Int J Clin Exp Med 2016;9(10):19074-19083
www.ijcem.com /ISSN:1940-5901/IJCEM0028925

*Original Article*

# Depletion of CCL27 inhibits cell proliferation, metastasis and adhesion in ectopic endometrial stromal cells

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Received March 22, 2016; Accepted August 10, 2016; Epub October 15, 2016; Published October 30, 2016

**Abstract:** Endometriosis represents a multifaceted disease with controversial pathogenesis. Effective medical therapy for endometriosis is still urgently needed. In this study, we attempted to reveal the association between CCL27 and endometriosis to investigate new approach in the endometriosis etiology and treatment. We examined the expression of CCL27 in endometriotic tissue and serum samples. Then we analyzed the effects of CCL27 on cell proliferation, migration, invasion, adhesion and signaling pathway in endometrial stromal cells (ESCs) separated from ectopic endometrial tissues after CCL27 knockdown. We found that CCL27 was aberrantly elevated in endometriosis. And the knockdown of CCL27 suppressed endometriosis progression by inhibiting cell growth, metastasis and adhesion in ESCs. And these repressed processes might be mediated by the JAK2/STAT3 pathway inactivated by silencing CCL27 in endometriosis. In conclusion, this study suggests that abnormal expressed CCL27 may function as a stimulative factor in the development of endometriosis.

**Keywords:** Endometriosis, CCL27, endometrial stromal cells (ESCs), JAK2/STAT3 pathway

## Introduction

Endometriosis is a benign gynecologic disorder, which affects 10-15% of women of reproductive age [1]. It is histologically characterized by the presence of endometrium-like tissue outside the uterine cavity containing the ovaries, the pelvic peritoneum and the fallopian tubes. The main symptoms of endometriosis are dysmenorrhea, dyspareunia, chronic pelvic pain and even infertility [2]. In recent years, increasing researches have been focused on this enigmatic disease, with the retrograde menstruation theory being the most accepted explanation. Whereas, many processes of endometriosis still cannot be totally explained. On the basis of the observation in various clinical settings, endometriosis is demonstrated sharing similar symptoms with several autoimmune diseases and malignancies for its chronic inflammatory process, infiltrative and destructive growth [3, 4]. By analogy with these diseases, more and more researchers agree that genetic and immunological factors may be related to the endometriosis pathophysiology [5]. To this point of view, factors that is involved in the regulation of cell proliferation, metastasis and immune function in endometriosis have gained increasing attention, such as growth factors, lectins and chemokines [6-8].

Chemokines are a family of 8-10 kDa cytokines or signal proteins, sharing homologous gene and amino acid sequences. These small polypeptide molecules are secreted by specific types of cells, and attract T cells, leukocytes or other cell types by interacted with their corresponding G-protein-linked receptors on the surface of target cells. Depending on the spacing of their first two cysteine residues, chemokines are classified into four sub-groups, including CC chemokine, CXC chemokine, C chemokine and CX3C chemokine [9]. The most important biological function of chemokines is to act as chemoattractant to mediate the migration of cells which is involved in homeostatic and inflammatory processes. Chemokine CC-motif ligand 27 (CCL27), also known as cutaneous T cell-attracting chemokine (CTACK), is a skin-specific chemokine that is mainly expressed in epider-

mal keratinocytes [10]. CCL27 acts as a key factor in T cell-mediated inflammation of skin by associated with homing of memory T lymphocytes to the skin [11, 12]. Studies on psoriasis have suggested that over-expressed CCL27 promotes the persistent deterioration in psoriasis by its essential role in immunoregualtory and inflammatory progressions [13]. Whilst in recent years, investigators have revealed that CCL27 is highly expressed in melanoma and SCC and is related to mediating immune evasion of tumor cells inducing tumor development [14, 15].

In this study, we analyzed the expression pattern and biological function of CCL27 in endometriosis for the first time. We examined the expression of CCL27 in serum samples and ectopic/eutopic endometrial tissues from patients with endometriosis. We further separated endometrial stromal cells (ESCs) from endometriosis, and assessed the effects of CCL27 on cell proliferation, metastasis and adhesion of ESCs by RNA interference. The data showed that CCL27 was highly expressed in serum and tissues of endometriosis as comparing to controls. And over-expressed CCL27 in ESCs promoted cell proliferation, migration, invasion and adhesion by facilitating phosphorylation of JAK-STAT signal pathway and upregulating relative protein levels of MMP2, MMP9, CD44 and ICAM1.

