

## Original Article

# Indoleamine 2,3-dioxygenase-1 (IDO1) enhances survival and invasiveness of endometrial stromal cells via the activation of JNK signaling pathway

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**Abstract:** Evidence for an immunosuppressive function of indoleamine 2,3-dioxygenase (IDO) has been accumulating. However, the unusual distribution of IDO1 in gynecologic cancer cells suggests that modulating immunity may not its only function. To clarify the physiological importance of IDO1 in endometriosis, a tumor-like benign disease, we have investigated the potential mechanism by which IDO1 modulated endometrial stromal cells (ESCs) proliferation and invasion. ESCs were obtained from 16 control women (normal) and 14 patients with ovarian endometrioma, then the normal ESCs were treated with plasmid pEGFP-N1-IDO1 or SD11-IDO1 short hairpin RNA (shRNA) alone, or in combination with c-Jun N-terminal kinase (JNK) inhibitor (SP600125), and subjected to cell viability, proliferation, apoptosis assay and Matrigel invasion assay. IDO1 mRNA expression was evaluated by quantitative real-time reverse transcription-polymerase chain reaction (real-time PCR), and protein levels of IDO1, survivin, protein 53 (p53), matrix metalloproteinase (MMP)-2, MMP-9, tissue-inhibitor of metalloproteinase-1 (TIMP-1) and cyclooxygenase-2 (COX-2) in IDO1-overexpressing and IDO1-deficiency ESCs were analyzed by in-cell Western. We found that IDO1 expression was higher in endometriosis-derived eutopic and ectopic ESCs, compared with endometriosis-free normal ESCs. As a result, IDO1-overexpression in ESCs was markedly linked to reduction of apoptosis and p53 expression, and upregulation of survival, proliferation, invasion, as well as expression of MMP-9, COX-2 expression, rather than expression of survivin, MMP-2 and TIMP-1. Reverse, JNK blockage could abrogate these alterations of ESCs in IDO1-overexpressing milieu, suggesting that JNK signaling pathway was indispensable for ESCs survival, proliferation and invasion enhanced by IDO1, which may contribute to the pathophysiology of endometriosis.

**Keywords:** Endometriosis, indoleamine 2,3-dioxygenase, endometrial stromal cells

## Introduction

Endometriosis, the presence of endometrium outside the uterine cavity, is a common gynecologic disorder, causing abdominal pain, dyspareunia and infertility [1]. As a tumor-like benign disease, endometriosis and cancer are similar in several aspects such as unrestrained growth, decreased apoptosis and aggressive invasion [2].

Indoleamine 2,3-dioxygenase (IDO) is an intracellular heme enzyme that catalyses the initial and rate-limiting step in the metabolism of the essential amino acid tryptophan along the kynurenine pathway. IDO plays crucial roles in vari-

ous infectious diseases, fetal rejection, organ transplantation, neuropathology, autoimmune disorder and cancer by reducing the availability of tryptophan [3-5]. IDO family consists of two members: IDO1 and IDO2. The corresponding genes have a similar genomic structure and are situated adjacent to each other on human chromosome 8. However, different enzymatic activities, diverse expression pattern in response to stimuli within tissues, suggest a distinct role for each protein [6]. Recent human studies indicate that, whereas the IDO2 gene seems to be functional in murine models, it was not found to be functional in humans [7]. Despite of the abundant evidence implicating a role for IDO1 in immunosuppression [3], the unusual distri-

bution of IDO1 in gynecologic cancer cells suggests that modulating immune response was not its only function [8]. IDO1 has been found to be present in the human female genital tract, and its level in endometrium is physiologically regulated by the menstrual cycle [9, 10]. Besides, our previous work demonstrated that IDO1 could also express in endometrial glandular, surface epithelial and stromal cells of endometrium [11]. Moreover, IDO1 was detected to be higher in eutopic endometrium from women with endometriosis by microarrays [12, 13]. Therefore, we decided to test whether IDO1 plays a role in the pathogenesis of endometriosis and also have interactions with other known abnormal factors in endometriosis.

Mitogen-activated protein kinase (MAPK), intracellular signal transducers, have been shown to participate in a diverse array of cell programs, including cell proliferation, cell death, cell movement [14]. Among five distinguishable MAPK modules, which have been identified so far in mammalian systems, the most common ones are the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade, which preferentially regulates cell growth and differentiation, as well as the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, which function mainly in stress responses like inflammation and apoptosis [15, 16]. Association of MAPK activity with the pathogenesis of endometriosis has been well described [17]. It has been reported that enhanced proliferation and survival of eutopic or ectopic endometrial cells from patients with endometriosis correlated with abnormal MAPK phosphorylation [18-20]. Previous work have demonstrated that, in many cell lines and tissues, IDO1 could be induced by lipopolysaccharide (LPS)-mediated effects [9], which related to activation of MAPK [21]. The racemic mixture of IDO1 inhibitor-1-methyl-tryptophan (1-MT) has also been reported to modify the polarization of dendritic cells (DC) by modulating MAPK [22]. Thus, MAPK might exist as the downstream of IDO1. So in the present study, we'd like to explore whether inhibition of MAPK signaling could affect the ESCs biologic characteristics regulated by IDO1.

Given the role of IDO1 and MAPK in endometriosis, the present study is undertaken to explore which MAPK signaling transduction pathway might mediate IDO1-induced ESCs proliferation and invasion, and the possible downstream sig-

nals of IDO1 participating in the modulation of ESCs.

## Materials and methods

### *Patients and tissue collection*

Endometrial or endometriotic samples were obtained from patients (age 23-40 years) who underwent laparoscopy and additional curettage for treatment of endometriosis (n=18) or ovary dermoid cyst (n=18). None of the women had taken medications or received hormonal therapy for at least 6 months prior to surgery. 4 negative samples for endometriosis and 2 for dermoid cyst were excluded after confirmation by laparoscopically and histological diagnosis. The median age was 30.1±5.9 years for the group of women (n=14) with endometriosis and 31.7±9.5 years for the control group (n=16). No significant difference was found between the parity of the endometriosis group (1.26±0.66) and control group (1.69±0.35). All samples were detected histologically to be in the secretory phase of menstrual cycle. Each subject completed a signed, written consent form approved by the Research Ethics Committee in Obstetrics and Gynecology Hospital, Shanghai Medical School, Fudan University. The tissue was collected under sterile conditions and transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium)/F-12 (Gibco, USA).

