### Original Article Depletion of NEDD9, a target gene of TGF-β, inhibits the proliferation and invasion of ectopic endometriotic stromal cells

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**Abstract:** Endometriosis is characterized by the growth of endometrial-like tissue outside the uterus. The proliferative and invasive ability of endometrial cells is believed to play a key role in the ectopic growth of endometrial implants. Neural precursor cell expressed developmentally down-regulated 9 (NEDD9, also known as HEF1/Cas-L), a target gene of transforming growth factor-b (TGF- $\beta$ ), has been identified as a metastasis gene in several cancer type. Here, endometriotic stromal cells (ESCs) were isolated from ectopic endometrium and NEDD9 expression was knocked down by small interfering RNA (siRNA) transfection. Silencing of NEDD9 in ESCs significantly inhibited cell proliferation and invasion as indicated by Cell Counting Kit-8 and Transwell assay, respectively. TGF- $\beta$ 1 (2.5 ng/mL) treatment enhanced the expression of NEDD9 in ESCs. Moreover, the mRNA and protein levels of NEDD9 and TGF- $\beta$ 1 were significantly increased in endometriotic tissues along with the severity of endometriosis. More importantly, a positive correlation between NEDD9 and TGF- $\beta$ 1 mRNA expression was observed in human endometriotic tissues. In conclusion, NEDD9, whose expression was enhanced by TGF- $\beta$ 1, may be involved in the proliferation and invasion of ESCs, thus contributing to the pathogenesis of endometriosis.

Keywords: Endometriosis, NEDD9, TGF-β, proliferation, invasion

#### Introduction

Endometriosis, first microscopically discovered by Karl von Rokitansky in 1860, is a chronic disease characterized by the growth of endometrial-like tissue outside the uterus [1]. Endometriosis affects 6% to 10% of reproductive-aged women, and 35% to 50% of women with pain or infertility [2]. It has attracted widespread attention because it causes chronic pelvic pain, dysmenorrhea and subfertility [3]. The most widely accepted explanation for the development of endometriosis is Sampson's implantation theory [4], which postulates that during menstruation, endometrial cells travel to the abdomen and form endometriotic lesions. The ability of endometrial cells to survive, adhere, invade tissues and proliferate is critical for this process. Epidemiologic, histopathological and molecular biology studies suggest that endometriosis has a malignant potential, and endometriosis is classified as a tumor like lesion by the World Health Organization [5, 6].

Neural precursor cell expressed developmentally down-regulated 9 (NEDD9, also known as HEF1/Cas-L) is a cytoplasmic docking protein of the Crk-associated substrate (CAS) family. In recent years, NEDD9 expression has been found elevated in several cancer types, such as colon cancer [7], gastric cancer [8], lung cancer [9], ovarian cancer [10] and cervical cancer [11] and breast cancer [12]. As a scaffolding protein, NEDD9 coordinates the Src-FAK (focal adhesion kinase), Rac, and AURKA (aurora A kinase) signaling cascades, and thus plays an important role in tumor metastasis [11, 13, 14]. However, whether NEDD9 plays a role in the proliferation and invasion of endometrial cells and whether NEDD9 is involved in the pathogenesis of endometriosis remains unclear.

To date many cytokines are suspected to be involved in endometriosis. Transforming growth factor-b (TGF- $\beta$ ) can regulate the growth, motility, and differentiation of various cell types [15]. TGF- $\beta$  levels in peritoneal fluid of women with

endometriosis are significantly higher than in controls and increased along with the severity of the disease [16]. Increased TGF- $\beta$  activity is observed at the sites of endometriosis [17]. Recently, studies on bone marrow macrophages and human dermal fibroblasts demonstrated that TGF- $\beta$ 1 can induce the transcription of NEDD9 [18-20]. However, whether TGF- $\beta$ 1 is able to regulate NEDD9 expression in endometrial cells is unknown.

In the present study, we isolated endometriotic stromal cells (ESCs) from ectopic endometrium, assessed the effects of NEDD9 knockdown on the proliferation and invasion of ESCs, and explored the effects of TGF- $\beta$ 1 stimulation on NEDD9 expression in ESCs. Moreover, we measured TGF- $\beta$ 1 and NEDD9 expression in eutopic, ectopic and control endometrial tissues, and tried to investigate the association between TGF- $\beta$ 1 and NEDD9.

