

# **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  $\alpha$ V*, forward 5'-ATGCTCCATGTAGATCACAAGAT-3' and reverse 5'-TTCCCAAAGTCCTTGCTGCT-3'; *integrin  $\alpha$ 6*, forward 5'-AGGTACAGTTGTTGGCGAGC-3' and reverse 5'-AGGCTCGCATGAGAATGTCC-3'; *integrin  $\beta$ 1*, forward 5'-GTCGTGTGTGTGAGTGCAAC-3' and reverse 5'-GCTGGGGTAATTTGTCCCGA-3'; *integrin  $\beta$ 3*, forward 5'-CTGCCGTGACGAGATTGAGT-3' and reverse 5'-TGCCCCGGTACGTGATATTG-3'; *integrin  $\beta$ 4*, forward 5'-GAGCTCACCAACCTGTACCC-3' and reverse 5'-GCCCAATAGGTCGGTTGTCA-3'; *integrin  $\beta$ 5*, 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';  *$\beta$ -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

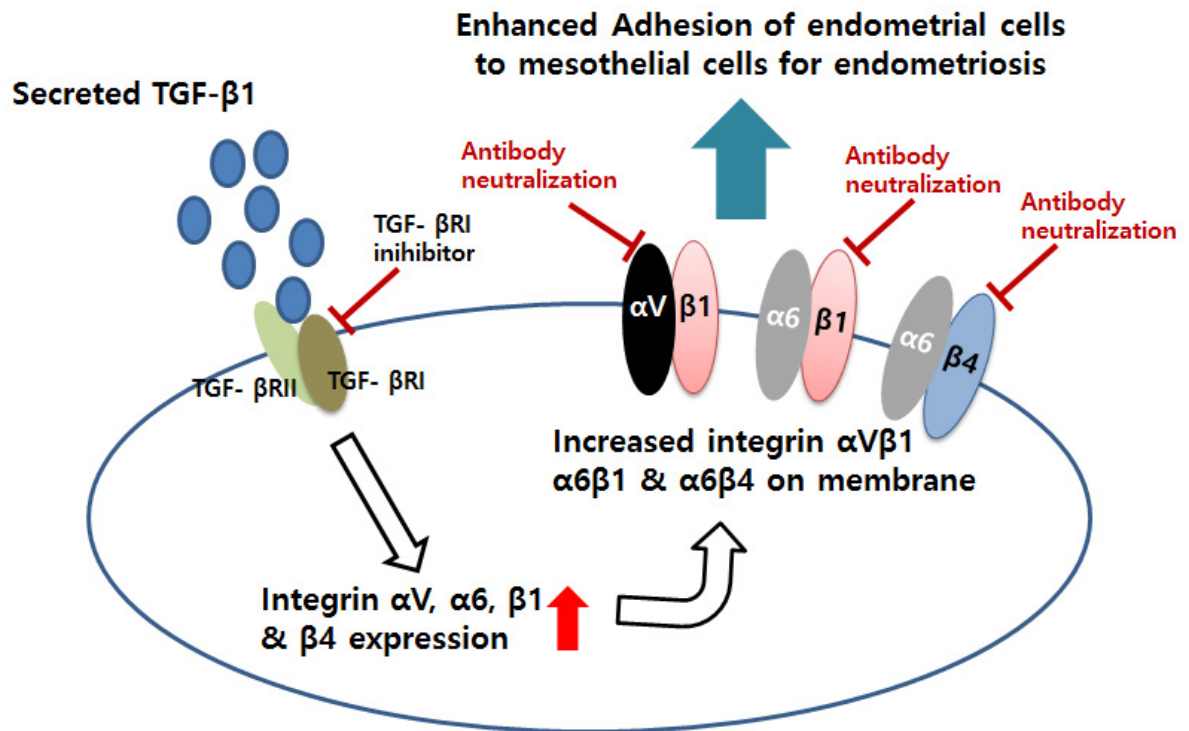
a protease inhibitor cocktail tablet (Roche, Germany). Equal amounts (20  $\mu$ g) of protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractionized proteins were transferred by electrophoresis onto nitrocellulose filters (Hybond ECL; GE 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  $\times$  10<sup>5</sup> cells) were seeded in a 6-well plate and cultured for 24 h. 12Z cells (3  $\times$  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- $\beta$  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  $\times$  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 three times with 1  $\times$  PBS to remove unbound cells. Attached HES and 12Z cells were visualized using a fluorescent microscope (200  $\times$  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  $\alpha$ V,  $\beta$ 1 and  $\beta$ 4 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.

## References

1. Desai NN, Kennard EA, Kniss DA and Friedman CI (1994) Novel human endometrial cell line promotes blastocyst development. *Fertil Steril* 61, 760-766
2. Krikun G, Mor G, Alvero A et al (2004) A novel immortalized human endometrial stromal cell line with normal progestational response. *Endocrinology* 145, 2291-2296
3. Zeitvogel A, Baumann R and Starzinski-Powitz A (2001) Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. *Am J Pathol* 159, 1839-1852