### *Cell culture*

We purified ESC as described previously elsewhere [23] with slight modification. Tissues were minced into 2- to 3-mm pieces and incubated in DMEM/F12 containing collagenase type IV (0.1%; Sigma, USA) and deoxyribonuclease type I (DNase I; 3000 U; Sigma, USA) with constant agitation for 70 min at 37°C. The resulting dispersed was filtrated through sterile 100 µm and 70 µm nylon strainers (Becton Dickinson) in turn to remove undigested tissue and epithelial cells. The filtrate was then centrifuged at 800×g for 15 min to further remove leukocytes and erythrocytes, and washed with phosphate-buffered saline (PBS). The ESCs were resuspended in DMEM/F-12 containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), and plated into culture flask in 5% CO<sub>2</sub> at 37°C. The culture medium was replaced every 3 days. Cell viability was assessed by

Trypan Blue exclusion assay (approximately 89.7%). The purity of ESCs was more than 95%, as judged by diffuse and strong immunostaining for vimentin and negative for cytokeratin-7 (CK7) in immunocytochemistry.

## *Real-time reverse transcriptase-polymerase chain reaction*

Total RNA was extracted from normal, eutopic and ectopic ESCs with Trizol reagent (Invitrogen; USA). The real-time PCR was performed using the SYBR Green PCR Mix (Fermentas Inc, Glen Burnie, Maryland), according to the manufacturer's instructions. The housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as the normalizer. The forward primer of IDO1 was 5'-TCACAGACCAC-AAGTCACAGC-3'. The reverse primer was 5'-AGT-TGGCAGTAAGGAACAGCA-3'. The forward primer of GAPDH was 5'-AACAGCGACACCCACT-CCTC-3'. The reverse primer was 5'-CATACC-AGGAAATGAGCTTGACAA-3'. The real-time PCR reaction was carried out for 40 cycles. Polymerase chain reactions were run on the Mx4000 and Mx3005 quantitative real-time PCR Stratagene systems (Agilent Technologies). Pair-wise comparisons between target and control at each time point were performed. All validation experiments used four-subject samples in each group. The values were normalized to the GAPDH controls.

## *IDO1 overexpression or shRNA plasmids transfection*

Normal ESCs were grown in culture medium with 10% FBS. When cells had reached confluency, Lipofectamine 2000 (Invitrogen; USA), OPTI-MEM (Gibco, USA) and plasmid pEGFP-N1-IDO1 or SD11-IDO1 shRNA (short hairpin RNA) (GeneChem, Shanghai, China) were mixed and incubated for 20 min and added to the cells at room temperature according to the manufacturer's protocol. The vector-only plasmid pEGFP-N1 and SD11 (GeneChem, Shanghai, China) were used as the negative controls, respectively. And the normal ESCs without plasmid transfection were treated as the blank control. After 6 h of incubation, these cells were then incubated in DMEM/F-12 containing 10% FBS in 5% CO<sub>2</sub> at 37°C.

## *In vitro treatment of ESCs*

To evaluate the effect of JNK MAPK signaling pathway on IDO1-overexpression (pEGFP-

N1-IDO1) or interference (SD11-IDO1 shRNA) normal ESCs survival, proliferation, invasion and target protein expressions, after serum starvation for 12h, the transfected cells were incubated with SP600125 (JNK inhibitor, 10 µM, Sigma, USA), or vehicle (1% dimethyl sulfoxide, DMSO; Sigma, USA) as negative control for 24h.

## *In-cell western*

According to the description by Egorina [24], we used a newly set-up assay called in-cell Western to determine the in-cell protein level of interest. Vector-only transfected ESCs, IDO1-overexpressing or interference ESCs were growing with DMEM/F-12 containing 10% FBS in 96-well plate for 36 h. After 12h serum starvation, the cells were incubated with SP600125 (10 µM) or vehicle (1% DMSO) for 24h, respectively. Then they were fixed with 4% formaldehyde 10 min, washed with 0.1% Triton in PBS for 5 times, and blocked by 150 µl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) for 90 min at room temperature. Subsequently, to detect the MAPK signaling pathway IDO1 activated, the cells were incubated with mouse anti-human phospho-Erk1/2 (1:50, Santa Cruz Biotechnology, USA), mouse anti-human phospho-JNK (1:50, Santa Cruz Biotechnology, USA), mouse anti-human phospho-p38 (1:50, Santa Cruz Biotechnology, USA). And rabbit anti-human Erk1/2 (1:80, Santa Cruz Biotechnology, USA), rabbit anti-human JNK (1:80, Santa Cruz Biotechnology, USA), rabbit anti-human p38 (1:80, Santa Cruz Biotechnology, USA) were added as homologous control, respectively. Additionally, the cells were incubated with mouse anti-human IDO1 (1:30; Abcam, USA), mouse anti-human monoclonal survivin (clone 91630, 20ug/ml; R&D Systems, Abingdon, UK), mouse anti-human monoclonal Protein 53 (p53; Clone 184721, 20ug/ml; R&D Systems, Abingdon, UK), mouse anti-human MMP-2 (clone 101721, 20ug/ml; R&D Systems, Abingdon, UK), mouse anti-human TIMP-1 (clone 151620, 20ug/ml; R&D Systems, Abingdon, UK). The polyclonal antibody of housekeeping protein actin, rabbit anti-human actin (1:50, Santa Cruz Biotechnology, USA) was meanwhile added to each well as an internal control. However, for rabbit anti-human polyclonal COX-2 (1:30, Cell Signaling Technology, USA), rabbit anti-human polyclonal

