

Original Article

Osteopontin promoting the pathogenesis of endometriosis and changes in cell invasion and migration through activation of NF- κ B signaling in endometrial epithelial cells

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Abstract: Background: Endometriosis, characterized by the appearance of active endometrial tissue (glands and stroma) outside the uterine cavity, leads to dysmenorrhea, chronic pelvic pain, infertility and/or clinical symptoms that seriously affect the health of women. The etiology and pathogenesis of endometriosis are largely unknown. Oncology studies have shown that osteopontin (OPN) binds to its receptor to activate nuclear factor- κ B p65 (NF- κ B p65) which relies upon the MAPK/PI3K pathway, thus promoting the secretion of urokinase-type plasminogen activator and matrix metalloproteinases (MMPs) which mediate cell invasion and migration to induce tumor formation and metastasis. Since the characteristics of endometriosis, such as adhesion, invasion and metastasis, are similar to those in cancer, vascular endothelial growth factor, OPN, MMP-9, and NF- κ B have been associated with the development of endometriosis. These factors may be closely associated with ectopic endometrial adhesion and invasiveness. However, the role of OPN in regulating the abnormal expression of endometrial epithelial cells (EECs) remains unknown. We tested the hypothesis that OPN plays an important role in regulating MMP-9 expression levels to change cell invasion and migration via NF- κ B-mediated signaling pathways. Methods: We used immunohistochemistry to detect the expression of OPN and NF- κ B p65 proteins and mRNA in eutopic and ectopic endometria of patients with endometriosis. Primary culture and identification of endometrial epithelial cells (EECs) was performed to investigate OPN and NF- κ B p65 expression levels in EECs after OPN-specific small interfering RNA (siRNA) downregulation and plasmid upregulation. Transwell assays were carried out to investigate the invasiveness and migration ability of EECs. Results: Expression levels of OPN and NF- κ B p65 proteins in eutopic and ectopic endometria of endometriosis patients were higher than in normal endometria. Treatment of EECs with siRNA directed against OPN reduced OPN, NF- κ B nuclear translocation proteins and mRNA expression. Cell invasion and migration and NF- κ B signaling was suppressed significantly by OPN siRNA. These effects of OPN and NF- κ B p65 on EECs were also demonstrated in OPN-overexpressing cells. Conclusion: Our results suggest that OPN regulates NF- κ B p65 and MMP-9 expression in EECs by activating NF- κ B signaling, providing new insights into the mechanism of invasion and metastasis in endometriosis.

Keywords: Endometriosis, osteopontin, NF- κ B p65, cell invasion, cell migration

Introduction

Endometriosis is a chronic, inflammation-driven, local environment disorder which results in infertility and pelvic pain in 35%-50% of women of reproductive age. It is associated with proliferation, invasion and metastasis of endometriotic lesions [1-4]. Although several theories

have been presented in an attempt to explain the etiology of endometriosis, such as the retrograde menstruation theory proposed by Sampson, the molecular mechanisms of the disorder remain unclear [5, 6]. Current management options for endometriosis are limited, mainly comprising surgical intervention and hormone therapy [7]. Thus, there is a need to

identify the target factors which are associated with endometriosis lesion growth at multiple locations.

Endometriosis is associated with the characteristics of adhesion, invasion and metastasis, similar to those observed in cancers, and these features can drive the eutopic endometrium to the extra-uterine location. Osteopontin (OPN), a secreted negatively-charged phosphoglycoprotein, plays an important role in cell invasion and migration [8-12]. According to oncology studies, the combination of OPN and its receptor CD44 or integrin can initiate a series of signal transduction pathways that regulate tumor progression and invasion [13-15]. A large body of evidence has confirmed that, in many malignant tumors such as breast cancer, gastric cancer, and cervical cancer, OPN expression levels are enhanced, indicating that OPN may be the key factor in the early invasion and metastasis of malignant tumors [16-18]. However, the molecular mechanisms for the role of OPN in the progression of endometriosis are not completely clear.

OPN, expressed and secreted by various cells, plays an important role in cell adhesion, invasion and apoptosis, and neovascularization, in addition to participating in the inhibitory activities of cell adhesion, chemotaxis, and stress-dependent angiogenesis [19]. In recent years, it has been reported that, in ovarian and breast cancer cells, OPN promotes the activation of urokinase-type plasminogen activator (uPA)/matrix metalloproteinase (MMP)-2 and increases cell migration and invasion via promoting NF- κ B subunit p65 from the cytoplasm to the nucleus, combined with a variety of cellular gene promoter or enhancer sequence specific sites [20]. Oncology studies show that OPN binds to its receptor to activate NF- κ B signaling, which relies upon the MAPK/PI3K pathway, thus promoting the secretion of uPA and MMPs and mediating cell invasion and cell migration, ultimately inducing tumor formation and metastasis [21]. Since the characteristics of endometriosis are similar to those in tumors (e.g. adhesion, invasion, and metastasis), many researchers associate vascular endothelial growth factor, OPN, MMP-9, and NF- κ B, with the development of endometriosis, indicating that these factors may be closely related with ectopic endometrial adhesion and invasiveness [22].

However, the relationship between OPN and NF- κ B, and the explicit mechanism by which OPN and NF- κ B promote cell invasion in endometriosis, are not completely understood. The aim of this study was to investigate the relationship between OPN and NF- κ B subunit p65 expression in endometriosis and their relationship with cell invasiveness.

