BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-17-097

Title: Transforming growth factor β1 enhances adhesion of endometrial cells to mesothelium by regulating expression of integrins

Article Type: Article

Keywords: Endometriosis; TGF-β1; Endometrial cells; Mesothelial cells; Integrin

Corresponding Author: Ki-Tae Ha

Authors: Hee-Jung Choi[1,2], Mi-Ju Park[2], Bo-Sung Kim[1,2,3], Hee-Jin Choi[1,2,3], Bosun Joo[2], Kyu Sup Lee[4], Jung-Hye Choi[5], Tae-Wook Chung[1,2], Ki-Tae Ha[1,*]

Institution: [1]Department of Korean Medical Science and [2]Healthy Aging Korean Medical Research Center and [3]Graduate Training Program of Korean Medicine for Healthy-aging, Pusan National University,
[4]Department of Obstetrics & Gynecology, Pusan National University Hospital,
[5]Department of Life and Nanopharmaceutical Sciences and Department of Oriental Pharmacy, Kyung Hee University,

Transforming growth factor β1 enhances adhesion of endometrial cells to mesothelium by regulating integrin expression

Hee-Jung Choi[1,2], Mi-Ju Park[2], Bo-Sung Kim[1,2,3], Hee-Jin Choi[1,2,3], Bosun Joo[2], Kyu Sup Lee[4], Jung-Hye Choi[5], Tae-Wook Chung[1,2,*], Ki-Tae Ha[1,2,3,*]

[1]Department of Korean Medical Science, School of Korean Medicine, [2]Healthy Aging Korean Medical Research Center and [3]Graduate Training Program of Korean Medicine for Healthy-aging, Pusan National University, Yangsan, Gyeongnam, Republic of Korea

[4]Department of Obstetrics & Gynecology, Pusan National University Hospital, Seo-gu, Busan, Republic of Korea

[5]Department of Life and Nanopharmaceutical Sciences and Department of Oriental Pharmacy Kyung Hee University, Seoul, Republic of Korea

* Correspondence to: Prof. Ki-Ta Ha

Department of Korean Medical Science, School of Korean Medicine and Healthy Aging Korean Medical Research Center, Pusan National University, Busandaehak-ro 49, Yangsan, Gyeongnam 50612, Republic of Korea.

Tel: +82-51-510-8464, E-mail: hagis@pusan.ac.kr

Dr. Tae-Wook Chung

Department of Korean Medical Science, School of Korean Medicine and Healthy Aging Korean Medical Research Center, Pusan National University, Busandaehak-ro 49, Yangsan, Gyeongnam 50612, Republic of Korea.

Tel: +82-51-510-8434, E-mail: twchung@pusan.ac.kr

ABSTRACT

Interestingly, transforming growth factor β1 (TGF-β1) 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-β1 directly induced the adhesion of endometrial cells to mesothelial cells through the regulation of integrin of αV, α6, β1, and β4 via the activation of the TGF-β1/TGF-βRI/Smad2 signaling pathway. Conversely, the adhesion of TGF-β1-stimulated endometrial cells to mesothelial cells was clearly reduced following treatment with neutralizing antibodies against specific TGF-β1-mediated integrins αV, β1, and β4 on the endometrial cell membrane. Taken together, our results demonstrate that secreted TGF-β1 enhances the adhesion of endometrial cells to mesothelial cells by inducing the expression of integrin heterodimers αVβ1, α6β1, and α6β4 via the activation of the TGF-βRI/Smad2 signaling pathway, leading to endometriosis formation outside the uterus.

Running Title: Cell-cell adhesion by TGF-β1 in endometriosis

Keywords: Endometriosis, TGF-β1, Endometrial cells, Mesothelial cells, Integrin

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 αvβ3, α4β1, 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-β (TGF-β) is a 25 kDa peptide that plays key roles in the progression of endometriosis (13). TGF-β 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-βs, it is generally regarded that TGF-β1 is a key player in the pathogenesis of endometriosis, due to its expressional pattern and correlation with disease progression (13, 17). TGF-β1 is involved in the suppression of immune surveillance, cell adhesion and invasion into the peritoneum, and in the growth of implants (13). In particular, peritoneal adhesion of endometrial cells is elevated in the presence of TGF-β1 (18, 19). The expression of several integrins in different cells is controlled by TGF-β1 (20-23), therefore it was suggested that the cell-cell interactions that are activated by TGF-β1 could be mediated by integrins (13). To date, there are no reports or the direct evidence for TGF-β1-mediated regulation of integrins and other adhesion molecules in endometrial cells, or its role in

peritoneal adhesion of retrograde endometrium.

