FOXP1 enhances fibrosis via activating Wnt/β-catenin signaling pathway in endometriosis

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Abstract: Endometriosis is a common gynecological disorder affecting 6-10% women. Endometriosis is associated with excess fibrosis, leading to chronic pain, scarring and aberrant tissue function. However, molecular and cellular mechanisms underlying fibrosis during endometriosis still remain elusive. In this study we used endometrial and endometriotic stromal cells isolated from patients, and employed siRNA to knockdown Forkhead box protein P1 (FOXP1) to investigate the effect of FOXP1 on collagen contraction, cell proliferation and mitigation. Western blot and quantitative PCR were applied for analysis of protein and mRNA levels, respectively. Compared to control stromal cells, endometriotic stromal cells from patients exhibited higher levels of FOXP1 expression and Wnt-related β-catenin acetylation. FOXP1 knockdown decreased not only Wnt signaling, but also the expression of fibrotic marker genes, including connective tissue growth factor, type I collagen, α-smooth muscle actin and fibronectin. Furthermore, FOXP1 knockdown reversed the endometriotic cellular phenotypes, including reducing collagen gel contraction, inhibiting cell proliferation and migration. Finally, Wnt signaling inhibitor AVX939 blocked β-catenin acetylation and endometrial stromal cell proliferation induced by ectopic FOXP1 expression. FOXP1 enhances fibrosis during endometriosis through upregulating Wnt signaling activity.

Keywords: Endometriosis, FOXP1, Wnt signaling, β-catenin acetylation, fibrosis

Introduction

Endometriosis is a common gynecological disorder affecting 6-10% of women, and is characterized by the expansion of stroma and endometrial glands in extra-uterine sites [1]. Endometriosis histologically manifests as excessive fibrous tissue surrounding the endometrial glands and stroma. Fibrosis is featured with proliferation and elaboration of localized fibroblasts, and accumulation of excess collagen [2]. Excess fibrosis leads to scarring, chronic pain, and more severe clinical outcomes including pelvic pain, dysmenorrhea and dyspareunia [3]. However, molecular mechanism underlying fibrosis in endometriosis is still elusive.

Fibrosis in endometriosis is regulated by the Wnt signaling pathway, which plays critical roles in various diseases [4]. Wnt signaling pathway is abnormally activated in endometriotic tissues of patients [5], and its inhibition blocks endometriotic lesion, cell proliferation and migration, as well as invasion of epithelial and stromal cells into the endometrial and endometriotic tissues [6]. Mechanically, Wnt signaling regulates expression of fibrotic marker genes, including connective tissue growth factor (CTGF), type I collagen (Col-I), α-smooth muscle actin (αSMA) and fibronectin (FN), all of which are implicated in fibrogenesis of endometrial and endometriotic tissues [7].

Wnt signaling is tightly regulated, and its aberrant activation leads to many diseases [1]. In the absence of Wnt stimulation, the downstream protein β-catenin is ubiquitinated by APC/Axin/GSK-3β complex and subsequently degraded. Wnt binds to Frizzled/LRP receptor complex to inactivate GSK-3β, which leads to cytosolic β-catenin accumulation and subsequent translocation into the nucleus to activate expression of Wnt-targeted genes [4]. The key output of Wnt signaling pathway is the acetylation at Lys 49 of β-catenin, which stabilizes the protein and promotes Wnt signaling activity [8]. It was reported that Forkhead box protein P1 (FOXP1) could interact with acetyltransferase...
CBP to promote β-catenin acetylation and thereby increase Wnt signaling activity [9]. However, it is unclear if FOXP1 is involved in fibrosis during endometriosis.

FOXP1 is a transcription factor, and exhibits various functions in development, adult tissue homeostasis, regeneration and disease [10]. A recent study found that FOXP1 expression level was greatly increased in endometriotic stromal cells from endometriosis patients [11]. Our current study further suggested that FOXP1 not only regulated expression of fibrotic marker genes, including αSMA, Col-I, CTGF and FN, but was also associated with stromal cell-stimulated collagen gel contraction, as well as proliferation and migration of endometriotic and epithelial cells. Wnt signaling inhibitor prevented ectopic FOXP1-induced proliferation of stromal cells. Thus, this study, for the first time, demonstrates that FOXP1 plays an enhancing role in fibrosis during endometriosis.