Methods

Patient recruitment and characterization

The study was authorized by the ethics committee of Ningxia Medical University. All study subjects provided written informed consent. All endometrial tissue was obtained from the Department of Obstetrics and Gynecology, General Hospital of Ningxia Medical University. The active group consisted of 35 patients (secretory phase, n=18; proliferative phase, n=17) selected from December 2013 to June 2014 at the General Hospital of Ningxia Medical University due to "ovarian endometriosis" surgery. Patient information, including age, menstrual cycle, and stage of endometriosis, was collected via access to medical records. Endometrial tissue was obtained by curettage during surgery in patients with pathologist-confirmed endometriosis. Patients were not permitted to have received hormonal therapy during the previous 3 months. All patients had stage III endometriosis (according to the American Fertility Association, 1985 amendment to the endometriosis staging act). All cases of endometriosis were confirmed by the Pathology Department of the Affiliated Hospital of Ningxia Medical University. The control group consisted of 35 patients who underwent laparoscopic bilateral tubal ligation (secretory phase, n=18; proliferative phase, n=17); these patients were confirmed by the pathologist not to have endometriosis.

Identification of primary endometrial glandular epithelial cells

Tissues were washed three times with sterile Hank's precooling phosphate buffered saline (PBS) three times, cut into pieces of approximately 1 mm³, and digested in 10 ml of PBS containing type IV collagenase (0.03%; Sigma, St. Louis, MO) and 10 U/ml DNase I (Sigma) at 37°C for 30 min to digest. The digested supernatant cell suspension was then transferred to

another centrifuge tube and centrifuged for 30 sec at 500 rpm; therefore, the suspended cells were separated by differential centrifugation of the endometrial glandular epithelial cells and the individual stromal cells. After repeated centrifugation and under microscopic observation, purified human endometrial glandular epithelial cells were obtained and added to the cell culture medium in a 100 mm Petri dish, using disposable pipettes. Cell culture fluid was added every other day to adjust cell intensity to the desired cell mass and cell cultures were maintained in a 5% CO₂ incubator until they reached 90% confluence.

Immunocytochemistry

Briefly, endometrial epithelial cells (EECs) were cultured on a sterile cover glass in six-well plates, fixed with 4% polyformaldehyde for 10 min, rinsed for 5 min in PBS, then 1% bovine serum albumin (BSA) for 30 min, before adding 1% BSA-diluted antibody at 37°C for 2 h. Dilution ratios were: Vimentin (1:500), pan CK (1:500), OPN (1:100) and NF-κB p65 (1:100). Cells were then incubated with a secondary antibody (PV9000) and visualized with 3, 3'-diaminobenzidine tetrahydrochloride. Cells were subjected to nuclear counterstaining (visualized blue) with hematoxylin.

Gene silencing

EECs were divided into two groups (small interfering RNA [siRNA]-OPN and siRNA-scrambled). Scrambled siRNA was used as a negative control. The OPN siRNA sequences were sense, 5'-GGUCAAAUUAAGAAGUUTT-3' and antisense, 5'-AACUUCUUAGAUUUUGACCTC-3'; scrambled siRNA sequences were sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. All siRNAs were designed and purchased from Shanghai GenePharma Inc. The siRNA oligonucleotides were transfected into EECs using X-tremeGENE reagent (Roche Diagnostics). The ratio of transfection reagent to siRNA was 10 µl:2.5 µg per 2×10⁵ cells. The transfection procedure was carried out according to the manufacturer's instructions. The efficiency of siRNA interference was assessed by Western blot 48 h later.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Trizol (Invitrogen), chloroform, isopropanol and ethanol were used successively to extract total

RNA from EECs. RNA concentration was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). For each sample, 1 µg of total RNA was used to synthesize cDNA with TIANscript Reverse Transcription Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was performed with SYBR Premix Ex Taq II (TaKaRa Biotech, Dalian, China) in triplicate for each sample according to the manufacturer's instructions using a Roche LightCycler480. Amplification of β-actin was used for normalization. Primers used in this assay were OPN: sense, 5'-ACAGCCGTGGGAAGGACAGTTA-3', antisense, 5'-CCTGACTATCAATCAGATCGGAATG-3'; NF-κB: sense, 5'-ATCTGCCGAGTGAACCGAACT-3', antisense, 5'-CCAGCCTGG TCCCGTGA-3'; β-actin: sense, 5'-AGCGAGCATCCCCCAAGTT-3', antisense, 5'-GGGCACGAAGGCTCATCATT-3'. The relative expression levels of each gene were determined using the 2^{-ΔΔCt} method.

Cell culturing and transfection

Drosophila S2 cell culture was grown in SFX medium (HyClone) at 25°C. Cells were transfected with Cellfectin II reagent (Invitrogen) according to the manufacturer's recommendations (approximately 8 × 10⁵ cells per transfection). Two hours before transfection, the cells were placed into the wells of a 12-well plate. DNA (0.5 µg) was used for one transfection. In all cases, co-transfection of the tested constructs (the firefly luciferase gene was used as a reporter gene) and a control construct (the jellyfish luciferase gene was under the control of the actin gene promoter at a 1:19 ratio) was performed. Cells were harvested 48 h after transfection.