In the present study, we demonstrated that autocrine secretion of TGF-β1 increased adhesion between endometrial and mesothelial cells through expression of integrin αV, α6, β1, and β4. Moreover, blocking these integrins with neutralizing antibodies suppressed the mesothelial adhesion of endometrial cells. Thus, we suggest that TGF-β1 may act to promote the initiation of endometriosis by enhancing integrin-mediated cell-cell adhesion.

RESULTS

TGF-β1 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-β1 expression is increased in the peritoneal fluid of women with endometriosis (13, 24). In the current study, we compared levels of TGF-β1 expression between normal human endometrial cells (HES cells) and human endometriotic epithelial cells from endometriosis lesions (12Z cells). As shown in Figure 1A, TGF-β1 expression was significantly higher in 12Z cells than in HES cells. A number of recent, studies reported 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 Figure 1B, the adhesion ratio of 12Z cells to Met-5A cells was approximately 3 times higher than that of HES cells. Then, because TGF-β1 expression and adhesion were higher in endometriotic epithelial cells than in normal endometrial cells, we examined whether TGF-β1-mediated signaling is involved in the adhesion of 12Z cells to Met-5A cells. Interestingly, adhesion of TGF-β receptor I (TGF-βRI) inhibitor-treated 12Z cells to Met-5A cells was lower than that of 12Z cells (Fig 1C). These results suggest that enhanced expression of TGF-β1 in 12Z cells affects the adhesion of endometrial cells to mesothelial cells for endometriosis

progression.

TGF-β1 induces the adhesion of endometrial cells to mesothelial cells through the TGF-βRI/Smad2 signaling pathway.

Secretion of TGF-β into the peritoneal fluid plays an important role in the establishment of endometriosis (13, 24). Thus, we investigated whether TGF-β1 directly induces the adhesion of endometrial cells to mesothelial cells for the initial stages of endometriosis formation outside the uterus. Adhesion of TGF-β1-stimulated HES and 12Z cells to Met-5A cells was clearly higher than that of untreated HES and 12Z cells (Fig. 2A). Furthermore, TGF-β1 significantly induced the adhesion of normal endometrial cells to mesothelial cells through activation of Smad-2 signaling. However, treatment with a TGF-βRI inhibitor markedly suppressed the TGF-β1-induced adhesion of HES cells to Met-5A cells by inhibiting TGF-βRI/Smad2 signaling (Fig. 2B and C).

TGF-β1 induces the expressions of cell adhesion molecules in endometrial cells

Although adhesion molecules including integrins, CD44, ICAM-1, L-selectin and E-cadherin (29) and TGF-β1 play pivotal roles in attachment of endometrial cells outside the uterus for endometriosis formation, 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 Figure 3A, TGF-β1 induced the expression of integrin αV, α6, β1, and β4. The expression of integrin β5, CD44s, ICAM-1, L-selectin was not increased by TGF-β1 treatment, and integrin β3 and E-cadherin were not detectable under the same conditions. To further investigate this, 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 αV, α6, β1, and β4 expression levels in HES cells induced by TGF-β1 (Fig. 3B). Thus, our data showed that

TGF-β1 induces adhesion of endometrial cells to mesothelial cells by enhancing the expression of integrin αV, α6, β1, and β4

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

Next, we shout to investigate whether integrin subunits αV, β1, and β4 regulate 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 Figure 4, TGF-β1 significantly induced the adhesion of endometrial cells to mesothelial cells. However, this adhesion was significantly reduced when the TGF-β1-mediated expression of integrin dimers αVβ1, α6β1, and α6β4 on endometrial cell surfaces was disrupted by neutralizing antibodies (Fig. 4). These results suggest that secreted TGF-β1 may affect adhesion of menstrual endometrial fragments to the mesothelium outside the uterus and thus initiate endometriosis formation by enhancing expression of integrin heterodimers αVβ1, α6β1, and α6β4 in endometrial cells.