## Materials and methods

### *Patients*

Endometriosis samples, including eutopic endometrial tissues (n=10), ectopic endometrial tissues (n=10) and peripheral blood samples (n=60) were collected from 60 patients with surgically confirmed endometriosis. Control endometrial tissues (n=10) and peripheral blood samples (n=60) were from 60 women without evident endometriosis. Endometrial tissues were obtained by biopsy during laparoscopy. All patients were aged between 25 and 45 years, and had no hormone treatment for at least two weeks before tissue collection. The patients enrolled in this study were treated at Women's Hospital, School of Medicine, Zhejiang University (Hangzhou, China). Informed and written consent was obtained from all patients according to the ethics committee guidelines.

### *Cell isolation*

The endometrial tissues from endometriosis patients were collected in Hank's balanced salt solution, and the ESCs were isolation according to the methods described previously [16, 17]. Briefly, the tissue was minced into 1 $mm^2$ pieces and digested with 0.125% collagenase (Xiaflex) in DMEM media supplemented with 5% fetal bovine serum, 200 U/ml penicillin and 0.2 mg/ml streptomycin without phenol red. Tissue digest was then passed through a sterile steel sieve of 140 μm followed by a 37 μm sterile steel sieve. The ESCs were retained in the filtered isolation medium and then collected by centrifugation. The separated cells were resuspended and seeded with a density of $5\times10^4$/ml in 24-well plates. Stromal cell culture medium consisted of DMEM/F12 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture was maintained at 37°C in a humidified 5% $CO_2$ atmosphere.

### *Enzyme-linked immunosorbent assay (ELISA)*

Serum obtained from peripheral blood samples by centrifugation was subjected to ELISA using 96-well plates coated with antibody against human CCL27. And the concentration of CCL27 in serum was measured according to the manufacture's instructions of CCL27 ELISA kit (Sigma).

### *Immunohistochemistry (IHC)*

The protein level of CCL27 in ectopic and eutopic endometrial biopsy were underwent IHC to evaluated positive areas under microscope. Briefly, antigen retrieval of sample slides was performed with high pressure pretreatment in 0.01 M citrate buffer (pH=6.0) for 15 min. After blocked by goat serum, the slides were incubated with goat polyclonal anti-CCL27 antibody (1:100, Abcam) followed by incubation with HRP-labeled secondary antibody (1:2000, Beyotime). Visualization was performed using commercial DAB reagent kit (Beyotime) according to the manufacture's methods. Nuclei was countered stained with hematoxylin.

### *siRNA transfection*

To silence the expression of CCL27 in separated ESCs, three human CCL27 mRNA (NM_

006664.3) siRNA targeting positions (158-180: GUACUCAGCUCUACCGAAA, 191-213: AGCUACUGAGGAAGGUCA and 330-352: CCAAGAGAGAAAGCUCCAU) were synthesized. A non-specific scramble siRNA sequence was performed as negative control (NC). Lipofectamine 2000 (Invitrogen) was used in the transfection of siRNAs into ESCs according to the manufacture's instructions. All the other assays were underwent at 48 h after siRNAs transfection.

*Reverse transcription and real-time PCR (qRT-PCR)*

Total RNA was extracted from ESCs using the TRIzol reagent (Invitrogen) according to the manufacture's methods. Reverse transcription was performed using cDNA Synthesis Kit (Fermentas). The qRT-PCR was conducted with SYBR Green PCR kit (Fermentas) in an ABI-7300 Real-Time PCR system (ABI) according to the instructions provided by the manufacture. The program of qRT-PCR was 10 min at 95°C, 40 cycles of 15 s at 95°C and 45 s at 60°C, followed by 1 min at 60°C, 15 s at 95°C and 15 s at 60°C. GADPH was served as internal control. The primer sequences were: CCL27 (NM_006664.3), Primer F: 5'-TCCTGCTGCTGTCATTGC-3', Primer R: 5'-GAGAGTGGCTTTCGGTAGAG-3'; and GADPH (NM_001256799.1), Primer F: 5'-CACCCACTCCTCCACCTTTG-3', Primer R: 5'-CCACCACCCTGTTGCTGTAG-3'.