Methods

Patients

Patients aged 19-39 years undergoing laparoscopy for endometriosis were recruited at Liaocheng People's Hospital for this study. The endometrial tissues from patients with uterine myomas acted as control samples. No research subject had received hormonal treatments or intrauterine contraception for more than 6 months prior to surgery. Tissues in deep endometriotic lesions (around 5 mm under the peritoneal surface) or ovarian endometriosis (ectopic endometrium), as well as paired ectopic endometrial tissues, were dissected out just before surgery with endometrial suction catheter (Pipelle). In this study, all patients with myomas had intramural and/or subserosal myomas, and no patients with uterine myomas or tubal infertility had endometriosis. The menstrual cycles (26-32 days) were normal in all patients. The endometriotic samples were categorized into the following five groups: proliferative (P) (days 8-14), early-secretory (ES) (days 15-19), mid-secretory (MS) (days 20-24), late-secretory (LS) (days 25-28) and menstrual (M) (days 1-3). All subjects have given informed consent. Ethics Committee of Liaocheng People’s Hospital had approved the protocol for the research project.

Cell culture

The endometrial and endometriotic tissues were dissected out and minced into 1-2 mm³ blocks. Then the tissues were digested with type I collagenase (0.25%) (Life Technologies, Pleasanton, CA, USA) and deoxyribonuclease I (15 U/mL) (Life Technologies, USA) in phenol red-free DMEM/F-12 for 1 h (endometrium) or 1 h 30 min (endometriosis) at 37°C. The digested tissues were filtered through 40 mm nylon cell strainers (BD, Le Pont de Claix, France) to isolate endometrial and endometriotic cells. The strainer separated the intact epithelial cells and dispersed stromal cells. The filtered cells were treated with hypotonic buffer (1 mM KHCO₃, 0.15 mM NH₄Cl, and 0.1 mM Na₂EDTA) (Life Technologies, USA) to remove red blood cells. The cells were seeded into Primaria flasks (BD, Franklin Lakes, NJ, USA) in phenol red-free DMEM/F-12 supplemented with 10% charcoal-stripped FBS and antibiotics, followed by incubation at 37°C in 95% air/5% CO₂. The epithelial cells were cultured in cell incubator for 1 h to remove the contaminated and wall-attached stromal cells. The floating epithelial cells were separated and seeded in complete culture medium as mentioned above. 2-3 days later, the cells reached confluence. The early passages of cells were used for experiments.

Western blot

The cells were lysed in RIPA buffer with protease inhibitor, and supplied with 1 μM TSA in samples for detection of β-catenin Lys49 acetylation. Proteins were separated by SDS-PAGE, followed by transfer onto PVDF membranes (ThermoFisher, Waltham, MA, USA). The membranes were sequentially blotted with primary antibodies and HRP-coupled secondary antibodies. Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) was used to visualize the bands. The antibodies against FOXP1 (1:1000) and β-catenin (1:1000) were all purchased from Cell Signaling (Danvers, MA).

Collagen gel contraction assay

500 μl of 1% bovine serum albumin (BSA) each well was added into 24-well plates and incubated for 1 h at 37°C to reduce surface stickiness and block attachment of gels to the dish-wall. After treatment with si-FOXP1 (50 ng/mL)
Mechanisms underlying fibrosis in endometriosis

or vehicle for 72 h, the endometriotic and endometrial stromal cells were digested with trypsin. After cell counting, 2.5×10⁶ cells/mL were mixed with 2.0 mg/mL Type I collagen solution (BD, Le Pont de Claix, France) in PBS containing 0.023 N NaOH. 500 μL collagen/cell suspension each well was added into the pre-coated 24-well plates, followed by incubation for 60 min at 37°C. For endometrial stromal cells treated with si-FOXP1, 500 μL of culture media plus 2% charcoal-stripped FBS was used. Collagen gel contraction was observed lasting for 24 h, and ImageJ software was applied to measure the surface area of the contracted gels at 0, 6, 12 and 24 h.

Cell proliferation assays

Cell proliferation was detected with the CellTiter 96H AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Charbonnie`res-les-Bains, France). In brief, 1×10⁴ cells per well were plated into 96-well plates. After culturing for 2 days, the cells were treated with si-FOXP at indicated concentrations or vehicle for 48 h. 20 μl CellTiter 96H AQueous One Solution Cell Proliferation Assay Reagent (Promega) was supplemented to the cells, followed by incubation for 3 h. The cell amount was measured by absorbance at 490 nm (Thermo Scientific, Illkirch, France).