Construction of plasmids and transfection

Full-length OPN cDNA and splice variants were amplified from EECs by reverse transcription polymerase chain reaction (RT-PCR). Total RNA and cDNA synthesis were performed as described above. The primer used for the OPN coding sequence (5'-GAAATTTGTGATGCTATTGC-3') was amplified and cloned into the mammalian expression vector mCherry using neomycin to generate pmCherry-C1. Plasmid pmCherry-C1-OPN was transfected into EECs by liposome-mediated transfection with a primary cell fusion degree of 70-90% for plasmid transfection. After respective dilution of DNA in 10 µl

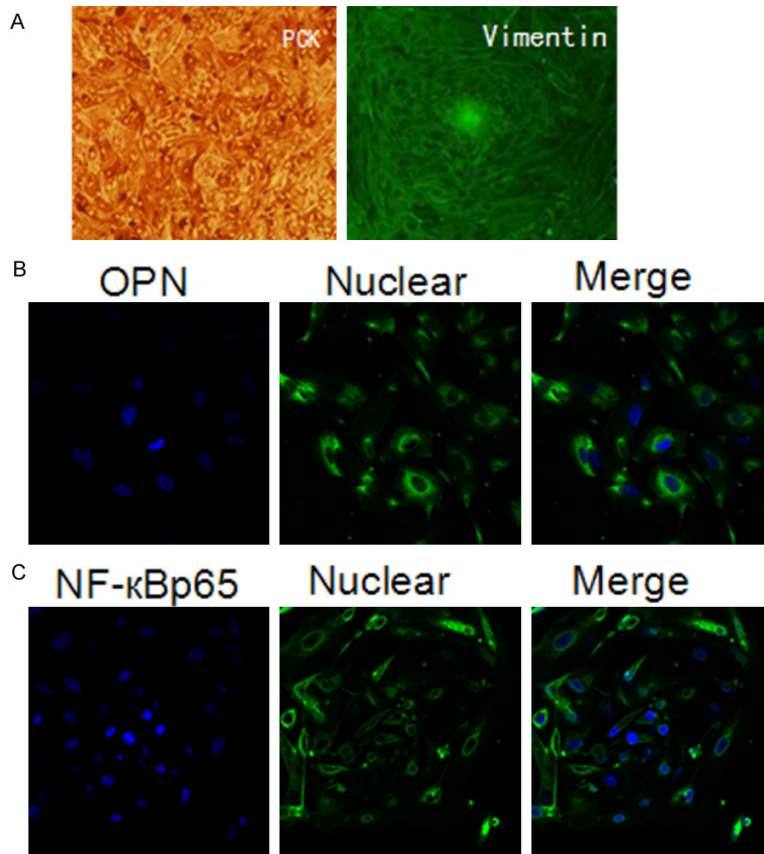


Figure 1. Primary endometrial epithelial cell identification, and NF-κB p65 and OPN expression in patients with endometriosis.

Opti-MEM serum-free culture medium and dilution of 500 μ l Lipofectamine 2000 in Opti MEM serum-free culture medium, mixtures were incubated at room temperature for 5 min then combined for a further 20 min at room temperature. Complexes were added to the cells in 60 mm culture dishes containing 5 ml DMEM/F12 medium and incubated for 4 to 6 h.

Western blot

A total of 1×10^7 EECs was washed twice with cold PBS, and then harvested in 250 μ l RIPA lysis buffer with 10 μ l protease inhibitor cocktail (Sigma-Aldrich). Protein aliquots (50 μ g) were run on a 10% SDS polyacrylamide gel, and then transferred to a nitrocellulose membrane (Amersham). The membrane was blocked in 5% milk powder in PBS containing 0.1% Tween 20 (PBS-T) at room temperature for 1 h. The membrane was incubated with 1% milk powder in PBS-T containing a mouse anti-human monoclonal OPN (1:100, Abcam) or NF-κB p65

(1:100, Abcam) antibody at 4°C overnight. A rabbit anti-human polyclonal β -actin antibody (1:500; Beyotime) was used as the internal control. The membrane was then probed with goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000 for both; Beyotime) in 1% milk powder in PBS-T for 1 h. Immunoreactive proteins were detected with an enhanced chemiluminescence reagent (Amersham) and exposed to X-ray film (FUJIFILM, Japan).

Transwell assay

One layer of 2% Matrigel was used to cover the top chamber of 24-well micropore polycarbonate membrane insert (8 μ m; Millipore, Billerica, MA). EECs were transfected with siRNA or treated with E2 as described above. Approximately 1×10^5 /well trypsinized cells were seeded into the top chamber using FBS-free

DMEM/Ham's F12. DMEM/Ham's F12 with 10% charcoal-dextran stripped FBS was added to the lower chamber as a chemoattractant. The plate was placed at 37°C for 24 h. Assays were then stopped by removing the non-invading cells in the top chamber with swabs. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with hematoxylin. Cells in five visual fields per insert were counted and photographed (400 \times magnification).

Statistics

All repeated data are shown as mean \pm SEM. The Student's t-test was used to compare the differences between each treatment and the control. *P* values < 0.05 were considered to be statistically significant. SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. PRISM 5.0 for Windows (GraphPad, San Diego, CA, USA) was used to create histograms.