*Proliferation analysis*

ESCs were seeded into 96-well plates and examined at 0 h, 24 h, 48 h and 72 h post RNA interference by using commercial Cell Counting Kit (7seabiotech) according to the procedure provided by manufacture. Absorbance of reacted cells were detected with excitation at 450 nm to evaluate cell proliferation.

*Migration and invasion assays*

Transwell assay was used to evaluate the migration and invasion of ESCs. Separated ESCs were serum starved for about 24 h post RNA interference followed by seeded into upper chamber of a 24-well transwell chamber (Trueline) with a 8 μm pore size. After 24 h cell culture, the cells migrating from upper class to the surface of lower class which was covered with culture medium supplemented with 30% fetal bovine serum beforehand. The migrating cells on the lower membrane were then fixed and stained with 0.05% crystal violet (Solarbio) and counted under the microscope.

Cell invasion assay was also performed using Trueline chamber. The inserts of upper chambers were coated with 50 μl Matrigel (1:2 dilution, BD Biosciences) of which containing a polycarbonate filter. And the rest of experiment processed as migration assay described above.

*Adhesion assay*

ESCs were harvested at 48 h after siRNA transfection and resuspended in DMEM/RPMI-1640 media. Cells were then seeded in 12-well plates covered with Fibronectin beforehand in the density of $1\times10^5$/well and incubated for 1 h at 37°C in 5% $CO_2$ atmosphere. Non-adhering cells were removed by washing 2 times with PBS. Adhering cells were fixed by 4% paraformaldehyde and stained with GIMSA for 15 min. the stained cells were finally counted under microscope.

*Western blotting*

ESCs were collected at 48 h post RNA interference for western blot assay. Cells were washed 3 times with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (Solarbio) supplemented with 0.01% protease inhibitor cocktail (Sigma) for 30 min. Cell lysis was then centrifuged at 4°C for 10 min at the speed of 12000 rpm. The supernatant was separated by SDS-PAGE followed by incubated with appropriate primary and secondary antibodies respectively. Visualization was performed using the enhanced chemiluminescence system. The antibody list was as follow: CCL27 (1:5000, Abcam), MMP2 (1:1000, Abcam), MMP9 (1:500, Abcam), CD44 (1:1000, CST), ICAM1 (1:1000, Abcam), p-JAK2 (1:1000, CST), JAK2 (1:1000, CST), p-STAT3 (1:1000, Abcam), STAT3 (1:1000, Abcam), GAPDH (1:1500, Abcam) and HRP-labeled secondary antibodies (1:1000, Beyotime). GAPDH was served as internal control.

*Statistical analysis*

At least three independent experiments were performed in every assay. The results were analyzed by SPSS 13.0 statistical package (SPSS). Data were xpressed as mean (± SD) and analyzed with t-test for multiple compari-

