

# Transforming growth factor $\beta$ 1 enhances adhesion of endometrial cells to mesothelium by regulating integrin expression

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Endometriosis is the abnormal growth of endometrial cells outside the uterus, causing pelvic pain and infertility. Furthermore, adhesion of endometrial tissue fragments to pelvic mesothelium is required for the initial step of endometriosis formation outside uterus. TGF- $\beta$ 1 and adhesion molecules importantly function for adhesion of endometrial tissue fragments to mesothelium outside uterus. However, the function of TGF- $\beta$ 1 on the regulation of adhesion molecule expression for adhesion of endometrial tissue fragments to mesothelium has not been fully elucidated. Interestingly, transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) expression was higher in endometriotic epithelial cells than in normal endometrial cells. The adhesion efficiency of endometriotic epithelial cells to mesothelial cells was also higher than that of normal endometrial cells. Moreover, TGF- $\beta$ 1 directly induced the adhesion of endometrial cells to mesothelial cells through the regulation of integrin of  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4 via the activation of the TGF- $\beta$ 1/TGF- $\beta$ RI/Smad2 signaling pathway. Conversely, the adhesion of TGF- $\beta$ 1-stimulated endometrial cells to mesothelial cells was clearly reduced following treatment with neutralizing antibodies against specific TGF- $\beta$ 1-mediated integrins  $\alpha$ V,  $\beta$ 1, and  $\beta$ 4 on the endometrial cell membrane. Taken together, these results suggest that TGF- $\beta$ 1 may act to promote the initiation of endometriosis by enhancing integrin-mediated cell-cell adhesion. [BMB Reports 2017; 50(8): 429-434]

## INTRODUCTION

Endometriosis is a common gynecological disorder defined as growth of endometrial tissues outside the uterus. Possible causes include retrograde menstruation, immunological disorders, invasive implantation, and ectopic growth of endometrial tissues (1, 2). However, the precise mechanisms that underlie the initial development and subsequent progression of endometriosis are not clear. At the initial stages of the disease, the attachment of retrograde endometrial tissues onto the pelvic mesothelium is a critical step. Several adhesion molecules, including integrin  $\alpha$ v $\beta$ 3,  $\alpha$ 4 $\beta$ 1, VCAM-1, and Nectin-4, have been suggested to be key factors in regulating the attachment of endometrial and mesothelial tissues (3-6). Furthermore, while previous studies have demonstrated that these adhesion molecules are regulated by cytokines and growth factors (7-12), the mechanisms that underlie this regulation are still not clear.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a 25 kDa peptide that plays key roles in the progression of endometriosis (13). TGF- $\beta$  expression is higher in the serum, peritoneal fluid, and cyst tissues of patients with endometriosis than in women without endometriosis (14-16). Among the three subtypes of TGF- $\beta$ s, TGF- $\beta$ 1 is generally considered to be a key player in the pathogenesis of endometriosis, due to its pattern of expression and correlation with disease progression (13, 17). TGF- $\beta$ 1 is involved in the suppression of immune surveillance, cell adhesion and invasion into the peritoneum, and in the growth of implants (13). Peritoneal adhesion of endometrial cells, in particular, is elevated in the presence of TGF- $\beta$ 1 (18, 19). The expression of several integrins in different cells is controlled by TGF- $\beta$ 1 (20-23); therefore it was suggested that cell-cell interactions that are activated by TGF- $\beta$ 1 could be mediated by integrins (13). To date, there have been no reports describing direct evidence for TGF- $\beta$ 1-mediated regulation of integrins and other adhesion molecules in endometrial cells, or its role in peritoneal adhesion of

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retrograde endometrium.

In the present study, we demonstrated that autocrine secretion of TGF- $\beta$ 1 increased adhesion between endometrial and mesothelial cells through expression of integrin  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4. Moreover, blocking these integrins with neutralizing antibodies suppressed the mesothelial adhesion of endometrial cells. Thus, we suggest that TGF- $\beta$ 1 may act to promote the initiation of endometriosis by enhancing integrin-mediated cell-cell adhesion.