#### Materials and methods

#### Patient recruitment and specimen collection

The study was approved by the Ethics Committee of Women's Hospital, Zhejiang University, and written informed consent was obtained from all participants. All participants were at reproductive age (27-45 years old), had not received hormones therapy for at least 3 months before specimen collection and had no prior history of malignancy or autoimmune diseases. Eutopic endometrial tissues (n=30) and ectopic endometrial tissues (n=30) were collected from 60 women with endometriosis undergoing laparoscopy. Control endometrial tissues were from 30 women with benign gynecological conditions and without evident endometriosis.

#### Immunohistochemistry (IHC)

IHC assay was conducted to measure the protein levels of TGF- $\beta$ 1 and NEDD9 in eutopic endometrial, ectopic endometrial and normal tissues. Briefly, the formalin-fixed and paraffinembedded tissue sections were deparaffinized, rehydrated and incubated with 0.01 M citrate buffer (pH 6) to unmask antigen. After incubation with 10% normal goat serum for 30 min to block non-specific antigens, the tissue sections were incubated with anti-TGF- $\beta$ 1 (Abcam, Cambridge, MA, USA; ab92486) or anti-NEDD9 (Abcam, ab18056) overnight at 4°C followed by horseradish peroxidase (HRP) conjugated secondary antibody (Beyotime, Shanghai, China) for 1 h at room temperature. Visualization was performed with the 3,3-diaminobenzidine (DAB) solution (Vector Laboratories, Burlingame, CA, USA), and nuclei were counterstained with hematoxylin.

### Isolation and culture of human endometriotic stromal cells (ESCs)

ESCs were isolated according to the previous method [21, 22]. Under sterile conditions, ectopic endometrium obtained at the time of laparoscopy were minced into small pieces (< 1 mm<sup>3</sup>) and digested with 1% collagenase solution (Serva, Heidelberg, Germany) for 2-3 hours in an atmosphere of 5% CO<sub>2</sub> at 37°C with occasional vortexing every 30 minutes. Cell suspension was filtered through 100 mm mesh (BD Biosciences, Franklin Lakes, NJ, USA) to remove the undigested tissues. After washing two times with culture medium, the obtained was seeded onto culture plates for 6 hours. The culture medium was changed to remove nonadherent and dead cells. The remaining adherent stromal cells were allowed to propagate. ESCs were cultured in DMEM/F12 medium (Hyclone, Logan, UT, USA) supplementary with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The purity of the ESCs were evaluated by immunocytochemical staining with anti-vimentin (Abcam, ab8978) [23].

#### Small interfering RNA (siRNA) transfection

To silence the expression of NEDD9 in isolated ESCs, siRNA targeting NEDD9 (GAAGCTCTATC-AAGTGCCA) [24] and a non-specific scramble siRNA (NC) were synthesized by Genepharma (Shanghai, China). ESCs were transfected with 400 nmol/L NEDD9 siRNA or NC by using lipo-fectamineTM 2000 (Invitrogen, Shanghai, China) per the instructions. At 48 hours post transfection, qRT-PCR and western blot analyses were carried out to confirm the knockdown of NEDD9.

Reverse transcription and quantitative realtime PCR (qRT-PCR) assay

Total RNA was extracted from collected tissue samples or ESCs using the TRIzol reagent



**Figure 1.** Suppressing of NEDD9 expression by siRNA transfection. (A) ESCs were isolated from ectopic endometrium, and immunocytochemical staining with anti-vimentin. Magnification:  $200 \times (B, C)$  ESCs were transfected with NEDD9 siRNA or a non-specific scramble siRNA (NC). ESCs without any treatment (Mock) were set as a control. Relative mRNA (B) and protein (C) expression levels of NEDD9 were detected at 48 hours after siRNA transfection. \*\*\*\**P* < 0.0001.



Figure 2. NEDD9 knockdown significantly suppressed the proliferation of ESCs. Rate of cell proliferation was assessed by CCK-8 assay when ESCs were transfected with NEDD9 siRNA or NC. \*\*\*P < 0.001 vs. NC.

(Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted using cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) according to the manufacture's protocol. gRT-PCR was used to examine the mRNA levels of indicated genes with GADPH as internal control. The qRT-PCR reaction was performed with SYBR Green PCR kit (Fermentas) on an ABI-7300 Real-Time PCR system (Applied Biosystem, Foster City, CA). The PCR procedure was 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 45 s at 60°C. The primer sequences were as follows: NEDD9 (NM\_001142393.1), 5'-TGTGAGGTCC-GTAATGTC-3' and 5'-GCCCTTGCCATAAGATTC-3'; MMP2 (NM\_004530.4), 5'-TTGACGGTAAGGAC-GGACTC-3'and5'-GGCGTTCCCATACTTCACAC-3': MMP9 (NM\_004994.2), 5'-AAGGGCGTCGTGG-TTCCAACTC-3' and 5'-AGCATTGCCGTCCTGGGT-GTAG-3'; TGF-B1 (NM\_000660.4), 5'-GACTACT-ACGCCAAGGAGGTC-3' and 5'-GAGAGCAACAC-

# GGGTTCAG-3'; and GADPH (NM\_001256799.1), 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACC-ACCCTGTTGCTGTAG-3'.

#### Western blot analysis

For tissue samples, about 100 mg of tissue was ground with a mortar and pestle under liguid nitrogen. Frozen tissue powder and ESCs were lysed in ice-cold radio immunoprecipitation assay buffer (JRDUN, Shanghai, China) supplemented with protease inhibitor cocktail (Sigma) for 30 minutes. Total protein extracts were separated by 10% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Millipore, Bredford, MA, USA). Anti-NEDD9 (Abcam, ab88584), anti-TGF-B1 (Abcam, ab92486), anti-MMP2 (Abcam, ab92-536), anti-MMP9 (Abcam, ab119906), anti-psmad2/3 (CST, Danvers, MA, USA; #8828), anti-smad2/3 (CST, #8685) and anti-GAPDH (CST, #5174) were used in Western analysis according to the manufacturers' instructions. Visualization was performed using the enhanced chemiluminescence system (Millipore).

#### Cell proliferation assay

Cell Counting Kit (CCK)-8 (Beyotime) was used to assess the proliferation property of ESCs. Briefly, ESCs were seeded in triplicate in 96-well culture plates at a density of 5,000 cells/well and transfected with NEDD9 siRNA or NC. The cultures were incubated for 0, 24, 48 and 72 hours, and then CCK-8 solution were added to each well and incubation was continued for 1 hour. The optical density of each well at 450 nm ( $OD_{450}$ ) was determined by a microplate reader (Bio-Rad, Richmond, CA, USA).



Figure 3. NEDD9 knockdown repressed the invasive ability of ESCs. (A) Invasion assays were performed in Matrigelprecoated Transwell chambers as described in Materials and Methods. (B, C) mRNA and protein levels of MMP2 and MMP9 were examined in ectopic ESCs by qRT-PCR (B) and Western bolt (C) at 48 hours post RNA interference. Data were presented as mean  $\pm$  SD from three independent experiments. \*\*\**P* < 0.001.



**Figure 4.** NEDD9 expression was significantly enhanced by TGF- $\beta$ 1 treatment. ESCs were treated with TGF- $\beta$ 1 (2.5 ng/mL) for 48 hours. Protein levels of p-smad2/3, smad2/3 and NEDD9 were detected by Western blotting. Data were presented as mean ± SD from three independent experiments. \*\*P < 0.01, \*\*\*P < 0.001.

#### Transwell invasion assays

The invasive ability of ESCs was evaluated by Transwell invasion assays using chamber with 8  $\mu$ m pore filters (Corning, New York, NY, USA).

Before assays, the inserts of upper chambers were coated with Matrigel (BD Biosciences). ESCs were serum starved overnight at 24 hours post siRNA transfection, and seeded into the upper chamber with serum-free medium. Then medium with 10% FBS were added to the lower chamber. After 24 hours, non-migrating cells were completely removed with a cotton swab, and the migrated cells were fixed and stained with 0.5% crystal violet and counted in five randomly selected fields under an inverted microscope (Olympus, Lake Success, NY, USA).

#### Statistical analysis

In vitro experiments were performed for three times with each time in triplicate. The results were analyzed by Graphpad Prism 6.0 software (GraphPad, San Diego, CA, USA). Data were presented as mean  $\pm$  SD and analyzed with ANOVA for statistical comparisons. *P*-value less than 0.05 was considered statistically significant.