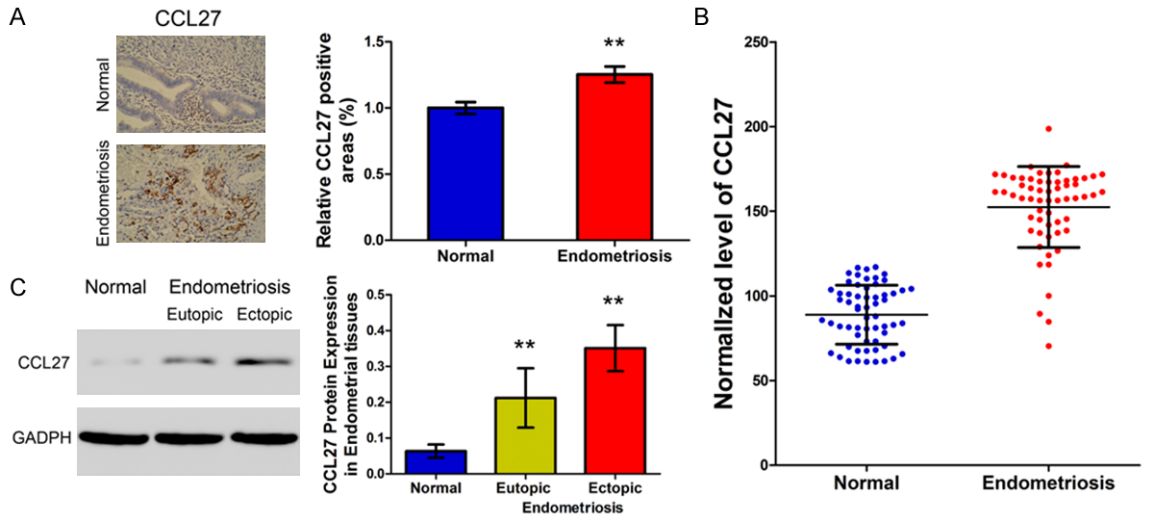


**Figure 1.** Expression of CCL27 in endometriotic tissue and serum samples. A. IHC staining and relative positive areas of CCL27 in normal and endometriotic tissues (n=10, original magnification ×200). B. Protein levels of CCL27 in serum samples from patients with and without endometriosis examined by ELISA (n=60, P<0.000001). C. Protein expression of CCL27 in eutopic and ectopic endometrial tissues (n=10). Data were shown as mean ± SD, *P<0.05, **P<0.01 (compared to controls).

sons. *P*-value less then 0.05 was considered statistically significant.

## Results

### *Over expressed CCL27 in endometriosis tissues*

To verify the expression pattern of CCL27 in endometriosis, we detected the mRNA and protein expression of CCL27 in ectopic (n=10)/eutopic (n=10) endometriotic and normal (n=10) endometrial tissues using western blot and IHC assays. Moreover, the protein level of CCL27 in endometriosis (n=60) and normal (n=60) serum samples was examined using ELISA assay. As shown in **Figure 1A** and **1B**, CCL27 was highly expressed in both tissue and serum samples of endometriosis as comparing to normal endometrial tissues. As **Figure 1C** showed, protein level of CCL27 in ectopic endometriosis tissues was significantly higher than that in eutopic endometriotic tissues. These results showed that CCL27 was abnormally highly expressed in endometriosis, especially in ectopic endometriosis tissues.

### *Knockdown of CCL27 in ESCs of endometriosis and the inhibition of cell proliferation*

To further evaluated the biological functions of CCL27 in endometriosis, siRNA transfection was used to knock down CCL27 expression in ESCs separated from ectopic endometriosis tissues. Three targeting positions of CCL27 mRNA was performed in RNA interference. After 48 h, transfected ESCs were examined by qRT-PCR and western blot. As **Figure 2A** showed, CCL27 expression was depressed significantly post RNA interference compared to mock. The inhibition rates of mRNA expression and protein level were 78.23% and 84.98%, in position 1, 46.79% and 58.73% in position 2, 66.84% and 67.62% in position 3. And the position1 (158-180: GUACUCAGCUCUACCGAAA) was selected as the targeting position to undergo further experiments with highest inhibition rate.

Then, we examined the proliferation of ESCs at 0 h, 24 h, 48 h and 72 h post RNA interference by the use of CCK-8 assay. As shown in **Figure 2B**, silencing of CCL27 inhibited proliferation of ESCs obviously. The data suggested that abnormal expression of CCL27 may facilitate cell growth of endometriosis.

### *Silencing of CCL27 inhibited metastasis of endometriosis ESCs*

Metastasis is a key symptom of endometriosis. To reveal the effects of CCL27 on endometriosis, we assessed migration and invasion of ESCs. In **Figure 3**, depletion of CCL27 suppressed ESCs migration and invasion obvious-