## RESULTS

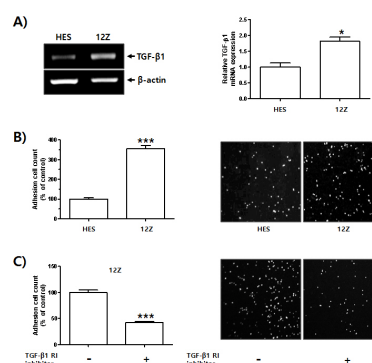
### TGF- $\beta$ 1 expression is increased in endometriotic epithelial cells and is associated with the adhesion of endometrial cells to mesothelial cells

It has been reported that TGF- $\beta$ 1 expression is increased in the peritoneal fluid of women with endometriosis (13, 24). In the current study, we compared levels of TGF- $\beta$ 1 expression in normal human endometrial cells (HES cells) and human endometriotic epithelial cells from endometrial lesions (12Z cells). As shown in Fig. 1A, TGF- $\beta$ 1 expression was significantly higher in 12Z cells than in HES cells. Results from several recent studies have suggested that proliferative, secretory, and menstrual endometrial fragments rapidly attach to the peritoneal mesothelium in case of endometriosis (25-28). Therefore, we also investigated differences in the adhesion of HES and 12Z cells to human mesothelial cells (Met-5A). As shown in Fig. 1B, the adhesion ratio of 12Z cells to Met-5A cells was approximately 3 times higher than that of HES cells. Because TGF- $\beta$ 1 expression and adhesion were higher in endometriotic epithelial cells than in normal endometrial cells, we examined whether TGF- $\beta$ 1-mediated signaling is involved in the adhesion of 12Z cells to Met-5A cells.

Interestingly, adhesion rate of TGF- $\beta$  receptor I (TGF- $\beta$ RI) inhibitor (SB-525334, Sigma, St. Louis, MO, USA)-treated 12Z cells to Met-5A cells was lower than that of 12Z cells (Fig. 1C). These results suggest that enhanced expression of TGF- $\beta$ 1 in 12Z cells affects the adhesion of endometrial cells to mesothelial cells, thus playing a role in the progression of endometriosis.

### TGF- $\beta$ 1 induces the adhesion of endometrial cells to mesothelial cells through the TGF- $\beta$ RI/Smad2 signaling pathway

Secretion of TGF- $\beta$  into the peritoneal fluid plays an important role in the establishment of endometriosis (13, 24). Thus, we investigated whether TGF- $\beta$ 1 played a role in the initial stages of endometriosis formation outside the uterus via direct induction of the adhesion of endometrial cells to mesothelial cells. Adhesion rates of TGF- $\beta$ 1-stimulated HES and 12Z cells to Met-5A cells were clearly higher than that of untreated HES and 12Z cells (Fig. 2A). Furthermore, TGF- $\beta$ 1 significantly induced the adhesion of normal endometrial cells to mesothelial