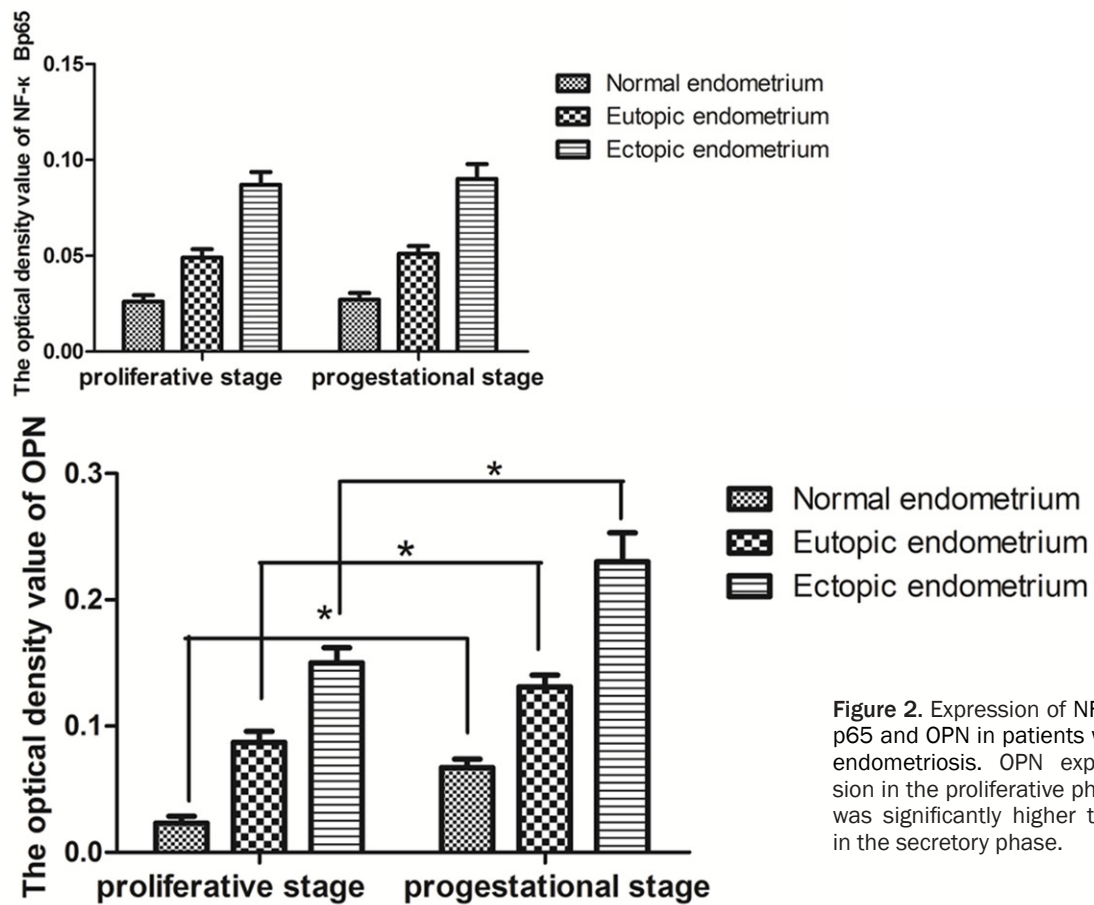
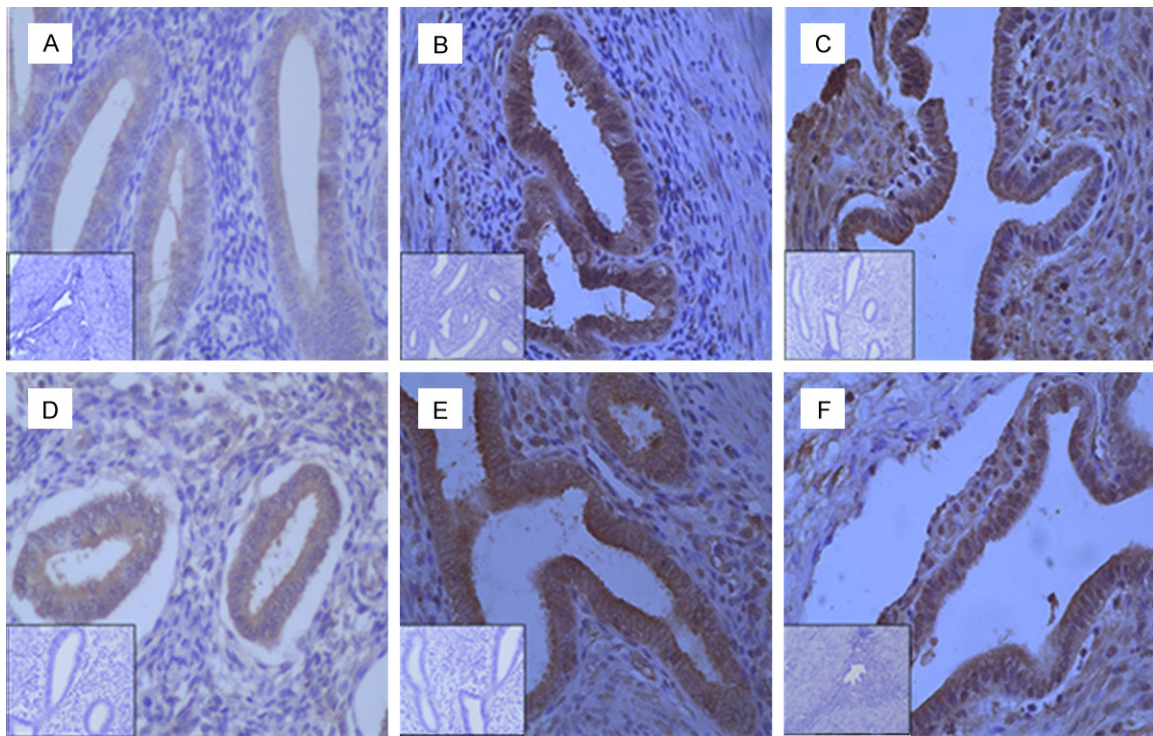