**Table 1.** Antibody used in in-cell Western

Target protein	Primary antibody		Fluorescence secondary antibody	
	Target	Internal control	Detect target protein	Detect internal protein
P-Erk1/2	mouse anti-human P-Erk1/2	rabbit anti-human Erk1/2	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
P-JNK	mouse anti-human P-JNK	rabbit anti-human JNK	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
P-p38	mouse anti-human P-p38	rabbit anti-human p38	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
IDO1	mouse anti-human IDO1	rabbit anti-human actin	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
survivin	mouse anti-human survivin	rabbit anti-human actin	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
p53	mouse anti-human p53	rabbit anti-human actin	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
MMP-2	mouse anti-human MMP-2	rabbit anti-human actin	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
MMP-9	rabbit anti-human MMP-9	mouse anti-human GAPDH	IRDye™ 800DX -goat anti-rabbit antibody (green)	IRDye™ 700DX -goat anti-mouse antibody (red)
TIMP-1	mouse anti-human TIMP-1	rabbit anti-human actin	IRDye™ 700DX -goat anti-mouse antibody (red)	IRDye™ 800DX -goat anti-rabbit antibody (green)
COX-2	rabbit anti-human COX-2	mouse anti-human GAPDH	IRDye™ 800DX -goat anti-rabbit antibody (green)	IRDye™ 700DX -goat anti-mouse antibody (red)

MMP-9 (20ug/ml, R&D Systems, Abingdon, UK) detection group, homologue mouse anti-human polyclonal GAPDH (1:50, Santa Cruz Biotechnology, USA) was served as an internal control. After overnight treatment at 4°C, the wells were then incubated with corresponding IRDye™ 700DX (red fluorescence)-conjugated goat anti-mouse fluorescence secondary antibody (1:50, Rockland Inc, USA) and IRDye™ 800DX (green fluorescence)-conjugated goat anti-rabbit fluorescence secondary antibody (1:50, Rockland Inc, USA) in the dark (as shown in **Table 1**). The signal was detected and the protein was analyzed semiquantitatively using the Odyssey Infrared Imaging System (LI-COR Biosciences German version of Ltd.). The expression level of the correspondent molecules was calculated as the ratio of the intensity of target proteins to actin or GAPDH.

#### Cell viability assay

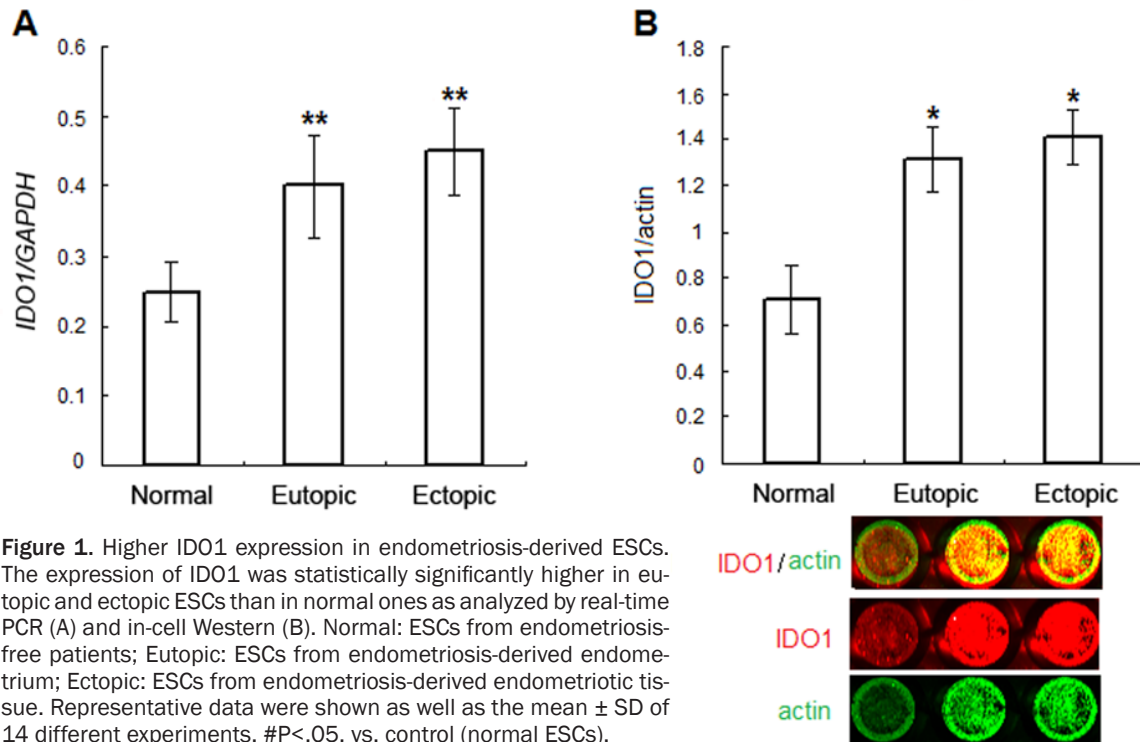
To detect cell viability, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay was used. The IDO1-overexpression or blockage ESCs ( $1 \times 10^4$  cell/ per well in 96 well plate) were cultured without serum for 12h and then incubated with

SP600125 (20  $\mu$ M) or vehicle for 24h in cell-growing media. Cells were then incubated for 4 h in the presence of 2.5 mg/ml MTT and thereafter 100  $\mu$ l DMSO was added. Absorbance (450 nm) was determined using the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria). These values were normalized to the vector-only controls whose absorbance was set to 1.

#### Proliferation assay

The ability of ESCs proliferation was detected by 5-bromo-2'-deoxyuridine (BrdU) cell proliferation enzyme-linked immunosorbent assay (ELISA) system (Millipore, USA) according to the manufacturer's instruction. The transfected ESCs ( $1 \times 10^4$  cell/ per well in 96 well plate) were cultured without serum for 12h and then incubated with SP600125 (20  $\mu$ M) or vehicle for 24h in cell-growing media. The proliferation assay was performed 12 h following the addition of BrdU reagent (10 ng/ml). The absorbance values measured at 450 nm wavelength represent the rate of DNA synthesis and correspond to the number of proliferating cells. These values were normalized to the experimental controls that set to 1.





#### Flowcytometry

The different stages of apoptosis were analyzed by flowcytometry with allophycocyanin (APC) conjugate-annexin V (eBioscience, USA) and propidium iodide (PI) staining. The ESCs transfected with pEGFP-N1-IDO1 or SD11-IDO1 shRNA were cultured without serum for 12h and then incubated with SP600125 (20  $\mu$ M) or not for 24h in cell-growing media. A minimum of 30,000 ESCs were harvested at the same concentration and washed in cold PBS. Then Annexin V-Alexa Fluor 750 and PI working solution were added into cell suspension for 15 min in the dark at room temperature. After staining, cells were washed twice with cold PBS and then applied to flowcytometry (Becton Dickson, San Jose, CA). Data were acquired in the list mode, and the relative proportions of cells within different areas of the fluorescence profile were quantified using the LYSYS II software program (Becton Dickson, Franklin Lakes, NJ). Data were revealed as a percentage of the controls.

