Overexpression of TGF-β enhances the migration and invasive ability of ectopic endometrial cells via ERK/MAPK signaling pathway

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Abstract. Endometriosis is a common gynecological disease with manifestations of endometrial-like tissue outside the uterus. Transforming growth factor-β (TGF-β) is known to facilitate a series of biological events in many cells, including migration. However, the roles of TGF-β in endometriosis still remain largely unknown. The aim of the present study was to discover the role of TGF-β1 in endometriosis development and progression and its associated mechanisms. It was demonstrated that the expression of TGF-β1 was significantly elevated in endometriosis in comparison with that in normal tissue. Overexpression of TGF-β increased the proliferation and upregulated proliferating cell nuclear antigen and cyclin D1 in endometrial stromal cells (ESCs). Furthermore, TGF-β overexpression also triggered a series of biological events occurring in ESCs, including cell migration and invasion, and activated the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway. The inhibition of the ERK/MAPK pathway reversed the previous effects of TGF-β overexpression. Collectively, the present results indicated that overexpression of TGF-β enhances the migration and invasion of ectopic ESCs via the ERK/MAPK signaling pathway, providing theoretical evidence for the development of new treatment methods targeting the TGF-β-ERK/MAPK signaling pathway for prophylaxis of endometriosis.

Introduction

Endometriosis is a gynecological disease typically manifested by the growth of endometrial tissues outside the uterus and affects 6-10% of fertile females (1). Various studies have investigated the pathogenesis of endometriosis (2-4), among which the widely recognized hypothesis is that endometriotic lesions are caused by attachment of detached endometrial cells to the peritoneal serous membrane during the menstrual period (5-7).

Currently, various factors contributing to endometrial lesions have been recognized, including their inheritance, immune responses, and anatomy (8,9). Attachment of detached endometrial cells to the peritoneal membrane, peritoneal invasion, angiogenesis and suppressed immune system are the major factors leading to the formation of peritoneal lesions (10). In addition, molecular changes are also involved in the development of peritoneal lesions (11-16).

Transforming growth factor-β (TGF-β), an inflammation-associated growth factor, modulates a variety of biological events that are involved in the formation of endometrial lesions (17,18). Upregulation of TGF-β has been identified in a variety of tumors, and through epithelial-to-mesenchymal transition, TGF-β promotes the migration and invasion of tumor cells in some cancers, which are mediated by the mothers against decapentaplegic homolog signaling pathway (19,20). Furthermore, TGF-β upregulates TWIST, N-cadherin, and vimentin in some cancers (21,22). It was also reported that endometriosis increases the level of TGF-β in females, and in the absence of TGF-β, the growth of endometrial lesions is hindered in mice. Thus, TGF-β contributes to the development of endometriosis (23-25), but how it affects endometriosis remains unknown.

In the present study, the expression pattern and functions of TGF-β were evaluated to elucidate the biological features of endometrial stromal cells (ESCs). Furthermore, the potential mechanism of TGF-β in the development of endometriosis was identified.

Materials and methods

Sample preparation. The present study was approved by the Ethics Committee of the Guangzhou Women and Children's Medical Center (Guangzhou, China) and all patients provided written informed consent prior to their participation. All subjects were enrolled and divided into two groups; the endometriosis group and the control group. Samples of
endometrial-like tissues were collected from patients with endometriosis, and of eutopic endometrial tissues from those without endometriosis. Patients in the endometriosis group (n=6) were aged between 27 and 44 years, and diagnoses were confirmed by histopathological examination, while those in the control group (n=6) were aged between 22 and 48 years. All subjects from Guangzhou Women and Children’s Medical Center (Guangzhou, China) had no history of hormone therapy within 3 months prior to the present study.

**Immunohistochemistry.** Tissue samples collected in the proliferative and secretory phases were fixed in 10% formaldehyde at 20°C for 24 h, embedded in paraffin and then sliced into sections of 5-µm thickness. The sections were dehydrated in ethanol in a graded concentration series, immersed in xylene at 100°C and alcohol, and then incubated with 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 20°C for 1 h and in hydrogen peroxide at 20°C for 30 min. Thereafter, they were probed with primary antibodies against TGF-β (cat. no. 2519; 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight, followed by three washes in TBS, and they were then probed with horseradish peroxidase-conjugated secondary antibodies (cat. no. 3900; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at 20°C. The sections were then treated with 3,3'-diaminobenzidine at 20°C for 5 min and counterstained with hematoxylin at 20°C for 20 min. Immunostaining was visualized at a magnification of x40 by the Nikon Optical TE2000-S inverted microscope (Nikon Corporation, Tokyo, Japan).