DISCUSSION

Integrins are heterodimeric membrane proteins composed of non-covalent associated α and β subunits, which are essential for linking the extracellular matrix to the cytoskeleton. In mammals, there 18 α subunits and 8 β subunits that can assemble into 24 different αβ 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 their involvement 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 identifying potential biomarkers for use in disease diagnosis and treatment (33).

Several integrins, including αvβ3, αvβ5, αvβ6, α4β1, and α6β1, 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 cannabinoid receptor agonist (7, 9-11, 37, 38). However, despite the obvious importance of TGF-β1 in endometriosis progression (13, 17), there have been no reports of the TGF-β1 function in the regulation of integrins in endometrial cells. Thus, we examined the regulation of integrins by TGF-β1 and their role in the initiation of endometriosis.

First, we confirmed the correlation between endometrial-mesothelial adhesion and TGF-β expression. Our results clearly showed that autocrine expression of TGF-β1 in endometrial cells positively regulates their attachment to mesothelial cells. In human endometriosis lesions, TGF-β1 expression has been found in macrophages, endometrial epithelial cells, endometrial stromal cells, and mesothelial cells (13, 24). Elevated levels of secreted TGF-β1 in peritoneal fluid influence many steps of endometriosis progression, including immune surveillance, cell adhesion and invasion into peritoneum, angiogenesis, and growth of implants (13, 17). The response of TGF-β1 measured by endometrial-mesothelial adhesion is different between normal endometrial HES cells and ectopic endometriosis 12Z cells. Rai *et al.* (39) reported that the expression of adhesion molecules is different between normal endometrium and endometriosis tissues. Thus, we proposed that it might be caused by elevated expression of TGF-β1 in 12Z cells. We further showed that inhibition of TGF-β1 activity using a specific TGF-β RI inhibitor significantly reduced mesothelial adhesion of TGF-β1-stimulated HES cells. From these results, we postulated that secreted TGF-β1 may act in an autocrine fashion on endometrial-mesothelial interactions.

To identify the factors that mediate TGF-β1-enhanced cell-cell interactions, we analyzed the expression of several adhesion molecules that are known to be important factors in the development of endometriosis (40, 41). Levels of integrin αv, α6, β1 and β4 mRNA were clearly increased following TGF-β1 treatment. The expression of integrin β5, CD44s, ICAM-1, L-selectin was not increased, and integrin β3 and E-cadherin were not detectable under the same conditions. We further examined whether the expressions of integrin αV, α6, β1, and β4 are dependent on TGF-β1 by suppressing the activity of TGF-β1.

Previous studies reported that several integrins, such as αV, β1, and β3, are positively regulated by TGF-β1 in fibroblast, glioblastoma, and kidney epithelial cells (23, 42, 43). In the present study, expression of integrin αV, α6, and β1 was increased in normal endometrial HES cells following TGF-β1 treatment, but integrin β3 expression was not. In A549 lung cancer cells, sustained ERK activity induced by TGF-β1 is involved the induction of integrin β3 (44). In the current study, phosphorylation of Smad2 was found to be a major signaling pathway involved in TGF-β1-stimulated integrin expression and endometrial-mesothelial adhesion. Previous studies demonstrated that integrin β4 is negatively regulated by TGF-β1 in fibroblast and mammary gland cells via epigenetic modification (45, 46). Contrary to this, we found that TGF-β1 increased the expression of integrin β4 in endometrial cells. Although the precise molecular machinery of integrin β4 modulation by TGF-β1 is not fully elucidated, we suppose that the differential expression of integrin β4 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 integrin αV, β1, and β4 significantly blocked the adhesion of endometrial cells onto mesothelium. To the best our knowledge, this is the first report to show that blocking the function of integrin αV, β1, and β4 using neutralizing antibodies reduces the development of endometriosis by inhibiting endometrial-mesothelial adhesion.