In vitro migration and invasion assays

To perform in vitro migration, 5×10⁴ cells per chamber in 500 μL DMEM/F12 medium without phenol red and FBS were seeded to the upper chamber of 24-well uncoated chambers/microfilters (BD, USA). The lower chamber contained 750 μl DMEM/F12 containing 10% charcoal-stripped FBS (Gibco, USA). 24 h later, cell motility/migration was determined according to the number of cells migrated through microfilter.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was purified with Trizol reagent (Thermo Fisher, USA). RNA concentration was determined with NanoDrop 2000 (Nanodrop). 1 μg RNA for each sample was reversely transcribed into cDNA with SuperScript IV (Thermo Fisher, USA). mRNA expression level was determined with qPCR on ABI 7500 RealTime PCR system using SYBR Green MasterMix kit (ABI). GAPDH acted as internal control. The designed primers were as follows: FOXP1 F: 5’-CGGTTCAGCCATCCAGAATGG-3’ R: 5’-GTCCACGGCGCGTCTCTCCG-3’; αSMA F: 5’-TGGCTGATGGACTTC-3’ R: 5’-GATAGAGAAGCCAGGATG-3’; CTGF F: 5’-TGGGTCGTCGTGGCTTGCTGCAGGC-3’ R: 5’-GCCAGACGAACGTCCATGCTGC-3’; Col-I F: 5’-GGATGGGCACGTCGAGGAGT-3’ R: 5’-GGATGGGAATCATATTGG-3’.

Statistical analysis

The SPSS version 16 was used for statistical analysis. Comparisons between groups were
Mechanisms underlying fibrosis in endometriosis

made with Student’s t-test, one-way analysis of variance or general linear model repeated measures. P<0.05 means statistical significance.

Results

**FOXP1 expression is upregulated at both mRNA and protein levels**

To investigate the genes involved in fibrosis during endometriosis, endometrial and endometriotic stromal cells were isolated from patients with or without endometriosis to establish an in vitro model. CTGF, Col-I, αSMA and FN are four fibrotic marker genes. As shown in Figure 1C, the expression of these genes was significantly increased in stromal cells from endometriotic patients, as expected. Moreover, the protein level of β-catenin was elevated in cells from patients with endometriosis (Figure 1B and Supplementary Figure 1). These data suggested that FOXP1 might be involved in fibrosis during endometriosis.

**Knockdown of FOXP1 lowers β-catenin acetylation and fibrotic gene expression**

To test the possibility of FOXP1 involvement in fibrosis during endometriosis, this gene was knocked down by siRNA treatment. As shown in Figure 2A and 2B, both FOXP1 mRNA and protein levels were decreased in response to FOXP1 siRNA treatment, but not to control siRNA treatment. Then we tested the expression of fibrotic genes. All four fibrotic marker genes, Col-I, CTGF, αSMA and FN, were significantly decreased (Figure 2C). In response to FOXP1 knockdown, the β-catenin acetylation at Lys49 was greatly reduced (Figure 2B and Supplementary Figure 2). These data suggested that FOXP1 was involved in regulating fibrotic gene expression mediated by Wnt signaling.

**Knockdown of FOXP1 weakens stromal cell-mediated collagen gel contraction**

To further investigate the function of FOXP1 on fibrosis during endometriosis. We performed

![Figure 2](image-url)
Mechanisms underlying fibrosis in endometriosis

FOXP1 knockdown weakens stromal cell-mediated collagen gel contraction. The endometriotic and endometrial stromal cells treated with si-FOX1 (50 ng/mL) or vehicle for 72 h, then, 2.5×10⁵ cells/mL were mixed with 2.0 mg/mL Type I collagen solution in 0.023 N NaOH-contained PBS. 500 µL collagen/cell suspension each well was added into the 500 µl 1% bovine serum albumin (BSA) pre-coated 24-well plates. A. Photographs of contracted gels obtained at 24 h in response to stromal cell treatment with or without FXOP1 siRNA. B. Time course of collagen gel contraction mediated by different stromal cells. ImageJ software was applied to measure the surface area of the contracted gels at 0, 6, 12, and 24 h. Abbreviations used in the figure: Endo(-): stromal cells from endometrium of patients free of endometriosis and without treatment; Endo(+): stromal cells from endometrium of endometriotic patients without treatment; si-FOX1: stromal cells isolated from endometrium of endometriotic patients treated with si-FOX1. Values are expressed as means ± S.E.M. N=6 biological repeats, *P<0.05 and **P<0.01, vs Endo(+).

Figure 3. FOXP1 knockdown weakens stromal cell-mediated collagen gel contraction.