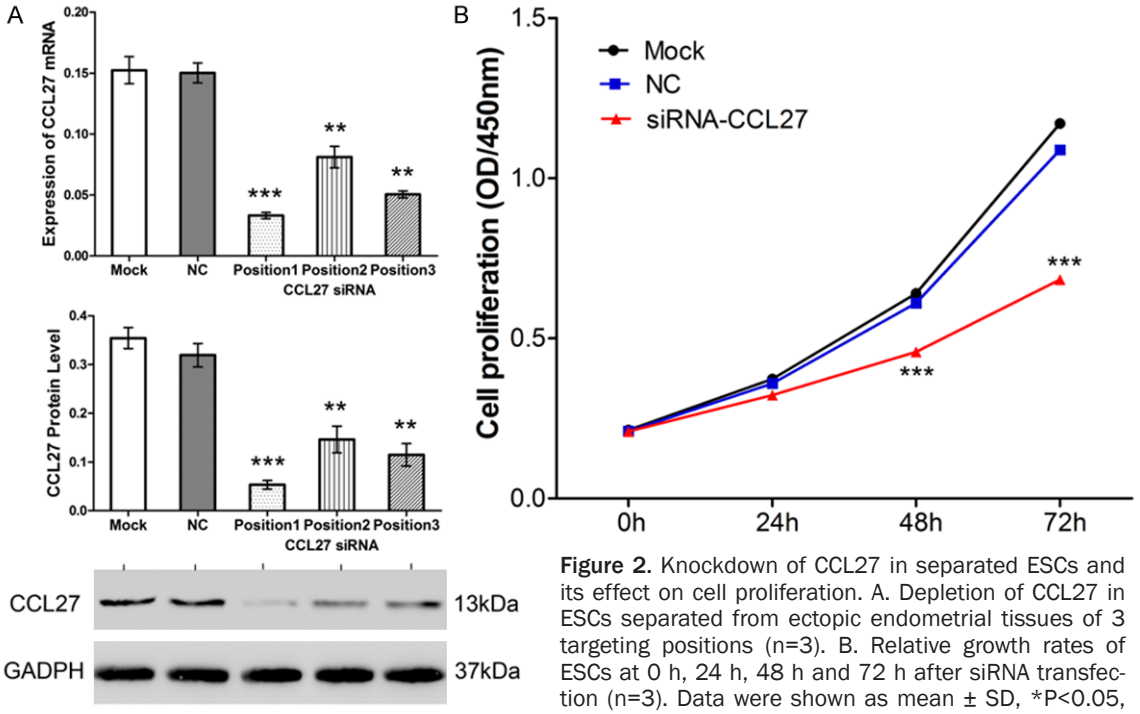


**Figure 2.** Knockdown of CCL27 in separated ESCs and its effect on cell proliferation. A. Depletion of CCL27 in ESCs separated from ectopic endometrial tissues of 3 targeting positions (n=3). B. Relative growth rates of ESCs at 0 h, 24 h, 48 h and 72 h after siRNA transfection (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (compared to controls).

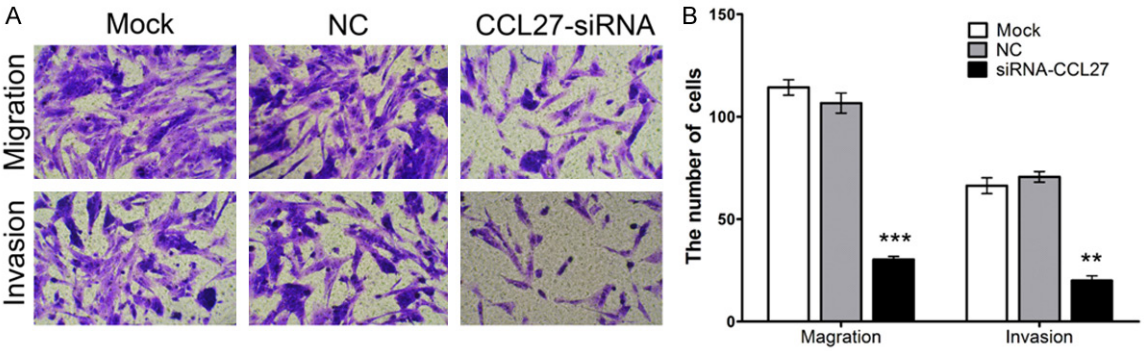


**Figure 3.** Depletion of CCL27 inhibited metastasis in ectopic ESCs. Migrating and invading cell numbers were counted under microscope after analyzed by transwell assay post RNA interference. A and B. Effects of CCL27 depletion on cell migration and invasion in ESCs (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (compared to controls).

ly. The cell numbers of migration and invasion were: 114 ± 7 and 66 ± 7 in Mock, 107 ± 9 and 71 ± 5 in NC, 30 ± 3 and 20 ± 4 in CCL27-siRNA. These data showed that depletion of CCL27 significantly inhibited ESCs metastasis of endometriosis.