Figure 2. Expression of NF-κB p65 and OPN in patients with endometriosis. OPN expression in the proliferative phase was significantly higher than in the secretory phase.

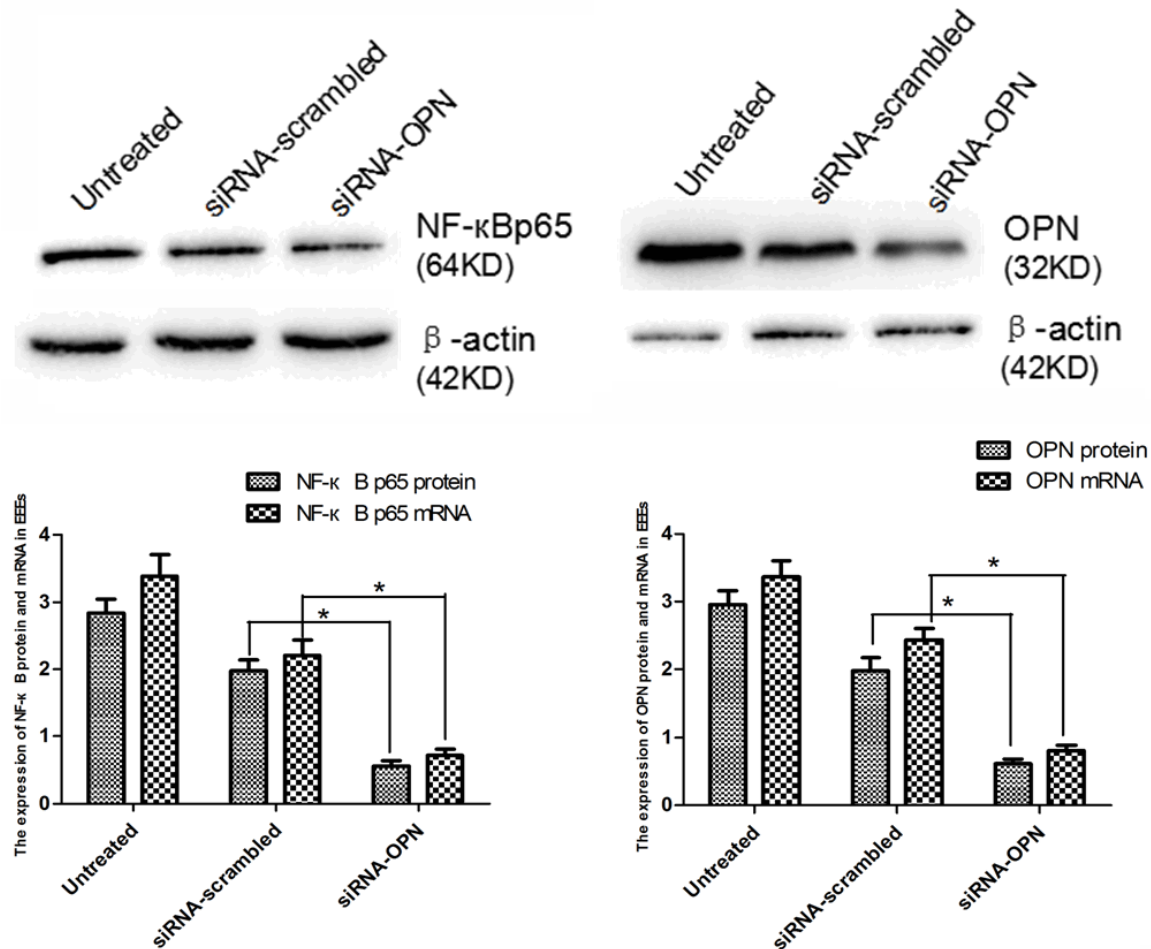


Figure 3. Changes in NF-κB p65 and OPN expression in epithelial cells before/after OPN siRNA intervention. The expression of NF-κB p65 and OPN protein and mRNA decreased significantly ($P < 0.05$) after intervention with OPN siRNA in eutopic primary EECs.

Results

Identification of primary endometrial glandular epithelial cells

Endometrial glandular epithelial cells were isolated successfully by primary cell culture. Endometrial glandular epithelial cells grew clonally in clumps. To determine that the cultured cells were endometrial glandular epithelial cells, we identified the primary cell by immunocytochemistry using the broad spectrum epithelial cell marker keratin, and cell-derived mesenchymal vimentin. As expected, most cultured primary cells were positive for pan CK (immunostaining in the cytoplasm), which is a feature factor for cells of epithelial origin; however, they were negative for vimentin, which represents cells of mesenchymal origin (**Figure**

1A). The successful isolation of EECs laid a solid foundation for subsequent experiments.