#### Matrigel invasion assay

Cells were analyzed for invasion using the Matrigel invasion assay with polycarbonate membranes as previously described [25]. An

equal number of transfected ESCs ( $5 \times 10^4$  resuspended in 200  $\mu$ l DMEM/F12 with 10% FBS) were seeded in the upper Matrigel-coated chambers (8  $\mu$ m pore size, 6.5 mm diameter, Corning, USA) and allowed to invasion for 24 h in 5% CO<sub>2</sub> at 37°C, while SP600125 (20  $\mu$ M) or vehicle was added in the lower chambers. The cells attached to the upper surface of filter were removed by scrubbing with cotton swab, and cells on the underside of the membrane were fixed, stained with hemotoxylin, and counted (five random fields) by two independent investigators. The results were expressed as a percentage of the controls.

#### Statistical analysis

Data were analyzed by Student's t-test and One-way analysis of variance (ANOVA) with post hoc test. Differences were considered as statistically significant at  $P < .05$ .

#### Results

*IDO1 expression in endometriosis-derived eutopic and ectopic ESCs was higher than the normal ones*

The expression of IDO1 in ESCs was determined by real-time PCR and in-cell Western.

## IDO1 regulates ESCs through JNK pathway

The level of IDO1 in eutopic and ectopic ESCs was higher than normal ones (**Figure 1A**,  $P<.05$ ). Moreover, the protein level of IDO1 in endometriosis-derived ESCs elevated significantly compared with that of endometriosis-free ESCs, indicating that IDO1 upregulation in ESCs may be involved in the pathogenesis of endometriosis. However, no statistically significant differences of IDO1 expression between eutopic and ectopic ESCs were observed here ( $P>.05$ ; **Figure 1A** and **1B**).

### *JNK pathway was involved in IDO1 expression of ESCs*

We then explored the signalling pathways involved in the upregulation of IDO1 in endometriosis-derived ESCs. To clarify IDO1's role in ESCs, we transfected normal ESCs with plasmid pEGFP-N1-IDO1 or SD11-IDO1 shRNA (ensemble ID ENSG00000131203) respectively. First we analyzed the effect of plasmid transfection on IDO1 protein expression in these ESCs. In-cell Western analysis showed that IDO1 protein level in ESCs was obviously increased to 1.81 fold after pEGFP-N1-IDO1 transfection, and on the contrary, it was markedly attenuated to 29.80% by the introduction of SD11-IDO1 shRNA, compared with vector pEGFP-N1 or SD11 transfection respectively (**Figure 2A**,  $P<.01$ ). Moreover, IDO1 protein level of IDO1-overexpression ESCs was similar to that of ectopic ones ( $P>.05$ , **Figure 1B** and **2A**), suggesting that the normal ESCs transfected by pEGFP-N1-IDO1 could well mimic the ectopic ESCs as respect of IDO1 expression. Compared with the normal ESCs without transfection (blank controls), pEGFP-N1 and SD11 vector transfected ESCs (negative controls) had effect on neither ESCs' expression of our detected proteins (such as IDO1, P-Erk1/2, Erk1/2, P-JNK, JNK, p38, P-p38, MMP2, MMP-9, TIMP-1, COX-2, survivin and p53) ( $P>.05$ , data not shown), nor ESCs viability, proliferation, apoptosis and invasion ( $P>.05$ , data not shown).

Since the higher MAPK phosphorylation in eutopic or ectopic endometrial cells from patients with endometriosis has been confirmed by others [18-20], then we studied whether IDO1 expression has any effect on change of MAPK phosphorylation in ESCs. As showed in **Figure 2**, P-JNK levels elevated to 1.60 fold in IDO1-overexpression ESCs, while

dramatically decreased to 47.5% in IDO1-deficient ESCs, compared with vector-only control ( $P<.01$ ; **Figure 2C**). No statistically difference of P-p38 or P-ERK1/2 levels upon IDO1 overexpression or knockdown was observed in ESCs (versus vector-only control;  $P>.05$ ; **Figure 2B** and **2D**), indicating that JNK pathway, but not ERK1/2 or p38 pathway, was activated by IDO1 overexpression in ESCs.

