CFL1 / endometriosis / eutopic endometrium / stromal cell / biological behaviour

Introduction

Endometriosis (EMs) is a common gynaecological disorder characterized by the implantation and proliferation of endometrial cells outside of the uterus. The incidence of EMs is ~10% in women during the reproductive period (Strathy et al., 1982). The recurrence rate after cessation of therapy or surgery is as high as 50–60% (Osteen et al., 2005). EMs can exhibit malignant-like behaviours when epithelial and stromal cells from abnormal endometria invade other tissues, such as peritoneum, ovary and intestines, via migration and adhesion (Witz et al., 2003). The pathophysiology of EMs remains an enigma in reproductive medicine. The theory of implantation posits that endometrial cells and fragments refluxed during the menstrual period contribute to the pathogenesis of EMs (Sampson, 1927). However, the incidence of retrograde menstruation (~70–80%) is much higher than that of EMs (<10%), implying that other factors may contribute to the pathogenesis of this disease. The eutopic endometria of EMs can proliferate, invade and develop on the surface of the peritoneum or ovary through extracellular matrix (ECM) degradation and angiogenesis (Chung et al., 2002). Our preliminary in vivo study confirmed...
that eutopic endometrium from patients with EMs has a much greater implantation capability than normal endometrium. Published evidence has revealed that the distinct biological behaviours of eutopic endometria are associated with the pathogenesis of EMs (Giudice and Kao, 2004). However, the mechanisms by which EMs modifies these biological activities have not been elucidated.

Cofilin-1 (CFL1), a small (19 kDa) and ubiquitous cytoskeletal protein, belongs to the actin depolymerizing factor family. CFL1 plays a vital role in promoting actin depolymerization/polymerization and rapid turnover of actin filaments (Chen, 2004). However, the mechanisms by which EMs modifies these biological activities are associated with the pathogenesis of EMs (Giudice and Kao, 2004). It has revealed that the distinct biological behaviours of eutopic endometrium (secretory phase) and 4.8-fold greater in endometriotic lesions of patients with EMs compared with the normal endometrium. Our results show that silencing of the CFL1 gene alters the pathogenesis of EMs.

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It is well accepted that cell motility, invasion and angiogenesis are critical to the pathogenesis and the malignant-like transformation of EMs. Surprisingly, to date, the role of CFL1 in EMs has not been reported. Studies show that RhoA, ROCK-I and ROCK-II are overexpressed in EMs (Yuge et al., 2005). Down-regulation of cofilin expression reduces the assembly and stability of invadopodia (Yamaguchi et al., 2000). Reorganisation of the actin cytoskeleton is crucial in tumour progression, cell motility, cell adhesion, cell invasion and angiogenesis. Inhibition of CFL1 activity in carcinoma cells depresses cell motility (Hotulainen et al., 2005). It has been identified as the target of some angiogenesis inhibitors (Keezer et al., 2003).

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Cofilin-1 (CFL1) is a small, ubiquitous protein that plays a critical role in cell motility, invasion and angiogenesis. It is known that cofilin is a key regulator of the actin cytoskeleton dynamics and its expression is controlled by a number of signaling pathways. Down-regulation of cofilin expression reduces the assembly and stability of invadopodia, which are important structures that play a role in cell invasion and angiogenesis. Inhibition of CFL1 activity in carcinoma cells has been shown to decrease cell motility (Hotulainen et al., 2005). It has been identified as a target of some angiogenesis inhibitors (Keezer et al., 2003).

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It has been identified as a target of some angiogenesis inhibitors (Keezer et al., 2003). Similarly, western blot showed that, compared with levels in normal endometria, CFL1 expression is 3.5-fold greater in eutopic endometrium (secretory phase) and 3.5-fold greater in endometriotic lesions of EMs than in normal endometrium (secretory phase). CFL1 protein levels were 3.4-fold greater in eutopic endometrium (secretory phase) and 3.5-fold greater in endometriotic lesions of EMs (Liu et al., 2008). We have also confirmed that there is no difference in CFL1 expression between the proliferative phase and the secretory phase (Liu et al., 2008). These results indicate that the overexpression of CFL1 is associated with EMs pathogenesis. However, the mechanism by which CFL1 affects the pathogenesis of EMs remains to be clarified. Therefore, in the current study, we selected the secretory phase of endometrium and explored how silencing the CFL1 gene would affect proliferation, apoptosis, invasion and adhesion of stromal cells isolated from EMs tissue. Our results show that silencing of the CFL1 gene alters the behaviours of these cells to be more like those from normal endometrium. To our knowledge, this is the first investigation to confirm that CFL1 is associated with the pathogenesis of EMs.