*Silencing of CCL27 depressed adhesion of endometriosis ESCs*

As described above, silencing of CCL27 inhibited metastasis of endometriosis, we further examined the effects of CCL27 on adhesion to fibronectin of ESCs. The numbers of adhering ESCs in mock, NC and siRNA were 45 ± 4, 39 ± 4 and 19 ± 3 respectively (**Figure 4**). And the data revealed that over expressed CCL27 promoted cell adhesion in endometriosis.

*Effects of CCL27 knockdown on key protein levels of cell adhesion*

To investigate the promotion mechanism of metastasis and adhesion of ESCs induced by highly expressed CCL27, we measured some key protein levels containing MMP9, MMP2,

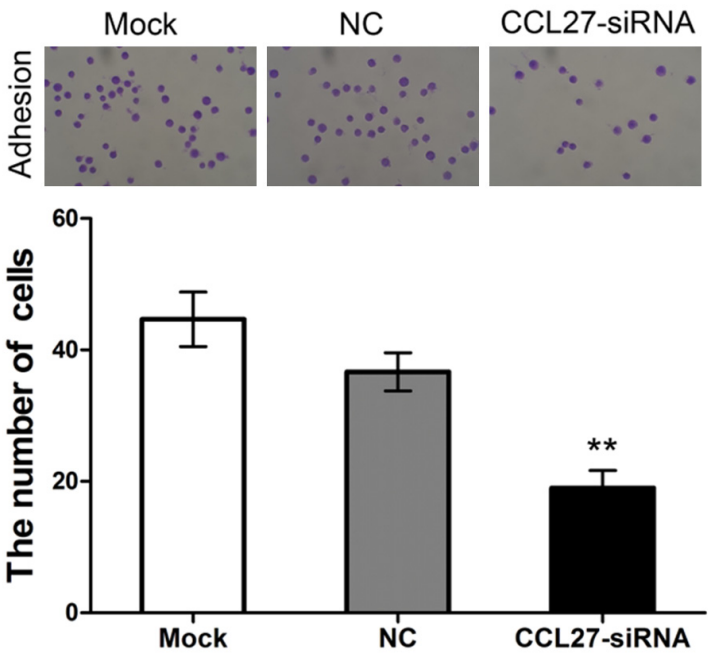


**Figure 4.** Silencing of CCL27 suppressed adhesion of ectopic ESCs. Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (compared to controls).

CD44 and ICAM1 using western blot at 48 h post RNA interference. As **Figure 5** showed, expression levels of all target proteins were notably depressed by silencing of CCL27. And the decrease rates were 82.46% in MMP9, 56.24% in MMP2, 55.90% in CD44 and 41.36% in ICAM1. As the results showed, depletion of CCL27 decreased the cellular protein levels relative to cell metastasis and adhesion.

*Effects of CCL27 knockdown on JAK2/STAT3 signal pathway*

As over expressed CCL27 may promote cell proliferation in endometriosis, we measured protein levels of some essential factors in JAK2/STAT3 signal pathway of ESCs. JAK2, p-JAK2, STAT3 and p-STAT3 were examined using western blot. As **Figure 6** showed, the ratios of p-STAT3/STAT3 and p-JAK2/JAK2 were 2- and 3-folds decrease in transfected ESCs as comparing to mocks. And protein levels of p-STAT3 and p-JAK2 were decreased obviously post RNA interference (decrease ratios: p-STAT3 54.87% and p-JAK2 77.91%, which were not shown in **Figure 6**). The cellular protein expression of STAT3 and JAK2 were slightly inhibited by depletion of CCL27 which did not reach statistical significantly. Summarily, knockdown of CCL27 depressed phosphorylation of STAT3 and JAK2.