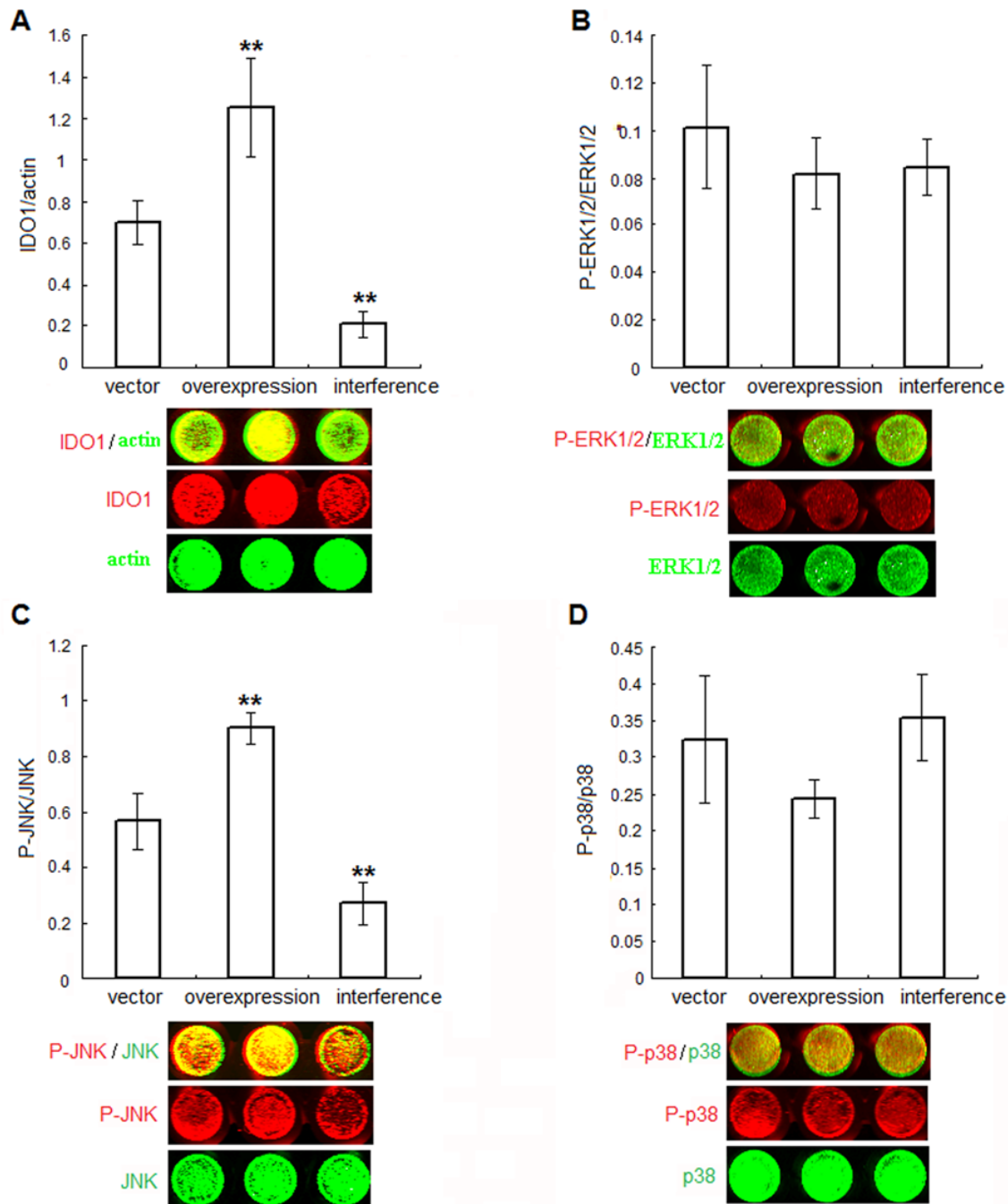
### *IDO1 regulated ESCs viability, proliferation, apoptosis and invasion via JNK signaling pathway*

Based on the results described above, and to further demonstrate the effect of JNK signaling pathway in IDO1-influenced ESCs biological behavior, we analyzed the effects of the JNK inhibitor, SP600125 (20  $\mu$ M) on transfected ESCs viability, proliferation, apoptosis and invasion 24h after its administration. Normal ESCs transfected with or without pEGFP-N1/SD11 vector had the similar effects on ESCs biological characteristics ( $P>.05$ , data not shown). Compared with vector-only transfected ESCs, IDO1-overexpressing ESCs was linked to upregulation of cell survival and growth levels to 128% and 159%, respectively. Additionally, overexpression of IDO1 in ESCs could reduce cell apoptosis to 43% (vs. control;  $P<.05$ ; **Figure 3A-C**). SP600125, an inhibitor of JNK, could reduce viability and proliferation of vector-only and pEGFP-N1-IDO1 transfected ESCs, while triggered their apoptosis ( $P<.05$ ; **Figure 3A-C**). However, SP600125 had no significant effect on IDO1-knockdown ESCs growth. Moreover, compared to the control, IDO1 overexpression significantly increased ESCs invasion ability ( $P<.01$ ; **Figure 3D**), and the migration could be attenuated by JNK signaling inhibitor SP600125 ( $P<.05$ ; **Figure 3D**). Collectively, these data strongly suggest that IDO1 affects cell viability, proliferation, apoptosis and invasion by a mechanism depended on JNK signaling.

### *P53 was indispensable for IDO1-regulated JNK dependent cell growth in ESCs*

To get an insight into the mechanism of JNK-dependent proliferation in IDO1-overexpressing or deficiency ESCs, we detected the proliferation-associated proteins survivin and apoptosis-related protein p53 in transfected ESCs by in-cell Western. Our data showed that IDO1-activated JNK signaling pathway suppressed

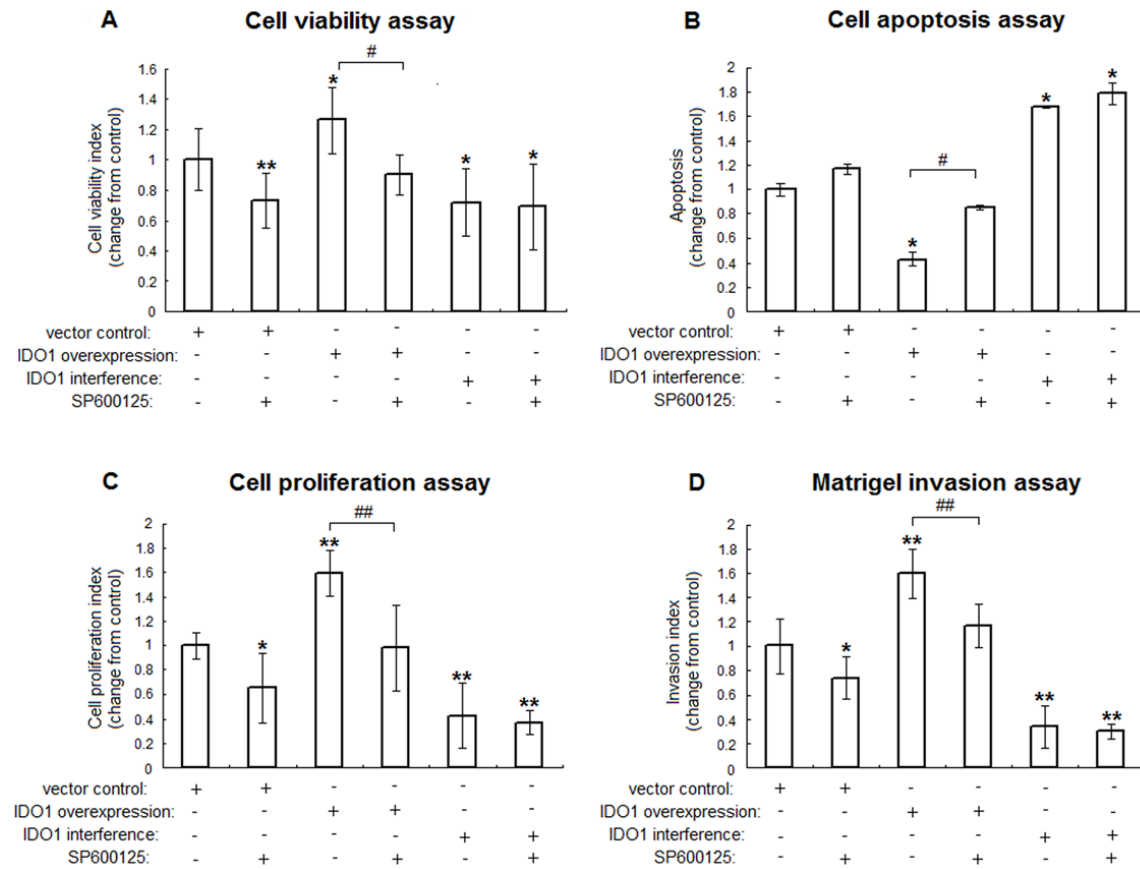
## IDO1 regulates ESCs through JNK pathway