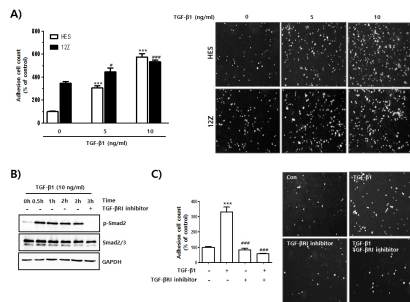


**Fig. 1.** Enhanced TGF- $\beta$ 1 expression in human endometriotic epithelial cells and its function in adhesion of endometrial cells to mesothelial cells. Total RNA was extracted from HES cells and 12Z cells. (A) Levels of TGF- $\beta$ 1 mRNA expression were examined using RT-PCR.  $\beta$ -actin was used as an internal control. Band intensity of TGF- $\beta$ 1 mRNA expression was quantified and normalized to  $\beta$ -actin internal control using densitometry (ImageJ software, NIH). Data obtained from densitometric analyses are shown as bar graph. Data are expressed as fold of control and shown as mean  $\pm$  SD for three independent experiments (\* $P$  < 0.05 in comparison between two groups). Differences between mean values and two groups were evaluated using Student's t-test and analysis of variance with an unpaired t-test. (B) HES cells ( $5 \times 10^5$  cells) were seeded onto 6-well plate and cultured for 24 h. 12Z cells ( $3 \times 10^5$  cells) were seeded onto 100  $\pi$  culture dish plate and cultured for 24 h. HES and 12Z cells were labeled with CMFDA for 15 min at 37°C, then washed in  $1 \times$  PBS and gently transferred onto a Met-5A cell monolayer. Number of HES and 12Z cells bound to confluent Met-5A cells was manually counted. Four pictures were taken per well and the number of adherent cells was calculated as a percentage of the control cell values and expressed as mean  $\pm$  SD for three independent experiments (\*\* $P$  < 0.01 in comparison between two groups). Differences between mean values and two groups were evaluated using Student's t-test and analysis of variance with an unpaired t-test. (C) 12Z cells ( $3 \times 10^5$  cells) were seeded onto 100  $\pi$  culture dish plate and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF- $\beta$ RI inhibitor for 24 h. Cells were then labeled with CMFDA for 15 min at 37°C, then washed in  $1 \times$  PBS and gently transferred onto a Met-5A cell monolayer. Number of cells bound to confluent Met-5A cells was manually counted. Four pictures were taken per well and the number of adherent cells was calculated as a percentage of the control cell values and expressed as mean  $\pm$  SD for three independent experiments (\*\* $P$  < 0.01 in comparison between two groups). Differences between mean values and two groups were evaluated using Student's t-test and analysis of variance with an unpaired t-test.

cells through activation of Smad-2 signaling. However, treatment with a TGF- $\beta$ RI inhibitor markedly suppressed the TGF- $\beta$ 1-induced adhesion of HES cells to Met-5A cells by inhibition of the TGF- $\beta$ RI/Smad2 signaling pathway (Fig. 2B and C).

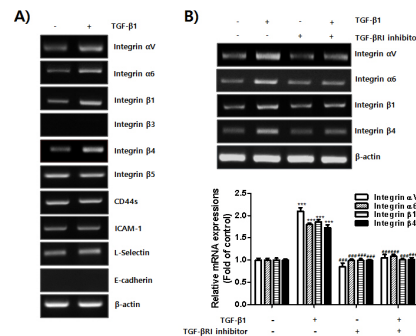
### TGF- $\beta$ 1 induces the expressions of cell adhesion molecules in endometrial cells

Adhesion molecules, including integrins, CD44, ICAM-1,



**Fig. 2.** Increased adhesion of endometrial cells to mesothelial cells by activation of TGF-β1-mediated signaling. (A) HES and 12Z cells were seeded and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β1 for 24 h. Cells were labeled with CMFDA for 15 min at 37°C, then washed in 1 × PBS and gently transferred onto a Met-5A cell monolayer. After gentle shaking at 20 rpm for 20 min at 37°C, Cells were washed three times with 1 × PBS to remove unbound cells. Attached cells were visualized using a fluorescent microscope, and quantified using ImageJ software. The number of cells in 4 randomly chosen areas in each well was used for statistical analysis. The results from 3 independent experiments were calculated as a percentage of the control cell values and presented as mean ± SD. \*\*\*P < 0.001 compared to control white bar graph (1<sup>st</sup> column). #P < 0.05 and ###P < 0.001 compared to control black bar graph (1<sup>st</sup> column). Differences between mean values of experimental groups were determined using one-way analysis of variance (one-way ANOVA) with a Tukey's post-hoc test, using GraphPad Prism software. (B) HES cells were seeded and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β1 in the presence or absence of TGF-βRI inhibitor for the indicated times. Phosphorylation levels of Smad2 were analyzed using western blot. GAPDH expression was used as an internal control. (C) HES cells were seeded and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β1 in the presence or absence of TGF-βRI inhibitor for the indicated times. Cells were labeled with CMFDA for 15 min at 37°C, then washed in 1 × PBS and then gently transferred onto a Met-5A cell monolayer. Number of cells bound to confluent Met-5A cells was manually counted. Four pictures were taken per well and the number of adherent cells was calculated as a percentage of the control cell values and shown as mean ± SD for three independent experiments. \*\*\*P < 0.001 compared to negative control (1<sup>st</sup> column). ###P < 0.001 compared to positive control (2<sup>nd</sup> column). Differences between mean values of experimental groups were determined using one-way ANOVA with a Tukey's post-hoc test, using GraphPad Prism software.