**Cell isolation, culture and treatment.** According to previously reported methods, primary ESCs were obtained from tissue samples collected from patients with endometriosis (26). Briefly, the tissues were ground sufficiently with collagenase IV for 1 h, followed by DNase I (Sigma-Aldrich; Merck KGaA) treatment for 30 min at 20°C. Then, filtration was performed to remove the debris, and ESCs were isolated by centrifugation at 800 x g for 5 min at 20°C. Sediment was collected and used to prepare a suspension. The ESCs were plated at a density of 1x10⁶ cells in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by regular culture of ESCs. To evaluate the role of the ERK/MAPK pathway in TGF-β-mediated migration and invasion, ESCs were primed with 5 µM U0126 (Sigma-Aldrich; Merck KGaA) for 30 min at 20°C. Then, filtration was performed to remove the debris, and ESCs were isolated by centrifugation at 800 x g for 5 min at 20°C. Sediment was collected and used to prepare a suspension. The ESCs were plated at a density of 1x10⁶ cells in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by regular culture of ESCs. To evaluate the role of the ERK/MAPK pathway in TGF-β-mediated migration and invasion, ESCs were primed with 5 µM U0126 (Sigma-Aldrich; Merck KGaA) for 30 min at 20°C and then transfected with TGF-β-pcDNA3.1 for 24 h.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from endometrial tissue with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was performed with PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) according to standard protocol. qPCR was performed using Quantitect SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) with an ABI 7300 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Regular extraction and purification of RNA from the tissue samples were performed in strict accordance with the instructions of the manufacturers, followed by reverse transcription to prepare cDNA, with primers as follows: GAPDH, forward 5'-GCA GAT TCA ACGGCA CAG-3' and reverse 5'-GCG AT GACT CCA GC AC A-3'; and TGF-β, forward 5'-CCC ACT GTAG CCGC TT G-3' and reverse 5'-TGA AG CAA AG GCC CTC GTGA-3'. The PCR cycling conditions were as follows: 5 min at 95°C, and 36 cycles of 10 sec at 95°C, 10 sec at 58°C and 20 sec at 72°C. miR-383 was normalized to U6. The expression levels were analyzed by Real-Time StatMiner™ Software (version 3.5; Integromics, Inc., Madison, WI, USA). GAPDH levels were used as an internal control and fold changes were calculated by relative quantification (the 2−ΔΔCq method) (27). Using RT-qPCR, the relative expression of mRNAs was detected in triplicate for each sample.

**Cell transfection.** Plasmids of TGF-β-pcDNA3.1 (TGF-β) and pcDNA3.1 (vector; both Shanghai Jima Industrial Co., Ltd., Shanghai, China) were used for cell transfection. Briefly, human ESCs from endometriotic tissue were inoculated on 6-well plates at 4x10⁵ cells/well at 37°C. After 24 h, 4 µg plasmid DNA and 3 µl of Turbofect reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the culture medium and incubated for 6 h at 37°C. Subsequently, the transfection mixture was removed and cells were further incubated with normal medium for a further 48 h at 37°C. The efficiency of cell transfection was verified by western blotting.

**Cell viability.** MTT assay was carried out in order to measure cell viability. Cells were inoculated at 37°C on a 96-well plate at a density of 5x10⁴ cells/ml for regular culture and transfection, and after 24, 48 and 72 h, MTT reagent (R&D Systems Europe, Ltd., Abingdon, UK) was added into the wells and incubated for 4 h at 37°C. Then, the MTT solution was aspirated and dimethylsulfoxide (200 µl/well) was added. Optical density of the supernatant was read at 490 nm using a microplate spectrophotometer. This procedure was conducted in triplicate.

**Cell migration.** Transwell assay was performed to measure cell migration. Briefly, a cell suspension was prepared using 50,000 cells in DMEM supplemented with mitomycin C (1 µg/ml; Zhejiang Hisun Pharmaceutical Co., Ltd., China). The cells were then inoculated on the upper chamber. In the lower chamber, DMEM containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) was added. Following 24 h of culture at 37°C, cells that failed to pass through the membrane between the upper and lower chambers were scraped while those in the lower chamber were fixed with 100% methanol at 20°C for 15 min and then stained with 1% crystal violet at 20°C for 30 min. Cells that passed through the membrane were counted at a magnification of x20 by the Nikon Optical TE2000-S inverted microscope in six microscopic fields that were selected randomly.