**Figure 2.** IDO1 activates JNK signaling pathway in ESCs. Normal ESCs from endometriosis-free women were treated with plasmid pEGFP-N1-IDO1 or SD11-IDO1 shRNA, and vector-only plasmid as control. The efficiency of plasmid transfection (A), as well as the phosphorylation level of Erk1/2 (B), JNK (C) and p38 (D) was demonstrated by in-cell Western analysis. Herein, vector control: normal ESCs transfected with vector-only plasmid; IDO1 overexpression: normal ESCs transfected with plasmid pEGFP-N1-IDO1; IDO1 interference: normal ESCs transfected with SD11-IDO1 shRNA. \* $P < .01$ , vs. normal group, \*\* $P < .01$ , vs. vector control group. Representative data as well as the mean  $\pm$  SD of 12 different experiments were shown.

expression of p53 to 77.1% (vector control without SP600125 vs. IDO1-overexpressing ESCs without SP600125,  $P < .05$ ; **Figure 4B**), and its

expression was elevated to 117% by SP600125 (IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125,



**Figure 3.** JNK signaling pathway responsible for enhancement of the ESCs viability, proliferation and invasion by IDO1. IDO1-overexpressing and IDO1-deficiency ESCs were treated with JNK inhibitor (SP600125, 20  $\mu$ M) or vehicle control (1% DMSO) for 24 h. Thereafter, MTT assay (A), apoptosis assay (B), BrdU assay (C) and Matrigel invasion assay (D) were applied to analyze the viability, apoptosis, proliferation and invasion of transfected ESCs, respectively. The columns represent the mean  $\pm$  SD calculus of 8 different experiments. \* $P < .05$  vs. vector control transfected ESCs without SP600125; \*\* $P < .01$  vs. vector control transfected ESCs without SP600125; # $P < .05$ , IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125; ## $P < .01$ , IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125.

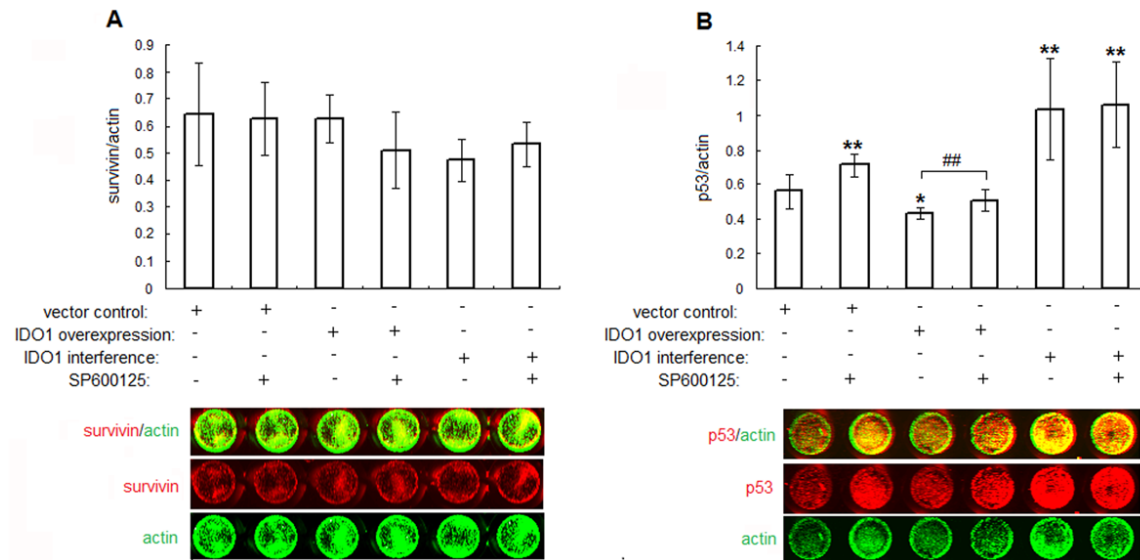
$P < .01$ ; **Figure 4B**). Besides, p53 expressions in IDO1-deficiency ESCs with or without SP600125 were stimulated to 185% and 190% ( $P < .01$  vs. vector control without SP600125; **Figure 4A**). Conversely, no statistical changes in survivin levels upon IDO1 transfection or JNK inhibitor were observed ( $P > .05$ , **Figure 4A**). Thus, IDO1 regulated p53 expression in normal ESCs via JNK signaling pathway.

#### JNK inhibitor on IDO1-induced MMP-2, MMP-9, TIMP-1 and COX-2 expression

To rule out how IDO1 participated in the regulation of ESCs invasion, we analyzed the influence of IDO1 overexpression or knockdown on ESCs MMPs, TIMP-1 and COX-2 expression. Data were presented in **Figure 5** that, JNK

inhibitor could abrogate IDO1-stimulated MMP-9 and COX-2 expression in the IDO1-overexpressing ESCs (IDO1-overexpressing with SP600125 administration vs. IDO1-overexpressing without SP600125 administration;  $P < .05$ ; **Figure 5B** and **5D**). Conversely, IDO1-deficiency ESCs had lower MMP-9, COX-2 expression ( $P < .01$ , **Figure 5B** and **5D**) compared with ESCs transfected with vector only, and that couldn't be influenced by SP600125 ( $P > .05$ ; **Figure 5B** and **5D**). Surprisingly, neither IDO1 nor JNK inhibitor could affect MMP-2, TIMP-1 expression ( $P > .05$ ; **Figure 5A** and **5C**). These findings suggested that IDO1 might be an upstream signal participating in the regulation of MMP-9 and COX-2, thereby possibly controlling the invasion of ESCs. However, further work should be done to confirm this causation.