L-selectin and E-cadherin (29) and TGF-β1, play pivotal roles in the attachment of endometrial cells outside the uterus i.e., the initiation of endometriosis; however, to the best of our knowledge, there is no direct evidence for a regulatory function of TGF-β1 on expressions of adhesion molecules. As shown in Fig. 3A, TGF-β1 induced the expression of integrins αV, α6, β1, and β4. The expression levels of integrin β5, CD44s, ICAM-1, and L-selectin were not increased by TGF-β1 treatment, and integrin β3 and E-cadherin were not detectable under the same conditions. To further investigate this result,

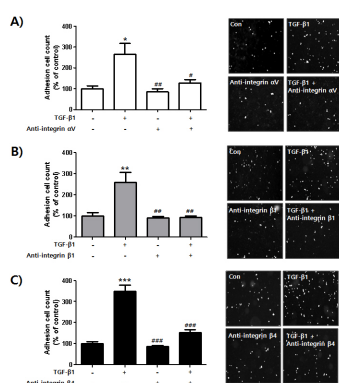


**Fig. 3.** Expressions of integrin αV, α6, β1, and β4 induced by TGF-β1 in endometrial cells. HES cells were seeded and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β1 for 24 h. Total RNA was extracted from the cells. (A) mRNA expression of adhesion molecules was examined using RT-PCR. β-actin was used as an internal control. (B) HES cells were seeded and cultured for 24 h. Medium was replaced and the cells were incubated in serum free-medium with or without TGF-β1 in the presence or absence of TGF-βRI inhibitor for 24 h. Total RNA was extracted from the cells. Expression levels of integrin αV, α6, β1, and β4 were examined using RT-PCR. β-actin was used as an internal control. Band intensity of each integrin mRNA expression was quantified and normalized to β-actin internal control using densitometry. Data obtained from densitometric analyses are shown as bar graph. Data are expressed as fold of control and are shown as mean ± SD for three independent experiments \*\*\*P < 0.001 compared to each negative control (1<sup>st</sup> column). ###P < 0.001 compared to each positive control (2<sup>nd</sup> column). Differences between mean values of experimental groups were determined using a one-way ANOVA with a Tukey's post-hoc test, using GraphPad Prism software.

we used a TGF-βRI inhibitor and measured levels of integrin αV, α6, β1, and β4 mRNA expression. Treatment with the TGF-βRI inhibitor clearly reduced integrin expression levels (αV, α6, β1, and β4) in HES cells induced by TGF-β1 (Fig. 3B). Thus, our data showed that TGF-β1 induced adhesion of endometrial cells to mesothelial cells by enhancing the expression of integrins αV, α6, β1, and β4.

### Neutralizing integrin αV, β1, and β4 inhibits adhesion of TGF-β1-stimulated HES cells to Met-5A cells

We then started to investigate whether integrin subunits αV, β1, and β4 regulated the adhesion of endometrial cells to mesothelial cells through TGF-β1-induced expression of integrin heterodimers αVβ1, α6β1, and α6β4 in endometrial cells. We assessed the adhesion of TGF-β1-stimulated endometrial cells to mesothelial cells in the presence of neutralizing antibodies against integrin subunits αV, β1, and β4. As shown in Fig. 4, TGF-β1 greatly enhanced the adhesion of endometrial cells to mesothelial cells. However, this adhesion was significantly reduced when the TGF-β1-mediated expression levels of integrin dimers αVβ1, α6β1, and α6β4 on endometrial cell surfaces were disrupted by neutralizing antibodies (Fig. 4). These results suggested that secreted TGF-β1 may play a role