#### Results

#### Isolation of endometriotic stromal cells (ESCs)

In order to explore the roles of NEDD9 on the progression of endometriosis, ESCs were isolated from ectopic endometrial tissues. Vimentin was abundantly expressed in ESCs, but not in epithelial cells [23]. The immunohistochemistry (IHC) staining demonstrated that vimentin was positively expressed in more than 90% of cultured cells (**Figure 1A**) and the cells can be used in the subsequent assays.

### Suppressing of NEDD9 expression by siRNA transfection

We then knocked down NEDD9 expression in the isolated ESCs by siRNA transfection. After 48 hours, NEDD9 mRNA and protein levels were examined in transfected ESCs by qRT-PCR and Western blot, respectively. As shown in **Figure 1B**, NEDD9 mRNA expression was significantly reduced after NEDD9 siRNA transfection compared to that with control siRNA transfection (NC) (P < 0.0001). The inhibition rate of mRNA expression was 79.8%. NC transfection had no effects on NEDD9 mRNA expression as compared with cells without any treatment (Mock). Similar results were obtained in the protein levels as illustrated by **Figure 1C**.

#### Knockdown of NEDD9 inhibited the proliferation of ESCs

To test the effects of NEDD9 on the proliferative capacity of ESCs, the CCK-8 proliferation assay was performed at 0, 24, 48 and 72 hours after siRNA transfection (**Figure 2**). ESCs with silenced NEDD9 exhibited a significantly lower proliferation rate compared with NC or Mock cells at 48 and 72 hours post transfection (P < 0.001).

## Silencing of NEDD9 inhibited the invasion of ESCs

The ability of endometrial cells to invade the extracellular matrix (ECM) is critical for the development of endometriosis. The ability of cells that digest matrigel and migrate through the membrane was then measured by Transwell assay (**Figure 3A**). ESCs transfected with NEDD9 siRNA ( $30 \pm 8$ ) exhibited significantly lower invasive properties compared with control cells (Mock,  $81 \pm 4$ ; NC,  $77 \pm 9$ ) (P < 0.001).

Matrix metalloproteinases (MMPs) play a key role in endometrial ECM breakdown, which is required for the ectopic growth of endometrial implants [25]. We then examined the mRNA and protein levels of MMP2 and MMP9 at 48 hours post RNA interference. As shown in **Figure 3B**, NEDD9 knockdown notably decreased the expression of both metalloproteinases in transcriptional and translational levels.

### NEDD9 expression was enhanced by TGF-β1 treatment

Previous studies demonstrated that TGF- $\beta$ 1 exposure can induce the transcription of NED-D9 in bone marrow macrophages and human dermal fibroblasts [18-20]. In order to explore the effect of TGF- $\beta$ 1 on NEDD9 expression in ESCs, we stimulated ESCs with TGF- $\beta$ 1 (2.5 ng/mL). As shown in **Figure 4**, TGF- $\beta$ 1 exposure caused a significant increase in smad2/3 phosphorylation and NEDD9 expression. These data demonstrated that NEDD9 expression was enhanced by TGF- $\beta$ 1 treatment.

### Positive correlation between mRNA levels of TGF-β1 and NEDD9 in endometriotic tissues

To study the expression pattern of TGF- $\beta$ 1 and NEDD9 in endometriotic tissues, we collected eutopic endometrial tissues (n=30) and ectopic endometrial tissues (n=30) from 60 patients and control endometrial tissues from 30 women without evident endometriosis. qRT-PCR analysis showed that the mRNA levels of TGF- $\beta$ 1 (Figure 5A) and NEDD9 (Figure 5B) were higher in eutopic (P < 0.01) and ectopic



**Figure 5.** Positive correlation between mRNA levels of TGF- $\beta$ 1 and NEDD9 in endometrial tissues. (A, B) mRNA expression of TGF- $\beta$ 1 and NEDD9 was assessed by qRT-PCR. \*\*P < 0.01, \*\*\*\*P < 0.0001. (C, D) Pearson correlation analysis between mRNA levels of TGF- $\beta$ 1 and NEDD9 in eutopic (C) and ectopic (D) endometrial tissues (n=30).

endometrial tissues (P < 0.0001) than in control endometrial tissues. A significant increase in the mRNA level of TGF- $\beta$ 1 and NEDD9 was also observed in ectopic endometriosis tissues as compared with eutopic endometriotic tissues (P < 0.0001).