### Materials and Methods

#### Tissue collection and cell culture

A group of 30 patients (37–49 years old) with advanced ovarian EMs and 30 control patients (35–49 years old) were entered into the study. The control patients had cervical intraepithelial neoplasia III without EMs and were undergoing total hysterectomy. All patients were from Shengjing Hospital, China Medical University. Tissue samples were obtained from the patients with informed consent in accordance with the requirements of the China Medical University Research Ethics Committee. None of these patients had received any GnRH analogue or other hormonal medication or antibiotics in the last 6 months prior to the surgery. All samples were obtained in the secretory phase of the menstrual cycle, which was confirmed histologically according to established criteria. Stromal cells derived from eutopic endometria of women with EMs (ESCs) and without EMs (NSCs) were isolated according to the method described previously (Tan et al., 2002; Nishida et al., 2004) with a slight modification. Briefly, the minced eutopic endometrium was digested with collagenase Type I (0.1%; Sigma, Santa Clara, CA, USA) for 40 min at 37°C. The suspension was filtered through a 100-μm nylon mesh to remove the undigested tissues. Following gentle centrifugation at 800g for 5 min, the supernatant was discarded, and the cells were resuspended in DMEM/F-12. The remaining suspension containing stromal cells was filtered through a 40-μm nylon mesh and centrifuged at 1000g for 10 min to remove leukocytes and erythrocytes. The cells were resuspended in DMEM/F-12 again. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to the culture plate for 30 min in a 5% CO2 atmosphere at 37°C. Then, the non-adhering epithelial cells were removed. The adherent stromal cells were cultured as a monolayer in flasks with DMEM/F-12 containing 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin and incubated in a 5% CO2 atmosphere at 37°C. Stromal cells in the monolayer culture after the third passage were >99% pure as confirmed by immunocytochemical staining with antibodies against vimentin, CD10, cytokeratin, factor VIII and leukocyte common antigen (all antibodies were from Zhongshan Goldenbridge Biotechnology, Inc., Beijing, China) and were used for the following experiments. The cells used in all experiments were from passage 3 to passage 5. Eutopic endometria from 30 EMs patients (ESCs) and control patients without EMs (NSCs) were all cultured.

#### Silencing of the CFL1 gene in ESCs

Three short hairpin RNA (shRNA) sequences targeting human CFL1 were designed using OligoEngine software (OligoEngine Inc., Seattle, WA, USA). A BLAST homology search indicated that the three shRNA sequences were specific to the human CFL1 gene. The shRNA sequences were as follows:

1. 5′-GGATCCGGTGTTAACATTCGAGAGTTTCTGCATGATATATTCTGAGGAATGAAGATGTAAGTTTCTAGCTTTTTTTCTTTTTTTTTTTTTTTTTTTTTT−3′
2. 5′-GGATCCGGTGTTAACATTCGAGAGTTTCTGCATGATATATTCTGAGGAATGAAGATGTAAGTTTCTAGCTTTTTTTCTTTTTTTTTTTTTTTTTTTTTT−3′
3. 5′-GGATCCGGTGTTAACATTCGAGAGTTTCTGCATGATATATTCTGAGGAATGAAGATGTAAGTTTCTAGCTTTTTTTCTTTTTTTTTTTTTTTTTTTTTT−3′

These sequences were ligated into plasmid pRNAT-U6.1/Neo, a eukaryotic expression vector (Genscript, Nanjing, China). The void pRNAT-U6.1/Neo plasmid and negative control pRNAT-U6.1/Neo plasmid including non-specific control (scrambled) shRNA were also from Genscript. The ESCs from each of the 30 patients were divided into six groups: CFL1 shRNA group 1 (pRT-CFL-s1), CFL1 shRNA group 2 (pRT-CFL-s2), CFL1 shRNA group 3 (pRT-CFL-s3), CFL1 shRNA negative control group (pRT-sNC), void pRNAT-U6.1/Neo and empty control group (pRNAT-U6.1/Neo).
plasmid group (pRT-V) and ESCs group (ESCs), which lacked plasmid and lipofectamine 2000. Thus, each of the six groups contained 30 samples. Transfection was performed with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a ratio of 0.8 μg of plasmid to 2 μl of lipofectamine 2000 according to the manufacturer’s recommendations. After 48 h of transfection, cells were harvested for analysis. The knockdown of CFL1 expression was confirmed by real-time fluorescent quantitative PCR and western blot analysis.