**Fig. 4.** Blocking adhesion of TGF- $\beta$ 1-stimulated endometrial cells to mesothelial cells using integrin  $\alpha$ V,  $\beta$ 1, and  $\beta$ 4 neutralizing antibodies. HES cells were seeded and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF- $\beta$ 1 in the presence or absence of integrin (A)  $\alpha$ V, (B)  $\beta$ 1, or (C)  $\beta$ 4 antibodies for 24 h. Cells were labeled with CMFDA for 15 min at 37°C, then washed in 1  $\times$  PBS and gently transferred onto a Met-5A cell monolayer. Number of cells bound to confluent Met-5A cells was manually counted. Four pictures were taken per well and the number of adherent cells was calculated as a percentage of the control cell values and shown as mean  $\pm$  SD for three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared to each negative control (1<sup>st</sup> column of each graph). #P < 0.05, ##P < 0.01, ###P < 0.001 compared to each positive control (2<sup>nd</sup> column of each graph). Differences between mean values of experimental groups were determined using a one-way ANOVA with a Tukey's post-hoc test, using GraphPad Prism software.

in the adhesion of endometrial fragments generated by menstruation and the passage of these fragments outside the uterus, where endometriosis is initiated through attachment to the mesothelium. The molecular mechanism involves enhanced expression of integrin heterodimers  $\alpha$ V $\beta$ 1,  $\alpha$ 6 $\beta$ 1, and  $\alpha$ 6 $\beta$ 4 in endometrial cells.

## DISCUSSION

Integrins are heterodimeric membrane proteins composed of non-covalently associated  $\alpha$  and  $\beta$  subunits, that are essential for linking the extracellular matrix to the cytoskeleton. In mammals, there are 18  $\alpha$  subunits and 8  $\beta$  subunits that can assemble into 24 different  $\alpha\beta$  combinations (30). The role of integrins in the reproductive system has been studied for over 20 years. A major focus of these studies has been the involvement of these transmembrane receptors in embryo-endometrial interactions during the implantation window (31). Many researchers have continued to work towards elucidating the function of integrins during endometriosis (32) and have identified potential biomarkers for use in diagnosis and treatment of the disease (33).

Several integrins, including  $\alpha$ V $\beta$ 3,  $\alpha$ V $\beta$ 5,  $\alpha$ V $\beta$ 6,  $\alpha$ 4 $\beta$ 1, and  $\alpha$ 6 $\beta$ 1, have been reported to mediate the attachment of

endometrial cells to the mesothelium (4, 34-36). The expression of these integrins is tightly regulated by diverse molecules, such as interleukin (IL)-1, IL-8, macrophage inhibitory factor (MiR)-183, prostaglandin E2, and a cannabinoid receptor agonist (7, 9-11, 37, 38). However, despite the obvious importance of TGF- $\beta$ 1 in the progression of endometriosis (13, 17), there have been no reports of the TGF- $\beta$ 1 function in the regulation of integrins in endometrial cells. Thus, we examined the regulation of integrins by TGF- $\beta$ 1 and their role in the initiation of endometriosis.

First, we confirmed the correlation between endometrial-mesothelial adhesion and TGF- $\beta$  expression. Our results clearly showed that autocrine expression of TGF- $\beta$ 1 in endometrial cells positively regulated their attachment to mesothelial cells. In human endometriosis lesions, TGF- $\beta$ 1 expression has been found in macrophages, endometrial epithelial cells, endometrial stromal cells, and mesothelial cells (13, 24). Elevated levels of secreted TGF- $\beta$ 1 in peritoneal fluid influenced many steps in the progression of endometriosis, including immune surveillance, cell adhesion and invasion into the peritoneum, angiogenesis, and growth of implants (13, 17). The response of TGF- $\beta$ 1 measured by endometrial-mesothelial adhesion is different between normal endometrial HES cells and ectopic endometrial cells (12Z). Rai et al. (39) reported that expression of adhesion molecules is different between the normal endometrium and endometriosis tissues. Thus, we proposed that it might be caused by elevated expression of TGF- $\beta$ 1 in 12Z cells. We further showed that the use of a specific TGF- $\beta$  RI inhibitor to inhibit the activity of TGF- $\beta$ 1 activity greatly reduced mesothelial adhesion of TGF- $\beta$ 1-stimulated HES cells. From these results, we postulated that secreted TGF- $\beta$ 1 may act in an autocrine fashion on endometrial-mesothelial interactions.