In conclusion, as illustrated in supplementary Figure 1, we demonstrated that TGF-β1 increases the endometrial-mesothelial adhesion via autocrine regulation. This TGF-β1-stimulated adhesion is mediated by integrin αV, α6, β1, and β4, and blocking these integrins with neutralizing antibodies reduces the mesothelial adhesion of endometrial cells. We therefore propose that TGF-β1-stimulation of integrin αV, α6, β1, and β4 could be a good target for new methods aimed at preventing and treating endometriosis.

MATERIALS AND METHODS

Cell adhesion assay and antibody neutralization

HES (5×10^5 cells) were seeded onto 6-well plate and cultured for 24 h. 12Z cells (3×10^5 cells) were seeded onto 100 π 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 labeled with 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen, 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 three times with 1 × PBS to remove unbound cells. Attached HES and 12Z cells were visualized using a fluorescent microscope (50 × 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) before being transferred onto a Met-5A cell monolayer and analyzed as described above.

ACKNOWLEDGEMENTS

This study was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (MISP), of the Korean Government (Grant no. NRF-2015R1D1A1A01060264 to K.T.H), and a grant of the Traditional Korean Medicine R&D Project, Ministry for Health & Welfare, Republic of Korea. (Grant no. HI15C0188 to T.W.C)

CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

References

1. Ping S, Ma C, Liu P et al (2016) Molecular mechanisms underlying endometriosis pathogenesis revealed by bioinformatics analysis of microarray data. Arch Gynecol Obstet 293, 797-804
2. Nothnick W and Alali Z (2016) Recent advances in the understanding of endometriosis: the role of inflammatory mediators in disease pathogenesis and treatment. F1000Res 5
3. Bedir R, Sehitoglu I, Balik G et al (2016) The role of the adhesion molecule Nectin-4 in the pathogenesis of endometriosis. Clin Exp Obstet Gynecol 43, 463-466
4. Schutt AK, Atkins KA, Slack-Davis JK and Stovall DW (2015) VCAM-1 on peritoneum and alpha4beta1 integrin in endometrium and their implications in endometriosis. Int J Gynecol Pathol 34, 85-89
5. Sundqvist J, Andersson KL, Scarselli G, Gemzell-Danielsson K and Lalitkumar PG (2012) Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis. Hum Reprod 27, 2737-2746
6. Surrey ES, Minjarez DA and Schoolcraft WB (2007) The incidence of aberrant endometrial alphavbeta(3) vitronectin expression in a high risk infertility population: could prolonged GnRH agonist therapy play a role? J Assist Reprod Genet 24, 553-556
7. Sanchez AM, Quattrone F, Pannese M et al (2017) The cannabinoid receptor CB1 contributes to the development of ectopic lesions in a mouse model of endometriosis. Hum Reprod 32, 175-184
8. Rakhila H, Girard K, Leboeuf M, Lemyre M and Akoum A (2014) Macrophage migration inhibitory factor is involved in ectopic endometrial tissue growth and peritoneal-endometrial tissue interaction in vivo: a plausible link to endometriosis development. PLoS One 9, e110434