**Transfection of CFL1-fused plasmid in NSCs**

The CFL1 expression vector pEGFP-N1 containing the total length of the CFL1 cDNA was designed and constructed by Genscript (Nanjing, China). The NSCs were divided into three groups: CFL1-fused plasmid group (pEGFP-N1-CFL1), void plasmid group (pEGFP-N1-V) and NSCs group (NSCs), which lacked plasmid and lipofectamine 2000. After 48 h of transfection, cells were harvested for analysis. CFL1 mRNA and protein levels were evaluated by real-time fluorescent quantitative PCR and western blot analysis.

**Real-time fluorescent quantitative PCR**

Successful knockdown of CFL1 at the mRNA level was confirmed by real-time fluorescent quantitative PCR. At 48 h after transfection, total RNA was extracted from an equal number of cells in each group with Trizol reagent (Takara, Otsu, Shiga, Japan) according to the manufacturer’s protocol. Genomic DNA contamination was removed by adding DNase I reagent (Takara, Otsu, Shiga, Japan) according to the manufacturer’s protocol. Genomic DNA contamination was removed by adding DNase I reagent (Takara, Otsu, Shiga, Japan) according to the manufacturer’s protocol. RNA purity and concentration were determined by spectrophotometry. RNA integrity was observed on a 1% agarose gel loaded with 1 μg of RNA. cDNA reverse transcribed from 2 μg of RNA was used in real-time quantitative PCR, which was carried out with the SYBR®Prime Script™ RT–PCR kit (Takara) on the ABI Prism 7500 Sequence Detection System according to the manufacturer’s protocol. The housekeeping gene encoding glyceraldehyde phosphate dehydrogenase (GADPH) was used for normalization. The primers used were 5′-TGCTGAGTGGAGCAACAG-3′ (forward) and 5′-GACAAAGGTGCGTAGGG-3′ (reverse) for CFL1 and 5′-GCACGTCGAAGGCTAGAAC-3′ (forward) and 5′-ATGGTGGTGAAGACGCCAGT-3′ (reverse) for GAPDH. The PCR protocol was: 50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15 s followed by 60°C for 1 min. Gene expression levels were calculated and determined by using the threshold cycle (CT) method (2^−ΔΔCT method) (Burger et al., 2003). Each experiment was repeated four times for each patient, and each of the four experiments was run in triplicate (3 × 4 = 12). Four averaged values were then averaged to yield a single value for each patient. One value per patient was used for statistical analysis.

**Western blot analysis**

Successful knockdown of CFL1 at the protein level was confirmed by western blot analysis at 48 h after transfection according to the manufacturer’s instructions (KeyGEN, Nanjing, China). The protein concentration was determined by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Samples of 50 μg of protein were loaded and separated by 15% SDS–PAGE and transferred to a PVDF membrane. After being blocked for 2 h at room temperature, the membranes were incubated with mouse anti-CFL1 monoclonal antibody (sc-53934, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, 1:2000 dilution) or rabbit anti-β-actin polyclonal antibody (sc-16166-R, Santa Cruz Biotechnology Inc., 1:5000 dilution) overnight at 4°C. The specific protein–antibody complex was detected by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Zhongshan Goldenbridge Biotechnology, Inc.). The signal was detected by the enhanced chemiluminescence kit (Beyotime, Shanghai, China). The bands were quantified by Image J software. β-Actin was used as a loading control. The individual CFL1 band intensities were then normalized to the corresponding β-actin absorbance value. Each experiment was repeated three times, and the three measurement values were averaged to yield a single value for each patient. One value per patient was used for statistical analysis.