To identify the factors that mediate TGF- $\beta$ 1-enhanced cell-cell interactions, we analyzed the expression of several adhesion molecules that are known to be important in the development of endometriosis (40, 41). Levels of integrins  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1 and  $\beta$ 4 mRNA were clearly increased following TGF- $\beta$ 1 treatment. The expression levels of integrin  $\beta$ 5, CD44s, ICAM-1, L-selectin were not increased, and integrin  $\beta$ 3 and E-cadherin were not detectable under the same conditions. We further suppressed the activity of activity of TGF- $\beta$ 1 by examining whether the expression levels of integrins  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4 were dependent on TGF- $\beta$ 1.

Previous studies reported that several integrins, such as  $\alpha$ V,  $\beta$ 1, and  $\beta$ 3, were positively regulated by TGF- $\beta$ 1 in fibroblast, glioblastoma, and kidney epithelial cells (23, 42, 43). In the present study, expression levels of integrins  $\alpha$ V,  $\alpha$ 6, and  $\beta$ 1 were increased in normal endometrial HES cells following TGF- $\beta$ 1 treatment, but integrin  $\beta$ 3 expression was not affected by the same conditions. In A549 lung cancer cells, sustained ERK activity induced by TGF- $\beta$ 1 is involved in the induction of integrin  $\beta$ 3 (44). In the current study, phosphorylation of Smad2 was found to be a major step in the signaling pathway

involved in TGF- $\beta$ 1-stimulated integrin expression and endometrial-mesothelial adhesion. Previous studies demonstrated that integrin  $\beta$ 4 is negatively regulated by TGF- $\beta$ 1 in fibroblast and mammary gland cells via epigenetic modifications (45, 46). By contrast, we found that TGF- $\beta$ 1 increased the expression of integrin  $\beta$ 4 in endometrial cells. Although the precise molecular machinery involved in the modulation of integrin  $\beta$ 4 by TGF- $\beta$ 1 is not fully elucidated, we hypothesize that the differential expression of integrin  $\beta$ 4 is mainly due to tissue specificity.

Next, we confirmed the role of induced integrins on endometrial-mesothelial attachment by neutralizing the action of integrins in endometrial cells. Addition of antibodies against integrins  $\alpha$ V,  $\beta$ 1, and  $\beta$ 4 significantly blocked the adhesion of endometrial cells onto mesothelium. To the best of our knowledge, this is the first report to show that blocking the functions of integrins  $\alpha$ V,  $\beta$ 1, and  $\beta$ 4 with neutralizing antibodies reduced the development of endometriosis by inhibiting endometrial-mesothelial adhesion.

In conclusion, as illustrated in supplementary Fig. 1, we demonstrated that TGF- $\beta$ 1 increased endometrial-mesothelial adhesion via autocrine regulation. This TGF- $\beta$ 1-stimulated adhesion is mediated by integrins  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4, and blocking these integrins with neutralizing antibodies reduced the mesothelial adhesion of endometrial cells. We therefore propose that TGF- $\beta$ 1-stimulation of integrins  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4 could be a good target for the development of new methods aimed at preventing or treating endometriosis.

## MATERIALS AND METHODS

See supplementary information.

## ACKNOWLEDGEMENTS

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## CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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# **Transforming growth factor $\beta$ 1 enhances adhesion of endometrial cells to mesothelium by regulating expression of integrins**

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## **Supplementary Materials and Methods**

### ***Cell culture***

Immortalized normal human endometrial cells (HES cells), established by Dr. Krikun (Yale University, New Haven, Connecticut), were kindly provided by Dr. Asgi Fazleabas (University of Illinois, Chicago) (1, 2). Immortalized human endometriotic epithelial cells (12Z) (3) were generously provided by Dr. Starzinski-Powitz (Johann-Wolfgang-Goethe-Universitaet, Germany). Human mesothelial Met5A cells were purchased from the American Type Culture Collection (ATCC, VA, USA). HES cells were cultured at 37°C in an atmosphere containing 5% CO<sub>2</sub>/air in Dulbecco's Modified Eagle Medium (DMEM; Welgene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). 12Z cells were cultured in RPMI1640 (Lonza, USA) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin. Met-5A cells were maintained as monolayers at 37°C in an atmosphere containing 5% CO<sub>2</sub>/air in Medium 199 (M199, Welgene, Daegu, Korea) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin.