Expression of NF-κB p65 and OPN in patients with endometriosis

NF-κB p65 is expressed mainly in the cytoplasm of epithelial cells, with a small amount of expression in the nucleus, and a trace in vascular endothelial epithelial cells. It is likely that OPN is also expressed in the cytoplasm of epithelial cells. In three groups, the expression of OPN and NF-κB p65 protein was mainly in the cytoplasm of EECs and stromal cells, with very little expression in vascular endothelial cells. The expression of NF-κB p65 protein in the ectopic endometrium of patients with endometriosis was higher than in eutopic and normal endometria, with significant between-group differences ($P < 0.05$). OPN expression in the pro-

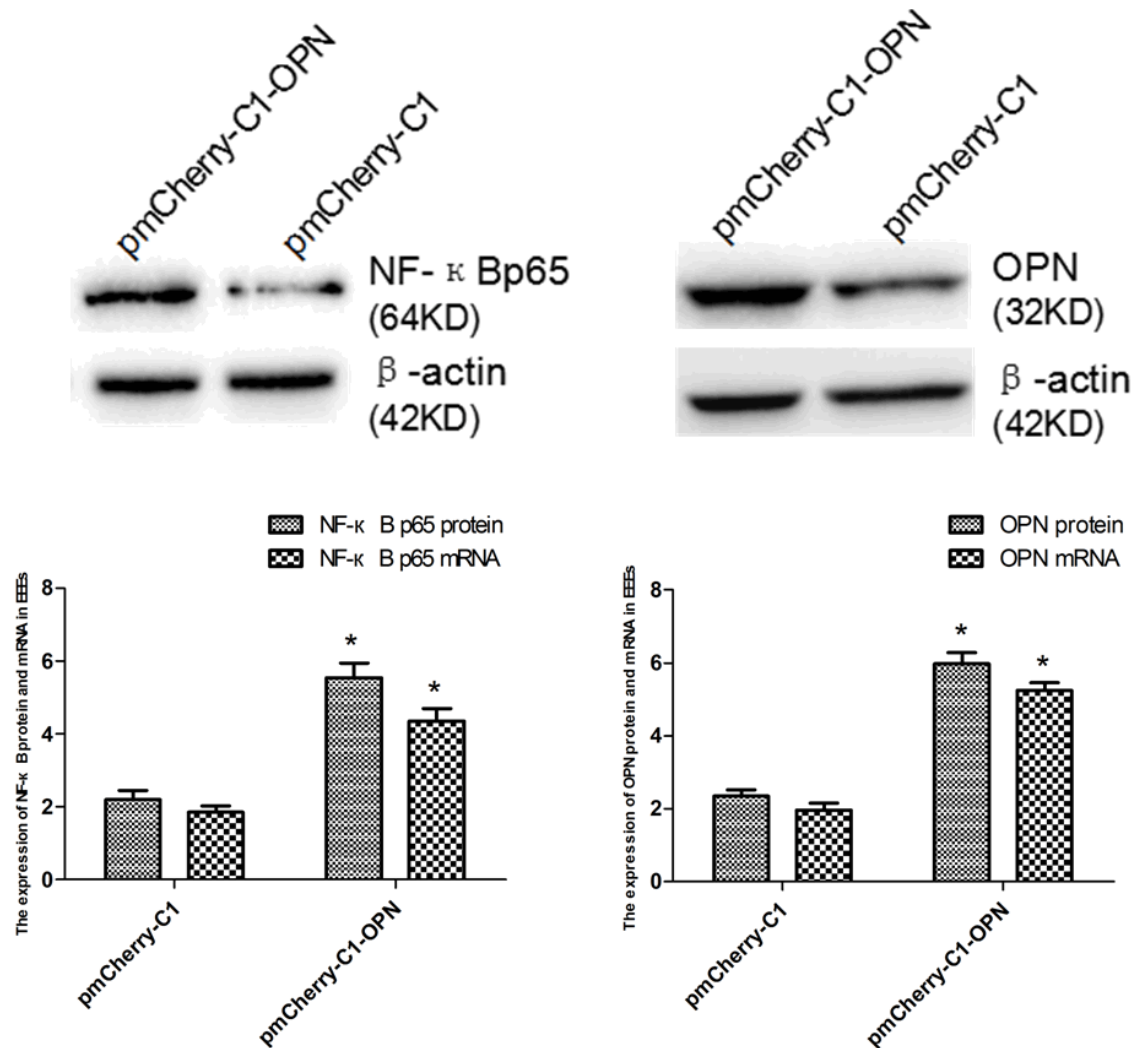


Figure 4. Changes in NF-κB p65 and OPN expression in epithelial cells before/after OPN plasmid upregulation. The expression of NF-κB p65 and OPN protein and mRNA increased significantly ($P < 0.05$) after intervention with pmCherry-C1-OPN in eutopic primary EECs.

liferative phase was significantly higher than in the secretory phase (**Figures 1B, 1C and 2**).