**Cell proliferation test**

Cell proliferation was estimated with a CCK-8 cell counting kit (Beyotime). Cells were seeded in 96-well plates at low density (5000 cells per well) in DMEM/F-12 culture medium. At 24, 48, 72 or 96 h after transfection, CCK-8 reagent was added to each well, and the plates were incubated at 37°C for 4 h. The absorbance at 570 nm was then measured on a microplate reader (Bio-Rad). Culture solution containing CCK-8 reagent without cells served as a blank control (BC). The results of the cell proliferation rate were described as the ratio of the absorbance value from the experimental group to that from the BC group. The sample information and the number of experiments were the same as those described in the real-time PCR section. The proliferation rate of the NSCs in 24 h was normalized to 1.0 and was used for comparison of all other groups.

**Flow cytometry analysis of apoptosis**

After 48 h of transfection, cells undergoing apoptosis were double stained with Annexin V-PE/7-AAD (KeyGEN) according to the manufacturer’s instructions and detected by flow cytometry. Cell pellets were suspended at a concentration of 1 × 10^6 cells/ml. Cells (1 × 10^5 cells in 100 μl) were incubated with Annexin V-PE/7-AAD for 15 min in the dark at room temperature. The stained cells were immediately analysed by fluorescence-activated cell sorting (BD Biosciences, San Jose, CA, USA). The sample information and the number of experiments were the same as those used for real-time PCR.

**Cell adhesion assays**

After 48 h of transfection, cells were plated at 2 × 10^4 cells per well onto a 96-well plate (BD, Franklin Lakes, NJ, USA) pre-coated with collagen. Then the plate was incubated at 37°C for 2 h. After a 30-min fixation in 4% paraformaldehyde, the adherent cells were stained with 1% borax/1% methylene blue for 5 min. The cells were rinsed, and absorbance at 570 nm was recorded. The cell adhesion results were expressed as the ratio of the absorbance value from the experimental group to that of the NSCs group. The sample information and the number of experiments were the same as those for real-time PCR.

**Transwell cell invasion assay**

To evaluate the effect of CFL1 on the invasion of ESCs or NSCs, transwell inserts (6.5 mm filters, 8 μm pore size; Corning, NY, USA) containing polycarbonate filters were used. The upper side was coated with 25 μl of diluted Matrigel (1:2 in serum-free DMEM/F12) and allowed to gel. After 48 h of transfection, cells (5 × 10^5) were resuspended in 200 μl of serum-free DMEM/F12. The lower chamber was filled with 600 μl of DMEM/F12 with 10% fetal bovine serum. After 36 h of incubation at 37°C, the cells from the upper surface of the filter were removed completely with gentle swabbing, and the invasive cells of the lower surface were fixed in 4% paraformaldehyde solution for 15 min at room temperature. Then the cells were stained with haematein. The extent of invasion was determined by counting the number of stained cells on the membranes in three randomly selected, non-overlapping fields at ×20 magnification.
under a light microscope. The results were expressed as the ratio of invasive cell numbers from different groups to that from the NSCs group. The sample information and the number of experiments were the same as those for the western blot section.

ELISAs for VEGF, ICAM-1 and MMP-9
Cells were seeded in 24-well plates at low density (2 x 10^5 cells/well) in DMEM/F-12 culture medium. After 48 h of transfection, the supernatant was collected. ELISA was carried out by a vascular endothelial growth factor (VEGF; Jingmei Biotech, Shenzhen, China), intercellular adhesion molecule-1 (ICAM-1; Jingmei Biotech) or matrix metalloproteinase (MMP)-9 (Rapidbio, Shanghai, China) ELISA kit according to the manual. Each experiment was repeated three times for each patient, and each of the three experiments was run in triplicate (3 x 3 = 9). The three averaged values were then averaged to yield a single value for each patient. One value per patient was used for statistical analysis.

Transmission electron microscopy
Cells were fixed in glutaraldehyde (2.5% in 0.1 mol/l sodium cacodylate buffer, pH 7.5) for 2 h, washed for 30 min, and post-fixed in osmium tetroxide for 2 h at 4°C. After being dehydrated in a graded series of alcohols (50–100%) and propylene oxide, the specimens were embedded in Araldite. Ultrathin sections were stained in uranyl acetate and lead citrate. The sections were viewed under the electron microscope.