### ***Reverse transcription-polymerase chain reaction (RT-PCR)***

Total RNA was isolated from HES and 12Z cells using a GeneJET RNA Purification Kit (ThermoFisher Scientific, USA). Equal amounts of total RNA (1  $\mu$ g) from each sample were

then subjected to reverse transcription with oligo-dT primers using M-MLV reverse transcriptase (ThermoFisher Scientific). cDNA was amplified by PCR using AccuPower®PCR PreMix (Bioneer Co., Daejeon, Korea). Primers used in this study were as follows: *integrin αV*, forward 5'-ATGCTCCATGTAGATCACAAGAT-3' and reverse 5'-TTCCCAAAGTCCTTGCTGCT-3'; *integrin α6*, forward 5'-AGGTACAGTTGTTGGCGAGC-3' and reverse 5'-AGGCTCGCATGAGAATGTCC-3'; *integrin β1*, forward 5'-GTCGTGTGTGTGAGTGCAAC-3' and reverse 5'-GCTGGGGTAATTTGTCCCGA-3'; *integrin β3*, forward 5'-CTGCCGTGACGAGATTGAGT-3' and reverse 5'-TGCCCCGGTACGTGATATTG-3'; *integrin β4*, forward 5'-GAGCTCACCAACCTGTACCC-3' and reverse 5'-GCCCAATAGGTCGGTTGTCA-3'; *integrin β5*, forward 5'-ACCTGGAACAACGGTGGAGA-3' and reverse 5'-AAAAGATGCCGTGTCCCCAA-3'; *CD44s*, forward 5'-AGGGATCCTCCAGCTCCTTT-3' and reverse 5'-AAAGGCATTGGGCAGGTCTGTGACT-3'; *ICAM-1*, forward 5'-CAGTGACCATCTACAGCTTTCCGG-3' and reverse 5'-GCTGCTACCACAGTGATGATGACAA-3'; *L-selectin*, forward 5'-AAACCCATGAACTGGCAAAG-3' and reverse 5'-CGCAGTCCTCCTTGTTCTTC-3'; *E-cadherin*, forward 5'-TACAATGCCGCCATCGCTTA-3' and reverse 5'-AGCTGTGAGGATGCCAGTTT-3'; *β-actin*, forward 5'-CAAGAGATGGCCACGGCTGCT-3' and reverse 5'-TCCTTCTGCATCCTGTTCGGCA-3'.

### **Western blot analysis**

Total protein was extracted from cells using 1% NP-40 lysis buffer (150 mM NaCl, 10 mM HEPES (pH 7.45), 1% NP-40, 5 mM NaPyrophosphate, 5 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>) containing