Discussion

In this study, we found that FOXP1 expression was upregulated in stromal cells from endometriotic patients. Further investigation discovered that FOXP1 knockdown decreased stromal cell-stimulated collagen contraction, and also prevented proliferation and migration of stromal and epithelial cells from endometriotic patients. Depletion of FOXP1 by siRNA decrea-
Mechanisms underlying fibrosis in endometriosis

Figure 4. FOXP1 knockdown retards cell proliferation of epithelial cells and stromal cells. The epithelial cells (A) and stromal cells (B) were isolated from patients with endometriosis at different phases, 1×10^4 cells per well were plated into 96-well plates. After cultured for 2 days, the cells were treated with si-FOXP (50 ng/mL) or vehicle for 48 h and subjected to cell proliferation assay. Abbreviations used in the figure: Endo(-): cells from endometrium of patients free of endometriosis and without treatment; Endo(+): cells from endometrium of endometriotic patients without treatment; si-FOXP1: cells isolated from endometrium of patients with endometriosis treated with si-FOXP1; M: menstrual phase; P: proliferative phase; ES: early secretory phase; MS: mid-secretory phase; LS: late secretory phase. Endo(-): M: n=7, P: n=9, ES: n=8, MS: n=10, LS: n=8. Endo(+): M: n=6, P: n=10, ES: n=7, MS: n=10, LS: n=7. *P<0.05 and **P<0.01, vs Endo(+).

Figure 5. FOXP1 knockdown decreases migration of epithelial cells and stromal cells. The epithelial cells (A) and stromal cells (B) were isolated from patients with endometriosis at different phases, 5×10^4 cells per chamber were plated into 24-well uncoated chambers and then treated with FOXP1 siRNA (50 ng/mL) or vehicle for 48 h and subjected to cell migration assay. 24 h later, cell migration was determined according to the number of cells migrated through microfilter. Abbreviations used in the figure: Endo(-): cells from endometrium of patients free of endometriosis and without treatment; si-FOXP1: cells from endometrium of patients with endometriosis treated with si-FOXP1; M: menstrual phase; P: proliferative phase; S: secretory phase. Endo(-): M: n=7, P: n=9, S: n=26. Endo(+): M: n=6, P: n=10, S: n=24. *P<0.05 and **P<0.01, vs Endo(+).
Mechanisms underlying fibrosis in endometriosis

FOXP1 acts as an activator of Wnt signaling by promoting β-catenin acetylation [9]. This study has, for the first time, showed that FOXP1 expression is upregulated in endometriotic stromal cells. In agreement with previous results [9], increased FOXP1 enhances acetylation of β-catenin, leading to increased Wnt signaling activity. In addition, FOXP1 knockdown significantly inhibits fibrotic marker gene expression, as well as proliferation and migration of endometriotic stromal cells, suggesting that FOXP1 is a potential target of therapy against endometriosis. Since CBP inhibitor ICG001 blocks FOXP1-enhanced β-catenin acetylation and Wnt signaling activity [9], it may be possible to alleviate clinical endometriotic symptoms of patients by delivering FOXP1 siRNA or CBP inhibitors specifically to the endometriotic sites.

Fibrosis also causes atherosclerosis [21] and heart failure [22]. FOXP1 protein is significantly increased in fibrous plaques in patients with atherosclerosis [23]. FOXP1 expression is increased during fibrosis due to heart failure [24]. These evidences support the involvement of FOXP1 function in fibrosis during endometriosis.

FOXP1 expression in endometriosis may be induced by TGF-β, which is reported to associate with the pathophysiology of endometriosis.
Mechanisms underlying fibrosis in endometriosis

[25]. Fibrosis mediated by TGF-β requires activation of Wnt signaling [25]. It was reported that TGF-β induced FOXP1 expression in various cell types in atherosclerotic lesion [26]. According to the above data, Wnt signaling, TGF-β and FOXP1 may form a network to regulate fibrosis during endometriosis. However, the mechanisms underlying FOXP1 induction in endometriosis need to be further investigated.

In summary, we present the first report that FOXP1 is upregulated in endometriotic cells and functions in fibrosis mediated by Wnt signaling.

Disclosure of conflict of interest

None.

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References

Mechanisms underlying fibrosis in endometriosis


Mechanisms underlying fibrosis in endometriosis

Supplementary information

Membranes were cut according to molecular weight of target proteins, probed with respective antibodies, and put together for visualization.

Supplementary Figure 1. Original Western blot film scanning for Figure 1B.

Supplementary Figure 2. Original Western blot film scanning for Figure 2B.

Supplementary Figure 3. Original Western blot film scanning for Figure 6A.