Statistical analyses
The data were analysed by one-way analysis of variance followed by an SNK (equal variances) or Dunnett’s (non-equal variances) post hoc test. Values were expressed as mean ± SEM. P <0.05 was considered statistically significant.

Results

Evidence for knockdown or up-regulation of CFL1 gene expression
Real-time PCR and western blot analysis showed that CFL1 mRNA and protein were overexpressed in ESCs under basal conditions (P <0.001 versus NSCs; Fig. 1A and B). After transfection of the three silencing vectors, the expression of CFL1 mRNA and protein decreased (P <0.001 versus ESCs; Fig. 1A and B). Transfection of vector 2 (pRT-CFL1-s2) had the strongest effect on the knockdown of CFL1 mRNA and protein level (Fig. 1A and B). The expression levels of CFL1 mRNA and protein did not change after transfection with control plasmids (pRT-sNC or pRT-V; P >0.05; Fig. 2A and B). The cellular proliferation and apoptosis rates did not change after transfection with control plasmids (pRT-sNC or pRT-V; P >0.05; Fig. 2A and B). CFL1 gene up-regulation decelerated the NSCs apoptosis rate (P <0.001 versus NSCs; Fig. 2B). The proliferation and apoptosis rates of NSCs did not change after the cells were transfected with the void pEGFP-N1 plasmid (P >0.05; Fig. 2A and B).

Silencing of the CFL1 gene impaired adhesion in ESCs, and up-regulation of the CFL1 gene enhanced adhesion in NSCs
Under basal conditions, the adhesion of ESCs was more intense than that of NSCs (P <0.001 versus NSCs; Fig. 3). Silencing the CFL1 gene significantly reduced the adhesion of ESCs (P <0.001 versus ESCs; Fig. 3). The adhesion of ESCs did not change after transfection with control plasmids (pRT-sNC and pRT-V; P >0.05; Fig. 3). After up-regulation of the CFL1 gene, the adhesion of NSCs was significantly enhanced (P <0.001 versus NSCs; Fig. 3). The adhesion of NSCs did not change after transfection with void pEGFP-N1 plasmid (P >0.05; Fig. 3).

Silencing of the CFL1 gene reduced the invasiveness of ESCs, and up-regulation of the CFL1 gene enhanced the invasiveness of NSCs
Under basal conditions, ESCs demonstrated a higher degree of invasiveness than did NSCs (P <0.001 versus NSCs; Fig. 4A). Silencing the CFL1 gene significantly reduced the number of invasive ESCs (P <0.001; Fig. 4A and C) compared with that at baseline (Fig. 4B). Transfection of ESCs with control plasmids (pRT-sNC and pRT-V) did not alter the degree of invasiveness (P >0.05; Fig. 4A). Up-regulation of the CFL1 gene significantly increased the number of invasive NSCs (P <0.001; Fig. 4A and E) compared with that at baseline (Fig. 4D). The level of invasiveness of NSCs did not change after transfection with void pEGFP-N1 plasmid (P >0.05; Fig. 4A).

Effect of CFL1 gene expression on ICAM-1, MMP-9 and VEGF secretion
To determine the level of secretion of ICAM-1, MMP-9 and VEGF, we measured the concentrations of these proteins in the cell culture media of NSCs and ESCs. Under basal conditions, ESCs secreted more ICAM-1, MMP-9 and VEGF than did NSCs (P <0.001 versus NSCs for all three proteins; Fig. 5). Silencing of the CFL1 gene led to a significant reduction in secretion of all three proteins (P <0.001 versus ESCs; Fig. 5). Transfection of ESCs with control
plasmids (pRT-sNC and pRT-V) did not alter the secretion levels ($P > 0.05$; Fig. 5). Up-regulation of the CFL1 gene in NSCs led to a significant increase in the secretion of ICAM-1, MMP-9 and VEGF (*$P < 0.05$ versus NSCs for all three proteins; Fig. 5). Transfection with void pEGFP-N1 plasmid did not alter secretion of the three proteins by NSCs ($P > 0.05$; Fig. 5).