## Discussion

Endometriosis is a multifaceted disease of unknown etiology and debated pathogenesis. Its complicated symptoms analogy with autoimmune diseases and cancers induce increasing researches of endometriosis focused on factors involved in genetic and immunological processes. Current evidence reveal that chemokine plays a key role in the pathogenesis of endometriosis [1]. And the recruitment and activation of immune cells induced by chemokines in implants may result in endometriosis-related pain and infertility [18]. Relative studies suggest that members of CC-chemokine subfamily are mainly associated with the pathogenesis of endometriosis. For examples, CCL5 is reported obviously elevated in patients with endometriosis. The stimulated expression of CCL5 in ESCs can be inhibited by progestin treatment, which is an effective therapy for pain relief and control of endometriosis [19-21]. And increased CCL2 level was detected in peritoneal fluid of endometriosis patients [22]. Li et al. demonstrated that CCL2 improved cell proliferation, invasion by activating PI3K/AKT and MAPK/ERK pathways in ESCs separated from endometriotic tissues [23]. Moreover, abnormally expressed CCL11 might promote angiogenesis and subsequence progression of endometriosis [24]. In this study, we attempted to reveal the association between endometriosis and CCL27, another important member of CC-chemokine subfamily. We examined the expression of CCL27 in tissue and serum samples from endometriotic patients using IHC, western blot and ELISA. And we found that protein level and positive areas of CCL27 were significantly higher in eutopic and ectopic endometriotic tissues compared to normal endometrial tissues. The elevated CCL27 was also detected in serum samples of endometriosis. These data implicated that CCL27 was highly expressed in endometriosis.

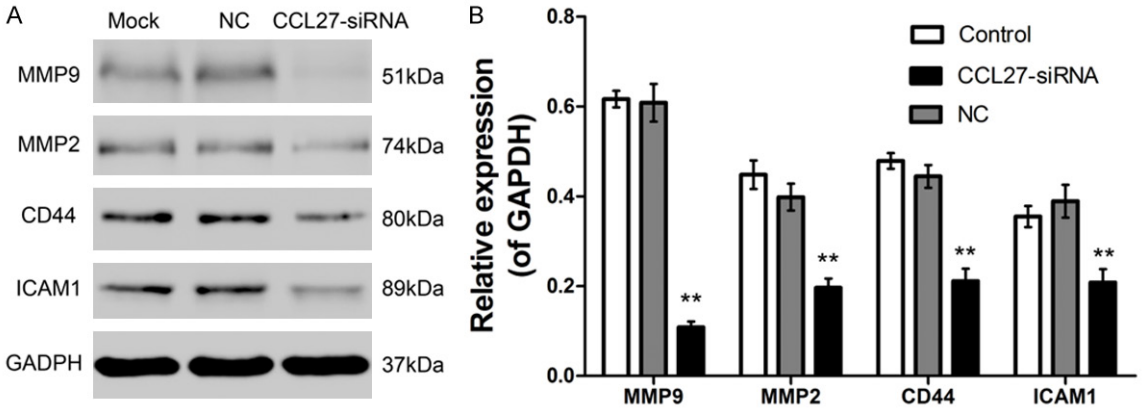


**Figure 5.** Silencing of CCL27 depressed the protein levels of factors related to cell metastasis and adhesion. A and B. Protein levels of MMP9, MMP2, CD44 and ICAM1 in ectopic ESCs examined by western bolt at 48 h post RNA interference (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (compared to controls).

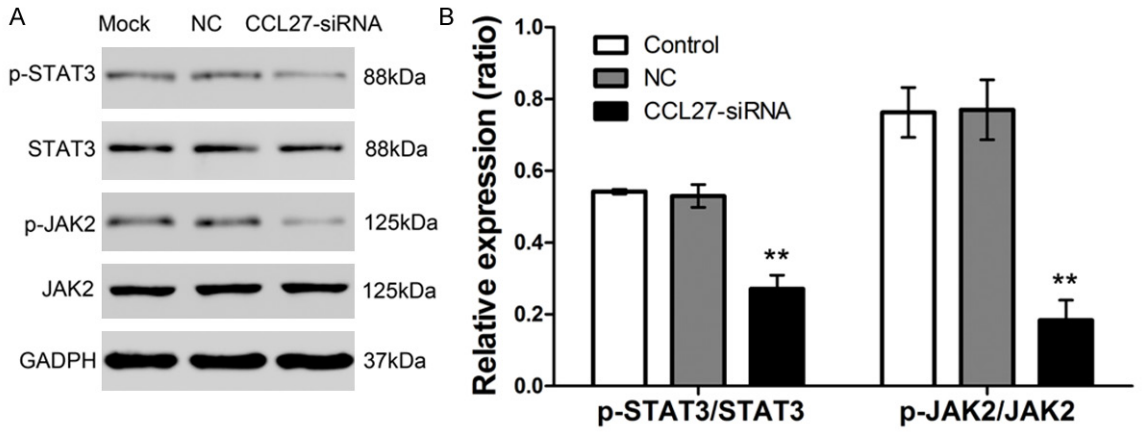


**Figure 6.** Knockdown of CCL27 inhibited the activation of JAK2/STAT3 signaling pathway. A and B. Protein levels of p-STAT3, STAT3, p-JAK2 and JAK2 in ectopic ESCs examined by western bolt at 48 h post RNA interference (n=3). Data were shown as mean ± SD, *P<0.05, **P<0.01, ***P<0.001 (compared to controls).