9. Lee J, Banu SK, Burghardt RC, Starzinski-Powitz A and Arosh JA (2013) Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits adhesion of human endometriotic epithelial and stromal cells through suppression of integrin-mediated mechanisms. Biol Reprod 88, 77
10. Khoufache K, Bondza PK, Harir N et al (2012) Soluble human IL-1 receptor type 2 inhibits ectopic endometrial tissue implantation and growth: identification of a novel potential target for endometriosis treatment. Am J Pathol 181, 1197-1205
11. Arici A (2002) Local cytokines in endometrial tissue: the role of interleukin-8 in the pathogenesis of endometriosis. Ann N Y Acad Sci 955, 101-109; discussion 118, 396-406
12. Garcia-Velasco JA and Arici A (1999) Interleukin-8 expression in endometrial stromal cells is regulated by integrin-dependent cell adhesion. Mol Hum Reprod 5, 1135-1140
13. Omwandho CO, Konrad L, Halis G, Oehmke F and Tinneberg HR (2010) Role of TGF-betas in normal human endometrium and endometriosis. Hum Reprod 25, 101-109
14. Tamura M, Fukaya T, Enomoto A, Murakami T, Uehara S and Yajima A (1999) Transforming growth factor-beta isoforms and receptors in endometriotic cysts of the human ovary. Am J Reprod Immunol 42, 160-167
15. Pizzo A, Salmeri FM, Ardita FV, Sofo V, Tripepi M and Marsico S (2002) Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis. Gynecol Obstet Invest 54, 82-87
16. D'Hooghe TM, Xiao L and Hill JA (2001) Cytokine profiles in autologous peritoneal fluid and peripheral blood of women with deep and superficial endometriosis. Arch Gynecol Obstet 265, 40-44
17. Dela Cruz C and Reis FM (2015) The role of TGFbeta superfamily members in the pathophysiology of endometriosis. Gynecol Endocrinol 31, 511-515
18. Chegini N (2008) TGF-beta system: the principal profibrotic mediator of peritoneal adhesion formation. Semin Reprod Med 26, 298-312
19. Sandoval P, Jimenez-Heffernan JA, Guerra-Azcona G et al (2016) Mesothelial-to-mesenchymal transition in the pathogenesis of post-surgical peritoneal adhesions. J Pathol 239, 48-59
20. Chin SL, Johnson SA, Quinn J et al (2003) A role for alphaV integrin subunit in TGF-beta-stimulated osteoclastogenesis. Biochem Biophys Res Commun 307, 1051-1058
21. Dou Q, Williams RS and Chegini N (1997) Inhibition of transforming growth factor-beta 1 alters the growth, anchor-dependent cell aggregation and integrin mRNA expression in human promonocytes: implications for endometriosis and peritoneal adhesion formation. Mol Hum Reprod 3, 383-391
22. Honda E, Yoshida K and Munakata H (2010) Transforming growth factor-beta upregulates the expression of integrin and related proteins in MRC-5 human myofibroblasts. Tohoku J Exp Med 220, 319-327
23. Moyano JV, Greciano PG, Buschmann MM, Koch M and Matlin KS (2010) Autocrine

transforming growth factor-{beta}1 activation mediated by integrin {alpha}V{beta}3 regulates transcriptional expression of laminin-332 in Madin-Darby canine kidney epithelial cells. Mol Biol Cell 21, 3654-3668

24. Young VJ, Brown JK, Saunders PT, Duncan WC and Horne AW (2014) The peritoneum is both a source and target of TGF-beta in women with endometriosis. PLoS One 9, e106773
25. Witz CA, Monotoya-Rodriguez IA and Schenken RS (1999) Whole explants of peritoneum and endometrium: a novel model of the early endometriosis lesion. Fertil Steril 71, 56-60
26. Witz CA, Allsup KT, Montoya-Rodriguez IA, Vaughn SL, Centonze VE and Schenken RS (2002) Culture of menstrual endometrium with peritoneal explants and mesothelial monolayers confirms attachment to intact mesothelial cells. Hum Reprod 17, 2832-2838
27. Witz CA, Thomas MR, Montoya-Rodriguez IA, Nair AS, Centonze VE and Schenken RS (2001) Short-term culture of peritoneum explants confirms attachment of endometrium to intact peritoneal mesothelium. Fertil Steril 75, 385-390
28. Lucidi RS, Witz CA, Chrisco M, Binkley PA, Shain SA and Schenken RS (2005) A novel in vitro model of the early endometriotic lesion demonstrates that attachment of endometrial cells to mesothelial cells is dependent on the source of endometrial cells. Fertility and Sterility 84, 16-21
29. Young VJ, Brown JK, Saunders PT and Horne AW (2013) The role of the peritoneum in the pathogenesis of endometriosis. Hum Reprod Update 19, 558-569
30. Campbell ID and Humphries MJ (2011) Integrin structure, activation, and interactions. Cold Spring Harb Perspect Biol 3
31. Merviel P, Challier JC, Carbillon L, Foidart JM and Uzan S (2001) The role of integrins in human embryo implantation. Fetal Diagn Ther 16, 364-371
32. Umezawa M, Saito Y, Tanaka-Hattori N, Takeda K, Ihara T and Sugamata M (2012) Expression profile of extracellular matrix and adhesion molecules in the development of endometriosis in a mouse model. Reprod Sci 19, 1365-1372
33. Gupta D, Hull ML, Fraser I et al (2016) Endometrial biomarkers for the non-invasive diagnosis of endometriosis. Cochrane Database Syst Rev 4, CD012165
34. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ and Mardon HJ (2007) Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. Hum Reprod 22, 3139-3147
35. Koks CA, Groothuis PG, Dunselman GA, de Goeij AF and Evers JL (2000) Adhesion of menstrual endometrium to extracellular matrix: the possible role of integrin alpha(6)beta(1) and laminin interaction. Mol Hum Reprod 6, 170-177
36. Puy LA, Pang C and Librach CL (2002) Immunohistochemical analysis of alphavbeta5 and alphavbeta6 integrins in the endometrium and endometriosis. Int J Gynecol Pathol 21, 167-177
37. Khoufache K, Bazin S, Girard K et al (2012) Macrophage migration inhibitory factor