**Figure 1** CFL1 mRNA and protein expression measured by (A) real-time fluorescence quantitative PCR and (B) western blot analysis. Data are expressed as mean ± SEM. The basal threshold cycle (CT) values of NSCs were: CFL1: 25.46 ± 0.13; and GAPDH: 22.45 ± 0.08. (C) Representative western blots showing CFL1 protein expression in ESCs and NSCs under basal conditions. (D) The effect of CFL1 gene silencing in ESCs on CFL1 protein as shown by western blots. Of the three vectors, pRT-CFL1-s2, displayed the strongest effect (A, B and D). Therefore, pRT-CFL1-s2 was used to knock down CFL1 gene expression for the remainder of the study. (E) Representative western blots showing CFL1 protein expression in NSCs after transfection with the CFL1 recombinant plasmid. pRT-CFL-S1/2/3, silencing vectors for CFL in ESCs; pRT-SNC and pRT-V, control vectors in ESCs; pEGFP-N1-V, control vector in NSCs; pEGFP-N1-CFL, expression vector for CFL in NSCs.

**Figure 2** Effect of CFL1 expression on (A) cell proliferation and (B) apoptosis in ESCs and NSCs. The basal absorbance value of NSCs was 0.27 ± 0.01. The basal apoptosis rate of NSCs was 13.93 ± 0.77%. Data are expressed as mean ± SEM. pRT-CFL-S2, silencing vector for CFL in ESCs; pRT-SNC and pRT-V, control vectors in ESCs; pEGFP-N1-V, control vector in NSCs; pEGFP-N1-CFL, expression vector for CFL in NSCs.
Effect of CFL1 gene expression on the ultrastructure of ESCs and NSCs

Silencing of the CFL1 gene led to an increase in apoptosis of ESCs. Under basal conditions, we observed prominences on the surface of the ESCs with irregular nuclear shape. Notches were visible in the nucleus. We did not detect chromatin margination (Fig. 6A). When the CFL1 gene was silenced, the prominences on the surface of the cell decreased. We then observed chromatin margination and shrinkage of the nucleus with a variable amount of condensed chromatin and dot-like/clump-like condensed nuclear fragments (Fig. 6B), known features of apoptosis.

In contrast to the ESCs, prominences were hardly visible on the surface of NSCs under basal conditions. However, chromatin margination and chromatin condensation were very obvious (Fig. 6C). After up-regulation of the CFL1 gene, prominences became visible on the surface of NSCs with irregular-shaped nuclei, notches became visible in the nucleus and chromatin margination disappeared (Fig. 6D). Thus, their structure became similar to that of ESCs under basal conditions.

Discussion

CFL1, an actin-binding protein that promotes actin depolymerization, is reported to be associated with the progression of malignant tumours in cell division, cell migration, cell invasion and angiogenesis. However, little is known about the role of CFL1 in the pathology and pathogenesis of EMs, despite the fact that ESCs present some malignant-like behaviours. Here, we report that CFL1 mRNA and protein are overexpressed in ESCs compared with their expression in NSCs. The most important finding is that this overexpression is associated with the malignant-like behaviours of ESCs, namely proliferation, adhesion, invasion, apoptosis and markers of angiogenesis. In vitro silencing of the CFL1 gene inhibits the proliferation, adhesion, invasion and markers of angiogenesis, but promotes apoptosis. These results indicate that CFL1 is a key molecule that regulates multiple malignant-like behaviours of EMs. Importantly, CFL1 gene silencing can attenuate these malignant-like behaviours.
It has been reported that platelet-derived growth factor (PDGF) and interleukin (IL)-1β can induce CFL1 mRNA and protein expression in canine pulmonary artery smooth muscle cells (Bongalon et al., 2004). In macrophages of the peritoneal fluid in EMs patients, the expression of IL-1β protein is significantly higher than that of control (Montagna et al., 2008). In addition, PDGF exerts a significant dose-dependent effect on stromal cell proliferation (Surrey and Halme, 1991). Whether PDGF and IL-1β induce CFL1 mRNA and protein expression in EMs remains to be elucidated in future studies.