Changes in the expression of NF-κB p65 and OPN in epithelial cells before and after OPN siRNA intervention

The expression of NF-κB p65 and OPN protein and mRNA decreased significantly ($P < 0.05$) after intervention with OPN siRNA in eutopic primary EECs (**Figure 3**).

Changes in the expression of NF-κB p65 and OPN in epithelial cells before and after OPN plasmid upregulation

The expression of NF-κB p65 and OPN protein and mRNA increased significantly ($P < 0.05$)

after intervention with pmCherry-C1-OPN in eutopic primary EECs (**Figure 4**).

Changes in cell invasion in epithelial cells before and after OPN siRNA intervention

After OPN siRNA intervention, the invasion of primary EECs was decreased significantly ($P < 0.05$). Simultaneously, after pmCherry-C1-OPN transfection, the invasion of primary EECs was increased significantly ($P < 0.05$) (**Figure 5**).

Discussion

Endometriosis is one of the most common gynecological disorders. Similar to the manner in which tumor cells metastasize, endometrial

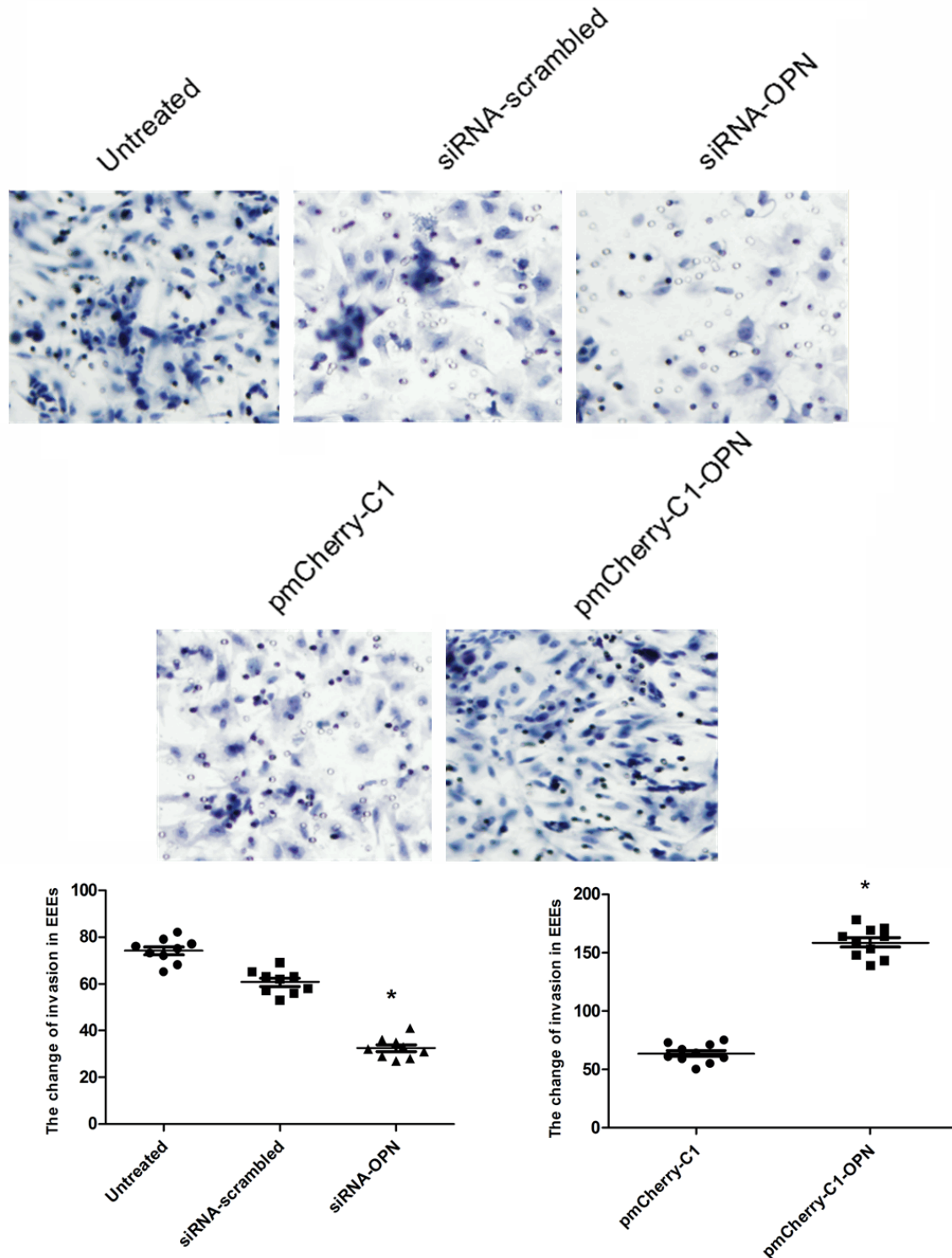


Figure 5. Changes in cell invasion in epithelial cells before and after OPN siRNA intervention. After OPN siRNA intervention, the invasion of primary EECs was decreased significantly ($P < 0.05$). Simultaneously, after pmCherry-C1-OPN transfection, the invasion of primary EECs was increased significantly ($P < 0.05$).

cells can invade the extracellular matrix and accelerate neovascularization [23]. According

to the theory of retrograde menstruation, endometrial cells can disaffiliate from the normal

position, and attach and invade the tissues outside the uterus, similar to the process of cancer cell metastasis [24]. That is, a series of actions including disaffiliation, attachment and invasion are of great importance for the development of early endometriosis. Understanding the molecular basis underlying early invasion and metastasis of endometrial cells may lead to new and more effective therapeutic interventions, resulting in an improved prognosis for patients with endometriosis.