We then assessed whether any relationship existed between mRNA levels of NEDD9 and TGF- $\beta$ 1 in endometriotic tissues. Pearson correlation analysis revealed that NEDD9 mRNA level was positively correlated with TGF- $\beta$ 1 mRNA level in eutopic (**Figure 5C**, r=0.7181, P < 0.0001) and ectopic endometrial tissues (**Figure 5D**, r=0.7651, P < 0.0001). These data indicated the association of NEDD9 and TGF- $\beta$ 1 during the pathogenesis of endometriosis.

To verify protein expression pattern of NEDD9 and TGF- $\beta$ 1, Western blot and IHC assays were performed. As shown in **Figure 6**, the same

trend was observed in the protein levels of both NEDD9 and TGF- $\beta$ 1 in three types of endometrial tissues.

#### Discussion

Although the detailed mechanism underlying the pathogenesis of endometriosis, a tumor like lesion [5, 6], is still unclear, the proliferative and invasive ability of endometrial cells is believed to play a key role in the ectopic growth of endometrial implants. During this process, TGF- $\beta$  is suspected to be involved [15]. NEDD9, a TGF- $\beta$ -smad2/3 target gene [18-20], has been regarded as a positive regulator for cancer cell proliferation and invasion [24, 26-29]. However, there is no study investigated the role of NEDD9 in the development of endometriosis until now. In the present study, we found that NEDD9 was associated with the proliferation and invasion of ESCs, and its expression was



**Figure 6.** Protein expression of TGF- $\beta$ 1 and NEDD9 in eutopic endometrial, ectopic endometrial and normal tissues. A. Immunohistochemistry staining. Magnification: 200×. B. Protein expression of TGF- $\beta$ 1 and NEDD9 was evaluated by Western blot analysis. N1, N2 and N3 represent normal tissues; Eut1, Eut2 and Eut3 represent eutopic endometrial tissues; Ect1, Ect2 and Ect3 represent ectopic endometrial tissues.

enhanced by TGF- $\beta$ 1. NEDD9 expression was increased in endometriotic tissues, which was positively related with TGF- $\beta$ 1 expression.

Firstly, suppressing NEDD9 expression resulted in an obvious decrease in cell proliferation (Figure 2) and invasion (Figure 3) of ESCs, which was consistent with previous studies on cancer cells [26-29]. MMPs can facilitate the breakdown of endometrial ECM and aberrant expression of MMPs will lead to the establishment and progression of endometriosis. Altered expression of MMP2 and MMP9 has been reported in eutopic and ectopic endometrial tissues [25]. In breast cancer cells, ectopic expression of NEDD9 upregulated the activity of MMP9 [24]. We then tested the effects of NEDD9 knockdown on the expression of MMP2 and MMP9. NEDD9 siRNA transfection notably down-regulated the mRNA and protein levels of MMP2 and MMP9. These data suggested that NEDD9 may promote ESCs invasion by regulating both proteinases.

TGF-β1, a multifunction cytokine, is suggested to play a role in the pathogenesis of endometriosis [15]. Previous studies have investigated the effect of TGF-β1 on NEDD9 transcription in normal human cell lines and cancer cell lines [18-20]. Here, in vitro experiment demonstrated that mRNA and protein expression of NEDD9 was enhanced by TGF-B1 treatment in ESCs (Figure 4). Moreover, the mRNA (Figure 5) and protein (Figure 6) levels of NEDD9 and TGF-β1 were significantly increased in endometriotic tissues along with the severity of endometriosis. More importantly, we found a positive correlation between NEDD9 and TGF-B1 mRNA expression in human endometriotic tissues (Figure 5). Our study firstly investigated the expression pattern of NEDD9 in human endometriotic tissues and uncovered the association between NEDD9 and TGF-β1.

In summary, NEDD9 played a critical role in the proliferation and invasion of ESCs. NEDD9 expression was up-regulated in endometriotic

tissues, and positively correlated with TGF- $\beta$ 1 expression. Meanwhile, TGF- $\beta$ 1 could induce the expression of NEDD9 in both mRNA and protein levels. Thus, we speculate that TGF- $\beta$ 1 might be involved in the up-regulation of NED-D9 expression in endometriotic tissues, which would probably contribute to the development of endometriosis. Our study may provide a novel therapy target for the treatment of endometriosis.

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#### Disclosure of conflict of interest

None.

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