Under physiological conditions, cell proliferation and apoptosis are well balanced. Increasing cell proliferation and decreasing cell apoptosis lead to abnormal cell activities as observed in the ESCs of EMs patients. Vigorous proliferation is one of the advantageous conditions in ectopic survival of ESCs. It has been reported that proliferation of ESCs is more vigorous than that of NSCs (Wingfield et al., 1995) and that the apoptotic rate of ESCs is lower than that of NSCs (Gebel et al., 1998). In addition, reduced ESCs apoptosis is critical in the pathogenesis of EMs (Jones et al., 1998). ESCs were shown to resist apoptosis induced by cytokine activation (Nishida et al., 2004). Our present study confirms that ESCs undergo less apoptosis than NSCs. However, silencing the CFL1 gene significantly increased the apoptosis rate and reduced the proliferation rate of ESCs, indicating that overexpression of CFL1 contributes to the abnormal proliferation of ESCs.

The mechanism by which CFL1 regulates cell proliferation and apoptosis is unclear. Studies have shown that in malignant T-lymphoma cells, cofilin is dephosphorylated by a serine phosphatase and translocates to the nucleus through constitutive activation. A serine phosphatase inhibitor, okadaic acid, blocks these processes and leads to apoptosis (Samstag et al., 1996). These results indicate that activated (dephosphorylated) cofilin exists constitutively in the malignant cells and that blockage of cofilin activation leads to apoptosis. If this is the case in ESCs, one could potentially increase ESCs apoptosis by blocking CFL1 activation. Additional study is needed to confirm our hypothesis. Knockdown of intracellular cofilin protein expression by antisense-cofilin transfection results in reduced cloning efficiencies (Samstag et al., 1996). Here, we showed a similar result in ESCs proliferation by silencing the CFL1 gene with an shRNA technique. These findings indicate that the overexpression of CFL1 may contribute to the pathogenesis of EMs. Therefore, the malignant-like proliferation of ESCs may be manageable by silencing the CFL1 gene.

![Figure 5](image-url) ICAM-1, VEGF and MMP-9 concentrations in culture medium. Concentrations of ICAM-1, VEGF and MMP-9 in the NSCs culture media were 124.37 ± 3.88 ng/ml, 156.72 ± 8.72 ng/ml and 11.50 ± 0.70 pg/ml, respectively. Data are expressed as mean ± SEM. pRT-CFL-S2, silencing vector for CFL in ESCs; pRT-SNC and pRT-V, control vectors in ESCs; pEGFP-N1-V, control vector in NSCs; pEGFP-N1-CFL, expression vector for CFL in NSCs.

![Figure 6](image-url) The effect of CFL1 expression on ESCs and NSCs ultrastructure. Transmission electron microscopy was used to observe the ultrastructure of ESCs (A, B) and NSCs (C, D) at baseline (A, C) and after CFL1 gene silencing (B) or up-regulation (D). Scale bar = 1 μm.
The first step in the process of eutopic endometria transplants away from the uterine cavity is adherence to the peritoneum or surface of other organs. Intense adhesion is favourable to cell survival in ectopia. ICAM-1 is a 90-kDa transmembrane glycoprotein that functions as a ligand for β2 integrin molecules expressed on leukocytes, lymphocyte function-associated antigen-1 and Mac-1. A soluble circulating form of ICAM-1 (sICAM-1), resulting from the proteolytic cleavage of membrane-bound ICAM-1, is also present in human serum and in peritoneal fluid. Cultured human ESCs are constitutively able to release sICAM-1 (Somigliana et al. 1996), suggesting that ESCs can be a source of sICAM-1 in vivo. In this study, we demonstrated that both ESCs and NSCs release sICAM-1. Furthermore, ESCs released more sICAM-1 than did NSCs under basal conditions. sICAM-1 can disturb adhesion between immune cells and ESCs (Viganò et al. 2001), a process that is thought to be important in the development of EMs. The antagonism occurs because sICAM-1 and membrane-bound ICAM-1 compete to bind to the same receptor. This competition might enable ESCs to escape normal immunological recognition and initiate the EMs. In the current study, we also demonstrated that silencing of the CFL1 gene decreases the secretion of sICAM-1. This decrease might recover the immunological recognition of ESCs by natural killing cells and lead to ESCs death. Future study is necessary to elucidate the mechanism by which CFL1 regulates the adhesion of ESCs.