In recent years, many factors have been reported to correlate with the pathological process of cancers, as well as the pathogenesis of endometriosis. In 1986, Sen and Baltimore published the first report of a eukaryotic transcription factor, NF- κ B, in mature lymphocytes [25]. NF- κ B belongs to the NF- κ B/Rel protein family, of which the most common of the P50/p65 heterologous dimerization forms exist in the cytoplasm. OPN, a protein found to be implicated in the adhesion and migration of cancer cells, has been evaluated in normal endometrium and endometrium with endometriosis [26]. In a recent study, OPN was shown to be highly expressed in the epithelial cells of the endometrium in patients with endometriosis [27]. OPN, rich in arginine, glycine, aspartic acid and ammonia, is an acidic glycoprotein with a molecular weight of 32 kD; it is expressed mainly by osteoclasts, macrophages, T lymphocytes, vascular smooth muscle cells and mammary epithelial cells [28]. As a substrate for thrombin and tissue glutamine aminotransferase, OPN has the ability to promote cell adhesion and migration. OPN binding to the cell surface receptors α v β 3 and CD44 through RGD-dependent or unique molecular structures, can mediate the chemotaxis of other cytokines and play a role in cell adhesion, migration and infiltration [29-31]. In addition, OPN gene expression is important in the vascular formation enhancement process, particularly in angiogenesis associated with stress, in the combination of Ras and Src activation, and via OPN binding to its receptor to activate the NF- κ B p65 pathway to promote cell proliferation, inhibit apoptosis, and induce the occurrence of various diseases [32].

In this study, we isolated primary eutopic EECs with endometriosis (EEEECs) and identified the primary cell by immunocytochemistry using the

broad spectrum epithelial cell origin marker keratin. We found that the expression of OPN in EEECs was increased significantly compared with in normal EECs (NEECs). We also found that NF- κ B p65 expression levels were increased, in synchrony with the incremental increases in OPN, which is in line with previously published reports [33-37]. These results suggest that a cause-and-effect relationship exists between the expressions of OPN and NF- κ B p65, and the elevated expression of OPN and NF- κ B p65 may initiate the disaffiliation, adhesion and migration of the endometrium to tissues outside the uterus. In cancer cells, it has been shown that OPN binding to its receptor activates the NF- κ B p65 pathway to promote cell proliferation, inhibit apoptosis, and induce the occurrence of various diseases [38]. To investigate whether a similar pathway exists in EEECs, we used siRNA interference to specifically downregulate OPN and plasmid to upregulate. Not surprisingly, in EEECs, the expression of NF- κ B p65 was downregulated and this was accompanied by decreased expression of OPN. Simultaneously, expression levels of OPN and NF- κ B p65 were significantly higher than the control group after EEECs were transfected with the pmCherry-C1-OPN overexpressing plasmid.

Hence, our data support the theory that an NF- κ B p65 signal pathway regulated by OPN exists in EEECs. The possible explanation for this phenomenon is that NF- κ B p65 is regulated by many different signal pathways and, with the biological and morphological transformation from NEECs to EEECs, some signal pathways are activated [39]. For example, TGF- β 1 and epidermal growth factor can promote EEECs migration through selectively activating Raf-1 [40, 41]; however, this regulation pathway has not currently been reported in NEECs. We infer that OPN may regulate NF- κ B p65 expression under the fluctuating local estrogen environment. To confirm that the migration of EEECs could be affected under the regulation of the OPN/NF- κ B p65 pathway, we performed a transwell assay with OPN-specific siRNA treatment. We found the number of cells on the lower surface of the membrane varied in line with the quantitative changes in OPN and NF- κ B p65 which were observed with the same treatment. Moreover, these effects of cell invasion on EEECs were also demonstrated in OPN-over-

expressing cells. These data show that the OPN/NF- κ B p65 pathway functions in EEECs. Our data suggest that there may be another signal pathway regulating cell migration in EEECs, which needs to be explored further.

Conclusion

In summary, we performed different assays to detect the differential expression levels of OPN and NF- κ B p65 under various treatments. The aim of these assays was to establish a novel signal pathway in which OPN plays a crucial cell invasion and migration role in endometriosis and regulates the activation of MMP-9 via NF- κ B-mediated signaling pathways. Now that the basic framework of this signal pathway has been established, further studies will be conducted in order to enhance our future understanding of the role of OPN in endometriosis.

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Disclosure of conflict of interest

None.

Authors' contribution

Wangshu Li and Zhimiao Bai conceived, designed and prepared the manuscript and figures. Qinchun Yang and Rui Wang performed experiments and contributed vital new reagents. Hua Chen and Weiwei Yao contributed to the clinical and treatment sections and analyzed the data. Chunfang Ha wrote the manuscript. All authors read and approved the final manuscript.

Abbreviations

BSA, Bovine serum albumin; EEC, Endometrial epithelial cell; EEEc, Eutopic endometrial epithelial cell; MMP, Matrix metalloproteinase; OPN, Osteopontin.

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