**Cell invasion.** For the invasion assay, a Transwell chamber coated with Matrigel was used. Following transfection, cells (1x10⁵ cells/chamber) were transferred to the upper chamber for incubation while in the lower chamber, DMEM with 20% FBS was added at 37°C. Following 24 h, cells that did not pass through the membrane between the upper and lower chambers were removed using a swab, and those in the lower chamber
were fixed with 100% methanol at 20℃ for 15 min and then stained with 1% crystal violet at 20℃ for 30 min. Cells that passed through the membrane were counted at a magnification of x20 by the Nikon Optical TE2000-S inverted microscope in six microscopic fields that were selected randomly.

Western blotting. Cell lysates and tissues were homogenized in NP-40 lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein (40 µg/lane) were loaded into the wells and separated by SDS-PAGE on 10-12% gels, and the proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane. Thereafter, the membrane was blocked with 10% bovine serum albumin at 20℃ for 1 h and incubated overnight at 4℃ with primary antibodies for various proteins (all from Cell Signaling Technology, Inc.), including TGF-β (1:1,000), proliferating cell nuclear antigen (PCNA; cat. no. 13110, 1:2,000), cyclin D1 (cat. no. 2978; 1:2,000), p38 (cat. no. 8690; 1:1,000), phosphorylated (p) -p38 (cat. no. 4511; 1:1,000), extracellular signal-regulated kinases (ERK; cat. no. 4695; 1:1,000), p-ERK (cat. no. 4370; 1:1,000), c-Jun N-terminal kinase (JNK; cat. no. 9252; 1:1,000), p-JNK (cat. no. 9255; 1:1,000) and β-actin (cat. no. 2519; 1:5,000). Following three washes in TBS/Tween 20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 14709; 1:10,000; Cell Signaling Technology, Inc.) at 20℃ for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence plus detection reagent (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using an Omega 16ic Chemiluminescence Imaging system (Ultra-Lum, Inc., Claremont, CA, USA). Proteins were analyzed with ImageQuant™ LAS 4000 imaging system and its associated software (version 17; both GE Healthcare, Pittsburgh, PA, USA).

Statistical analysis. Data are expressed as mean ± standard error of the mean. For data with unequal variance, comparisons were carried out with Student’s t-test or one-way analysis of variance and Tukey’s post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β is upregulated in endometrial tissues. To investigate how TGF-β regulates the biological events occurring in ESCs, the expressions of TGF-β were measured in endometrial samples with or without endometriosis. In Fig. 1, RT-qPCR, immunohistochemistry, and western blotting indicated that the mRNA and protein expressions of TGF-β were upregulated significantly in patients with endometriosis in comparison with those in the control group. Thus, upregulation of TGF-β in patients with endometriosis may be associated with endometriosis.

TGF-β overexpression enhances proliferation of ESCs and increases the expression of PCNA and cyclin D1. To further clarify how TGF-β affects the biological features of ESCs, the role of increased TGF-β expression in cell proliferation was investigated. In Fig. 2A, TGF-β was evidently increased when ESCs were transfected with the TGF-β plasmid as compared
TGF-β overexpression significantly promoted cell proliferation (Fig. 2B). PCNA and cyclin D1 have roles in modulating cell growth (28). Consistently, TGF-β overexpression markedly upregulated PCNA and cyclin D1 expression, which are associated with cell proliferation (Fig. 2C-E). These results demonstrated that TGF-β increased the proliferation of ESCs.

**TGF-β overexpression increases the migration and invasion ability of ESCs.** The results of migration assay demonstrated that TGF-β overexpression significantly increased cell migration (Fig. 3A and B), while the invasion assay revealed that the number of ESCs that successfully passed through the Transwell membrane following TGF-β transfection exceeded that of the empty control (Fig. 3C and D), indicating that TGF-β enhanced the migration and invasion of ESCs.