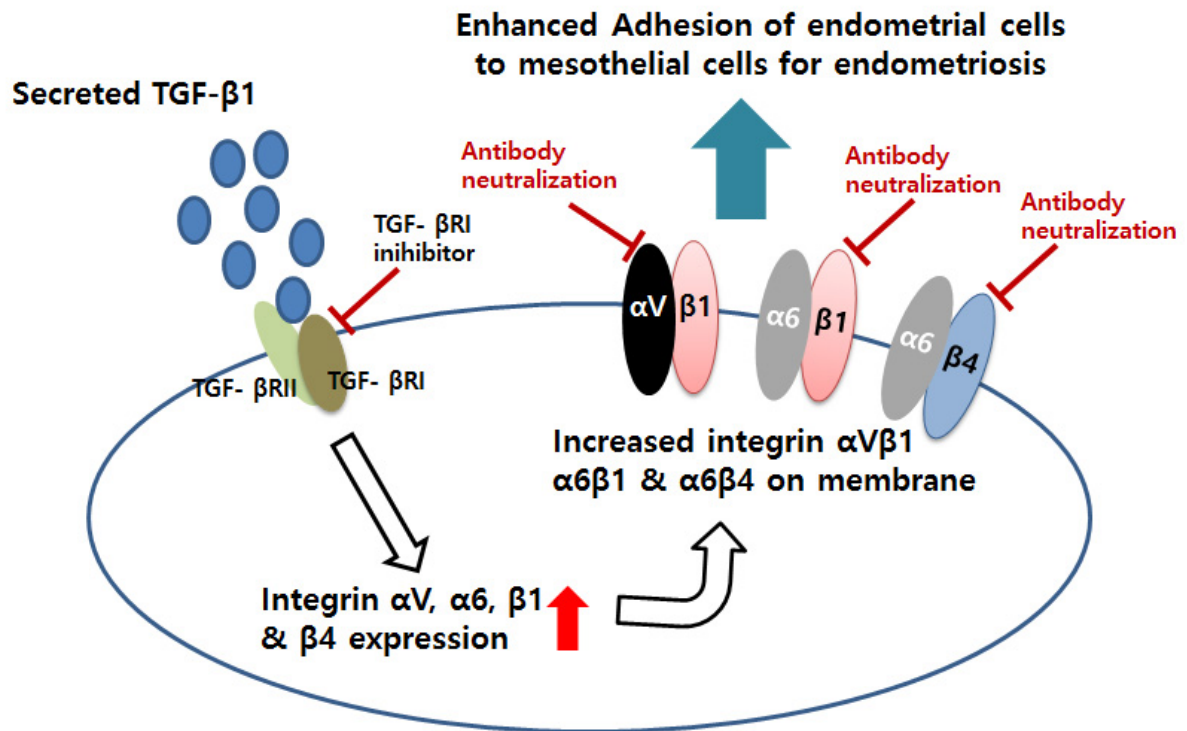
a protease inhibitor cocktail tablet (Roche, Germany). Equal amounts (20 µg) of protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractionized proteins were transferred by electrophoresis onto nitrocellulose filters (Hybond ECL; GE Healthcare). Filters were blocked with 5% nonfat dry milk at room temperature for 1 h and incubated with primary antibodies against p-Smad, Smad or GAPDH at 4°C overnight. Filters were washed three times and incubated with horseradish peroxidase conjugated-secondary antibodies. Bands representing target proteins were detected using ECL Plus and ImageQuant LAS 4000 (GE Healthcare).

#### ***Cell adhesion assay and antibody neutralization***

HES (5 × 10<sup>5</sup> cells) were seeded in a 6-well plate and cultured for 24 h. 12Z cells (3 × 10<sup>5</sup> cells) were seeded onto into a 100 mm -culture dish plate and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β for 24 h. HES and 12Z cells were first labeled with 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C, then washed in 1 × phosphate-buffered saline (PBS) and gently transferred onto a Met-5A cell monolayer. After gentle shaking at 20 rpm for 20 min at 37°C, cells were washed 3three times with 1 × PBS to remove unbound cells. Attached HES and 12Z cells were visualized using a fluorescent microscope (200 × magnification), and quantified using ImageJ software (NIH, Bethesda, MD, USA). The number of cells in 4 randomly chosen areas in each well was used for statistical analysis. In experiments using neutralizing antibodies, HES cells were treated with monoclonal integrin αV, β1 and β4 antibodies (Abcam, Cambridge, UK) or a rabbit IgG control antibody (Abcam, Cambridge, UK) before being transferred onto a Met-5A cell monolayer and analyzed as described above.

#### ***Statistical analysis***

Statistical analysis was performed using a Student's t-test or one-way analysis of variance with Tukey's post-hoc test using GraphPad Prism Software (GraphPad, CA, USA). Values are expressed as mean  $\pm$  SD. Minimum significance level was set at a *P* value of 0.05. At least 3 independent replications were performed for each experiment.



**Supplementary Fig. 1. Schematic representation describing adhesion of endometrial cells to mesothelium by TGF- $\beta$ 1-mediated adhesion molecules expression.**

This study show that the secreted TGF  $\beta$ 1 is involved in the increases of endometrial cell adhesion to mesothelium by enhancing expression of adhesion molecules including integrin  $\alpha$ V,  $\alpha$ 6,  $\beta$ 1, and  $\beta$ 4.

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