Appropriate expression levels and activation status of CFL1 are essential to the recombination of the actin cytoskeleton and formation of lamellipodia. ESCs invasion and penetration of the peritoneum are also essential to the pathogenesis of EMs (Yoshida et al. 2004). Studies have shown that overexpression of CFL1 is related to the powerful invasion of malignant tumour cells such as pancreatic cancer cells (Whiteman et al. 2007), ascites hepatoma cells, glioblastoma cells (Gunnersen et al. 2000) and gastric carcinoma cells (Wang et al. 2007). CFL1 can stabilize and maturate the process of invadopodia (Yamaguchi et al. 2005). Suppression of CFL1 expression results in the formation of short-lived, less invasive invadopodia, thus weakening the invasion of malignant cells. This may partially, if not entirely, explain the mechanism by which silencing of the CFL1 gene weakens the invasion of ESCs.

ECM degradation is the critical step of cell invasion. MMPs are responsible for degrading all kinds of ECM proteins. MMP-9 appears to be implicated in the invasion of tumours, and tumour metastasis is significantly reduced in MMP-9-knockout mice (Itoh et al. 1999). In addition, the expression of MMP-9 is much higher in eutopic endometrium with EMs than in normal endometrium. Furthermore, MMP-9 facilitates the invasion of shedding eutopic endometrium in EMs (Collette et al. 2006). In this study, we found that silencing of the CFL1 gene inhibits MMP-9 secretion. Taken together, overexpression of CFL1 in ESCs may enhance the ESCs invasion by increasing MMP-9 secretion, whereas silencing the CFL1 gene may weaken the ESCs invasion.

Eutopic endometrium with EMs is highly angiogenic. VEGF is a heparin-binding angiogenic growth factor, the most potent mediator of angiogenesis (Ferrara and Davis-Smyth 1997). Evidence indicates that VEGF is involved in the pathophysiology of EMs (Becker and D’Amato 2007). This study demonstrates that ESCs secrete more VEGF protein than do NSCs. Silencing the CFL1 gene inhibits the secretion of VEGF from ESCs. These results support the previous finding that CFL1 is an angiogenic molecule (Martoglio et al. 2000).

Our findings reveal that the intense angiogenesis in ESCs may be manageable by silencing the CFL1 gene.

Cell function is associated with the internal structure and morphology of the cell. Here, we showed that ESCs display many cell prominences with irregular-shaped nuclei and many notches in the nucleus. The ESCs ultrastructure was almost identical to that described in a previous report (Yu et al. 2006). The prominences are associated with cell invasion and are consistent with our finding that ESCs have a high invasion capacity. One of the most prominent features of malignant cells is the irregular nuclear shape with notches. The similar findings on ESCs could partially explain their potential malignant-like capacity. After silencing of the CFL1, ESCs apoptosis occurs as indicated by chromatin margination, chromatin condensation and nuclear shrinkage. These results indicate that the overexpression of CFL1 is associated with the lower apoptotic capacity in ESCs.

Cell prominences are barely detectable in NSCs under basal conditions. Without these features, NSCs are unable to invade other tissues with the intensity of ESCs. Furthermore, under basal conditions, apoptosis occurs in NSCs as indicated by reduced prominences and the presence of prominent chromatin margination. After up-regulation of CFL1, the chromatin margination disappears and the prominences become apparent, indicating that NSCs are empowered with the ability of invasion. These findings are consistent with our results showing increased invasive capability.

Inhibition of CFL1 expression in ESCs attenuated their malignant-like behaviours, altered their ultrastructural morphology and reduced their release of ICAM-1, MMP-9 and VEGF. Increasing the expression of CFL1 in NSCs had the opposite effects. Such results indicate that regulation of actin filament formation and stabilization affects the behaviour and differentiation of cells, as CFL1 plays a vital role in promotion of actin depolymerization/polymerization and rapid turnover of actin filaments (Chen et al. 2000). Our findings are consistent with a previous report showing that changes in actin dynamics, particularly the stabilization of F-actin, have a significant negative impact on decidualization, and that the translocation of cofilin to the nucleus is a key feature of this process in the primate (Ihnatowych et al. 2009).

In conclusion, our results provide the first evidence that overexpression of CFL1 is associated with enhanced cell proliferation, adhesion, invasion and angiogenesis. These malignant-like cellular behaviours are manageable by silencing of the CFL1 gene. To our knowledge, this is the first study to confirm that CFL1 is associated with the pathogenesis of EMs.

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