**TGF-β overexpression enhances the activation of ERK/MAPK signaling pathway in ESCs.** The involvement of the MAPK pathway in modulating the growth and invasion of cells has been demonstrated previously (29). The effect of TGF-β overexpression on MAPK signaling pathways was investigated via immunoblotting. In Fig. 4, TGF-β overexpression increased the phosphorylation level of ERK without any significant influence on the phosphorylation of p38 and JNK. These results demonstrated that TGF-β contributed to the activation of the p38 MAPK signaling pathway in ESCs.

**Discussion**

In the present study, the expression of TGF-β was upregulated in endometrial tissues compared with that in normal tissues. The functions of TGF-β in ESCs were also illustrated using overexpression assays. The results of the present study demonstrated that TGF-β overexpression increased the proliferation, migration and invasive ability of ESCs, and these effects were also reversed by U0126 (Fig. 5B-E). Thus, TGF-β increases the proliferation, migration and invasive ability of ESCs via the ERK/MAPK pathway.
Figure 3. TGF-β overexpression increases the migration and invasive ability of ESCs. (A) ESCs successfully reached the lower chamber when transfected with empty vector and TGF-β plasmid. Scale bar, 100 µm. (B) Number of ESCs successfully migrating to the lower chamber in different groups. (C) ESCs successfully reached the lower chamber when transfected with empty vector and TGF-β plasmid. Scale bar, 100 µm. (D) Number of ESCs successfully invading the lower chamber in different groups. Data are presented as the mean ± standard error of the mean, and this procedure was performed in triplicate. **P<0.01 vs. vector group. TGF, transforming growth factor; ESC, endometrial stromal cell.

Figure 4. TGF-β overexpression enhances the activation of ERK/MAPK in ESCs. (A) Representative immunoblots and quantitative analysis of (B) ERK phosphorylation, (C) p38 and (D) JNK expression in ESCs following transient transfection of TGF-β plasmid or empty vector. Data are presented as the mean ± standard error of the mean, and this procedure was performed in triplicate. **P<0.01 vs. vector group. TGF, transforming growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ESC, endometrial stromal cell; JNK, c-Jun N-terminal kinase; p, phosphorylated.
The present results provide novel insights to the role of TGF-β in the pathogenesis of endometriosis and also in the development of reagents for endometriosis treatment. Endometriosis results from increased cellular proliferation, adhesion and invasion of the retrograde endometrium (30,31). It has been demonstrated that the biological phenotype of patients with endometriosis is different from that of controls, which contributes to the development of endometriosis (32,33). In the present study, it should be noted that TGF-β overexpression is associated with the enhanced proliferation, migration and invasion of ESCs, which are dependent on the upregulation of downstream molecules, such as PCNA and cyclin D1 via MAPK/ERK signal-dependent pathways.

It has been demonstrated that TGF-β1 is involved in the regulation of various biological events (34,35), which also take place in the development of endometriotic lesions (36-38). In patients with endometriosis, alterations in TGF-β1 and its downstream molecules were observed; indicating that upregulation of TGF-β1 may increase the resistance of ESCs to apoptosis in patients with endometriosis (39-41). Thus, in addition to the increased resistance to apoptosis, TGF-β can enhance ectopic tissue survival through decrease in the number and activity of immune cells (42,43). However, the molecular mechanism remains unclear. Growing evidence has proposed the involvement of the MAPK signaling pathway in the modulation of cell growth and invasion of endometriosis (44). In the present study, TGF-β1 overexpression enhanced the activation of ERK/MAPK but not p38 and JNK in vitro. Furthermore, treatment with the ERK inhibitor U0126 almost abolished the enhanced proliferation, migration and invasion of ESCs induced by TGF-β1 overexpression. These findings suggest that TGF-β1 increased the proliferation, migration and invasion of ESCs via activation of the ERK/MAPK signaling pathway. The effect of TGF-β1 knockdown on endometriotic tissue requires investigation in future studies.

In conclusion, the present findings demonstrate that the overexpression of TGF-β enhances the migration and invasive ability of ESCs via the ERK/MAPK signaling pathway. These findings are expected to aid in the development of novel strategies targeting the TGF-β/ERK/MAPK signaling pathway in the prophylaxis of endometriosis.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors' contributions
ZL conceived the project, designed and performed the experiments, analyzed the data, and wrote the manuscript. LY and MD performed the experiments and analyzed the data. GG performed the experiments and revised the manuscript. YZ conceived the project, designed the experiments, and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the Guangzhou Women and Children's Medical Center (Guangzhou, China) and all patients provided written informed consent prior to their participation.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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