**Figure 4.** IDO1 inhibits p53 expression through JNK signaling pathway. The transfected ESCs were treated with JNK inhibitor (SP600125, 20  $\mu$ M) or vehicle control (1% DMSO) for 24 h. Thereafter, the expressions of proliferation associated proteins survivin (A), p53 (B) were determined by in-cell Western. The columns represent the mean  $\pm$  SD calculus of 8 different experiments. \* $P < .05$  vs. vector control transfected ESCs without SP600125; \*\* $P < .01$  vs. vector control transfected ESCs without SP600125; # $P < .05$ , IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125.

## Discussion

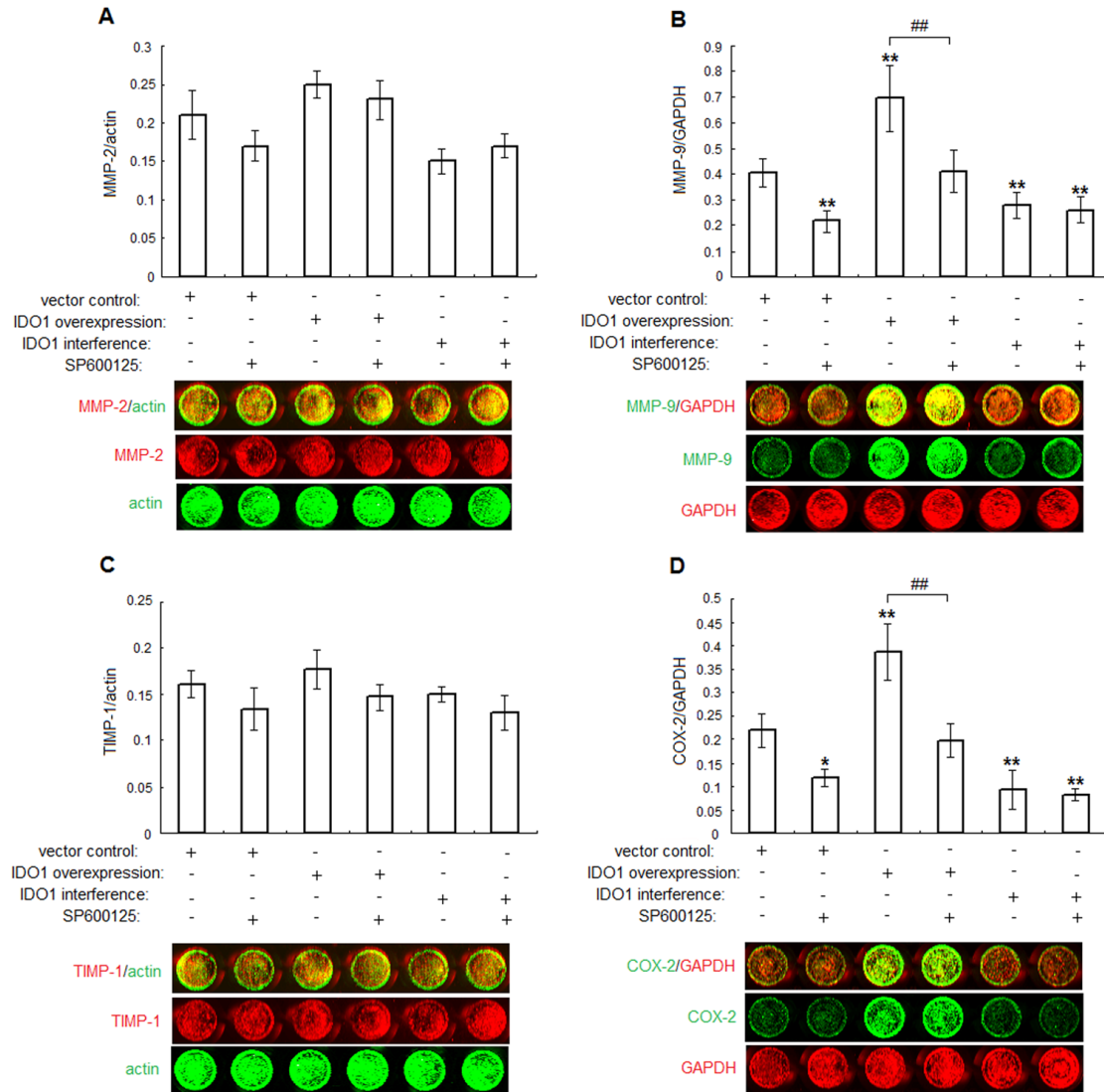
The results presented establish unambiguously that IDO1 highly expresses in eutopic and ectopic ESCs from patients with endometriosis than normal ones, and overexpression of IDO1 in normal ESCs elicits an increase in the phosphorylation of the JNK signaling pathway. Via JNK pathway, IDO1 regulates ESCs expression of p53, MMP-9 and COX-2, which were accompanied by the enhancement of cell survival, proliferation, invasion, and coupled to inhibitory effects on cell apoptosis.

Traditionally, IDO is thought to be an immune modulator through tryptophan depletion and via the generation of proapoptotic metabolites [4]. It has also been pointed out to be participating in tumor progression [8]. Since endometriosis is a gynecological tumor-like disease, we supposed that IDO1 is a potential candidate which facilitates endometriosis development. Burney [12] and Aghajanova [13] have mentioned that IDO1 gene expression increased in endometriosis-derived eutopic endometrium, and was relevant to the patients' clinical stage. And our previous result also revealed that IDO1 present in the stromal cells of endometrium or endometriotic tissue, and especially highly

expressed in endometriosis-derived ESCs [11]. To further test the mechanism of IDO1 in origin of endometriosis, we regulated IDO1 expression by transfection of plasmid pEGFP-N1-IDO1 or SD11-IDO1 shRNA, which could well reflect the role of IDO1 in endometriosis-derived ESCs, and re-evaluated the effect of IDO1 on ESCs biologic functions. We found that overexpressing of IDO1 significantly increase the P-JNK in ESCs, which is in agreement with others' work in CD11+ dendritic cells [26].