To further investigated the function of CCL27 in endometriotic progression, we transfected CCL27-siRNA into ESCs separated from ectopic endometrial tissues to silence the CCL27 expression. And we analyzed cell proliferation, migration, invasion and adhesion of ESCs at 48 h post RNA interference. The results suggested that silenced CCL27 inhibited cell growth, metastasis and adhesion of ESCs obviously. These result showed that CCL27 may act as a promoting factor in endometriosis development.

As cell metastasis and adhesion in ESCs of endometriosis were promoted significantly by over-expressed CCL27. We then selected four relative proteins including MMP2, MMP9, CD44 and ICAM1 to examine their intracellular levels. MMPs are important factors in metastasis for their degradation capacity of extracellular matrix. ICAM1 and CD44 are surface glycoproteins promoting adhesion in immunological and inflammatory reactions. All of them play role in pathogenesis of endometriosis [25, 26]. Researches have reported that expression of MMPs and ICAM1 is much greater in ectopic endometrium than that in normal endometrium [27]. Moreover highly expressed ICAM1 may be involved in the promotion of susceptibility to endometriosis [28, 29]. At 48 h after siRNA transfection, target protein levels were detected using qRT-PCR. And as results showed, knockdown of CCL27 notably down-regulated the intracellular levels of MMP2, MMP9, CD44 and ICAM1 of ESCs. So over-expressed CCL27 may contribute to abnormally elevated expression of MMPs and ICAM1 in endometriosis.

Finally, to reveal the functional mechanism of CCL27 in endometriosis, we analyzed the effects of CCL27 on JAK/STAT pathway in ESCs. The levels of p-STAT3, STAT3, p-JAK2 and JAK2 were examined using qRT-PCR. We found that the values of p-JAK2/JAK2 and p-STAT3/STAT3 were obviously down-regulated in ESCs post RNA interference. Activation of JAK/STAT signaling pathway is involved in mediating the regulation of various aspects of response, such as defense, differentiation, proliferation, hematopoiesis and oncogenesis [30, 31]. For example, previous study have implicated that activation of JAK2/STAT3 pathway is associated with leptin-mediated promotion of invasion and migration in hepatocellular carcinoma cells [32]. And Wu et al. indicated that JAK/STAT3 signaling was involved in metastasis in endometrial cancer cells by regulating intracellular levels of MMP2 and MMP9 [33]. In endometriosis, the activation of JAK/STAT pathway is indicated related to the pelvic pain by regulating the expression of pro-inflammatory factors including IL-1β and tumor necrosis factor-α [34-36]. Oh et al. also demonstrated that the leptin-induced proliferation in epithelial endometriotic cells was mediated by the activation of JAK2/STAT3 pathway [37]. The results of present study indicated that highly expressed CCL27 may act as a signaling molecule to activate JAK2/STAT3 pathway in ESCs. And the promotion of cell proliferation and metastasis may induced by the JAK2/STAT3 pathway activated by CCL27 in ESCs.

Summarily, this study for the first time uncovered the expression pattern and molecular mechanism of CCL27 in endometriosis. We found that CCL27 was abnormally elevated in endometriotic tissue and serum samples. And over-expressed CCL27 contributed to the promotion of proliferation, metastasis and adhesion in ESCs separated from endometriotic patients, through up-regulating MMP2, MMP9, CD44 and ICAM1 and activating JAK2/STAT3 signaling pathway. This may provide a novel therapy target for the treatment of endometriosis.

## Acknowledgements

This work was supported by research funds from 2013 Zhejiang Province Natural Science Funds Grant (No. Y13H040003) and 2014 National Natural Science Funds of Youth Science Foundation Grant (No. 81401181).

## Disclosure of conflict of interest

None.

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