JNK belong to the MAPK family, which is critical for cellular functions in eukaryotic cells. Each pathway is preferentially recruited by distinct sets of stimuli, thereby allowing cells to response to multiple divergent inputs in a coordinate manner. Recently, clusters of researches have indicated the importance of MAPK in functions of human eutopic and ectopic endometrial cells [17, 27, 28]. And the enhanced proliferation and survival of eutopic or ectopic endometrial cells from endometriosis patients have been confirmed to correlate with higher level of MAPK phosphorylation [18-20]. The JNK protein kinases are collectively referred to as stress-activated MAP kinase (SAPKs), and encoded by three distinct genes. JNK1 and JNK2 are ubiquitously expressed, while JNK3 is

## IDO1 regulates ESCs through JNK pathway



**Figure 5.** IDO1 stimulates COX-2, MMP-9 expression in ESCs through activation of JNK signaling pathway. IDO1 in normal ESCs was overexpressed or blockaded by plasmid transfection, and treated with or without JNK inhibitor (SP600125, 20 $\mu$ M). The expression of invasion-related proteins MMP-2 (A), MMP-9 (B), TIMP-1 (C) and COX-2 (D) were determined by in-cell Western. Data were shown as the mean  $\pm$  SD calculus of 8 different experiments; \* $P < .05$  vs. vector control transfected ESCs without SP600125; \*\* $P < .01$  vs. vector control transfected ESCs without SP600125; # $P < .05$ , IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125; ## $P < .01$ , IDO1-overexpressing ESCs with SP600125 vs. IDO1-overexpressing ESCs without SP600125.

selectively expressed in the brain [29]. JNK phosphorylation and activation occur in response to a variety of environmental, developmental, and inflammatory stimuli [30]. In the canonical JNK pathway, activated JNK acts to phosphorylate the transcriptional activation domain of c-Jun, which then constitutes the activator protein-1 (AP-1) transcription factor with c-Fos [31]. Subsequently, G protein-coupled receptors regulate MAPK signaling path-

ways that result in the expression of specific response genes involved in cell proliferation, invasion and apoptosis [32]. Since the regulatory elements such activator protein-1 (AP-1) was located on the human IDO gene promoter region, it could better explain the role of JNK in IDO1-regulated ESCs [21]. Because JNK has been shown to be required for IDO1 expression, we used SP600125 as it is a potent, cell-permeable and selective inhibitor of JNK. It com-

petitively targets the AP binding site of JNK1, JNK2 and JNK3, exhibiting over 300-fold greater selectivity for JNK [33].

Endometriotic cells are identified with altered growth potency and lower susceptibility to apoptosis. However, SP600125, the blocker of JNK, led to the inhibitory activity in survival and proliferation, while offered greater level of apoptosis, as well as the expression of p53 in IDO1-overexpressing ESCs. The role of apoptosis in the physiopathology of endometriosis is increasingly apparent [34, 35]. It can be initiated by extracellular and intracellular “death signals” that increase p53 protein expression [36]. Evidences for p53 as a marker of anomalous apoptosis in endometriosis has been accumulating, especially in ovarian endometriosis [37, 38]. And experiments also suggested that JNK pathway is associated with inhibition of p53 in human [39, 40]. Similarly, our findings suggest that IDO1 could downregulate the expression of p53, as well as ESCs apoptosis through JNK pathway. Survivin has also been revealed to participate in the endometriosis, and correlated with apoptosis and invasive phenotype of endometriotic tissues [2]. It has been defined to be regulated mainly through the Raf-1/MEK/ERK pathway in human cells but not JNK pathway [41, 42], indicating that increase of survivin in endometriotic tissue may due to the other factors rather than IDO1.

Invasion, controlled by cross-talk mechanisms between cells and extracellular microenvironment, has been investigated in the pathogenesis of endometriosis [43]. We demonstrated that IDO1-overexpression ESCs had an elevated invasiveness compared to that of normal ESCs. Moreover, JNK inhibitor could abolish the increase invasion capability and MMP-9, COX-2 expressions of ESCs induced by IDO1 in a significant manner. Our findings were in line with previous findings that MMPs and COX-2 are involved in the regulation of endometriotic cells [44, 45]. It has been reported that product of COX-2, prostaglandins (PGs), can explain most of the symptoms of endometriosis [46]. Conversely, selective inhibition of PGE2 receptors could decrease migration and invasion of human immortalized endometriotic epithelial and stromal cells into Matrigel [47]. Another essential proteinase-MMP, the enzymes for extracellular matrix (ECM) degradation was also play a vital role in the invasion of endome-

triotic lesions. The retrograde endometrial tissue could be more prone to peritoneal implantation and invasion due to the altered production of MMPs in eutopic endometrium from endometriosis affected women [2]. Upregulation of COX-2 and MMPs secretion response to various stimuli through JNK pathway has been reported yet [48, 49]. We conjecture that, MMP-9 and COX-2 secreted from IDO1-stimulated ESCs may contribute to the invasion of ESCs and may be activated in the disease of ESCs via JNK pathway, though a further study needed to reinforce the thesis.

In conclusion, abnormal expression of IDO1 in ESCs is associated with aberrant activation of JNK pathway, which contributed to the down-regulation of p53 and coupled to inhibitory of cell apoptosis. Besides, through JNK pathway, IDO1 induced the expression of MMP-9 and COX-2, and led to the increased invasion of ESCs. Based on our previous work, the present study further probed into the potential signaling pathway through which IDO1 involved in the origin of endometriosis, as well as its downstream effect molecules. However, the evidences are still insufficient to confirm that, whether increased IDO1 in eutopic endometrium of women with endometriosis precedes the development of disease or results afterwards from development of ectopic lesions. So animal model should next be established to help us to understand and elude how IDO1 participates in the pathophysiology of endometriosis after all. Therefore, this information would be helpful in further investigation on the pathogenesis and therapeutics of endometriosis.

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