antagonist blocks the development of endometriosis in vivo. PLoS One 7, e37264

38. Chen J, Gu L, Ni J, Hu P, Hu K and Shi YL (2015) MiR-183 Regulates ITGB1P Expression and Promotes Invasion of Endometrial Stromal Cells. Biomed Res Int 2015, 340218
39. Rai V, Hopkisson J, Kennedy S, Bergqvist A, Barlow DH and Mardon HJ (1996) Integrins alpha 3 and alpha 6 are differentially expressed in endometrium and endometriosis. J Pathol 180, 181-187
40. Singh H and Aplin JD (2009) Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. J Anat 215, 3-13
41. Witz CA (2003) Cell adhesion molecules and endometriosis. Semin Reprod Med 21, 173-182
42. Silginer M, Burghardt I, Gramatzki D et al (2016) The aryl hydrocarbon receptor links integrin signaling to the TGF-beta pathway. Oncogene 35, 3260-3271
43. Gutierrez J, Droppelmann CA, Contreras O, Takahashi C and Brandan E (2015) RECK-Mediated beta1-Integrin Regulation by TGF-beta1 Is Critical for Wound Contraction in Mice. PLoS One 10, e0135005
44. Hong SK, Park JR, Kwon OS, Kim KT, Bae GY and Cha HJ (2016) Induction of integrin beta3 by sustained ERK activity promotes the invasiveness of TGFbeta-induced mesenchymal tumor cells. Cancer Lett 376, 339-346
45. Yang X, Pursell B, Lu S, Chang TK and Mercurio AM (2009) Regulation of beta 4-integrin expression by epigenetic modifications in the mammary gland and during the epithelial-to-mesenchymal transition. J Cell Sci 122, 2473-2480
46. Scardigli R, Soddu S, Falcioni R, Crescenzi M, Cimino L and Sacchi A (1996) The beta 4 integrin subunit is expressed in mouse fibroblasts and modulated by transforming growth factor-beta 1. Exp Cell Res 227, 223-229

FIGURE LEGENDS

Figure 1. Enhanced TGF-β1 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-β1* mRNA expression were examined using RT-PCR. β-actin was used as an internal control. Band intensity of TGF-β1 mRNA expression was quantified and normalized to *β*-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 ± 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 (5×10^5 cells) were seeded onto 6-well plate and cultured for 24 h. 12Z cells (3×10^5 cells) were seeded onto 100 π 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 × 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 ± 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 (3×10^5 cells) were seeded onto 100 π culture dish plate and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-βRI inhibitor for 24 h. Cells were then labeled with CMFDA for 15 min at 37°C, then washed in 1 × 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 ± 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.

Figure 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 (1st column). #P<0.05 and ###P<0.001 compared to control black bar graph (1st 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 (1st column). ###P<0.001 compared to positive control (2nd 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.

Figure 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 24h. 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 24h. 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 (1st column). ###P<0.001 compared to each positive control (2nd 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.

Figure 4. Blocking adhesion of TGF-β1-stimulated endometrial cells to mesothelial cells

using integrin αV, β1, and β4 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-β1 in the presence or absence of integrin **(A)**αV, **(B)**β1, or **(C)**β4 antibodies for 24h. 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. 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*<0.05, ***P*<0.01, ****P*<0.001 compared to each negative control (1st column of each graph). #*P*<0.05, ##*P*<0.01, ###*P*<0.001 compared to each positive control (2nd 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.

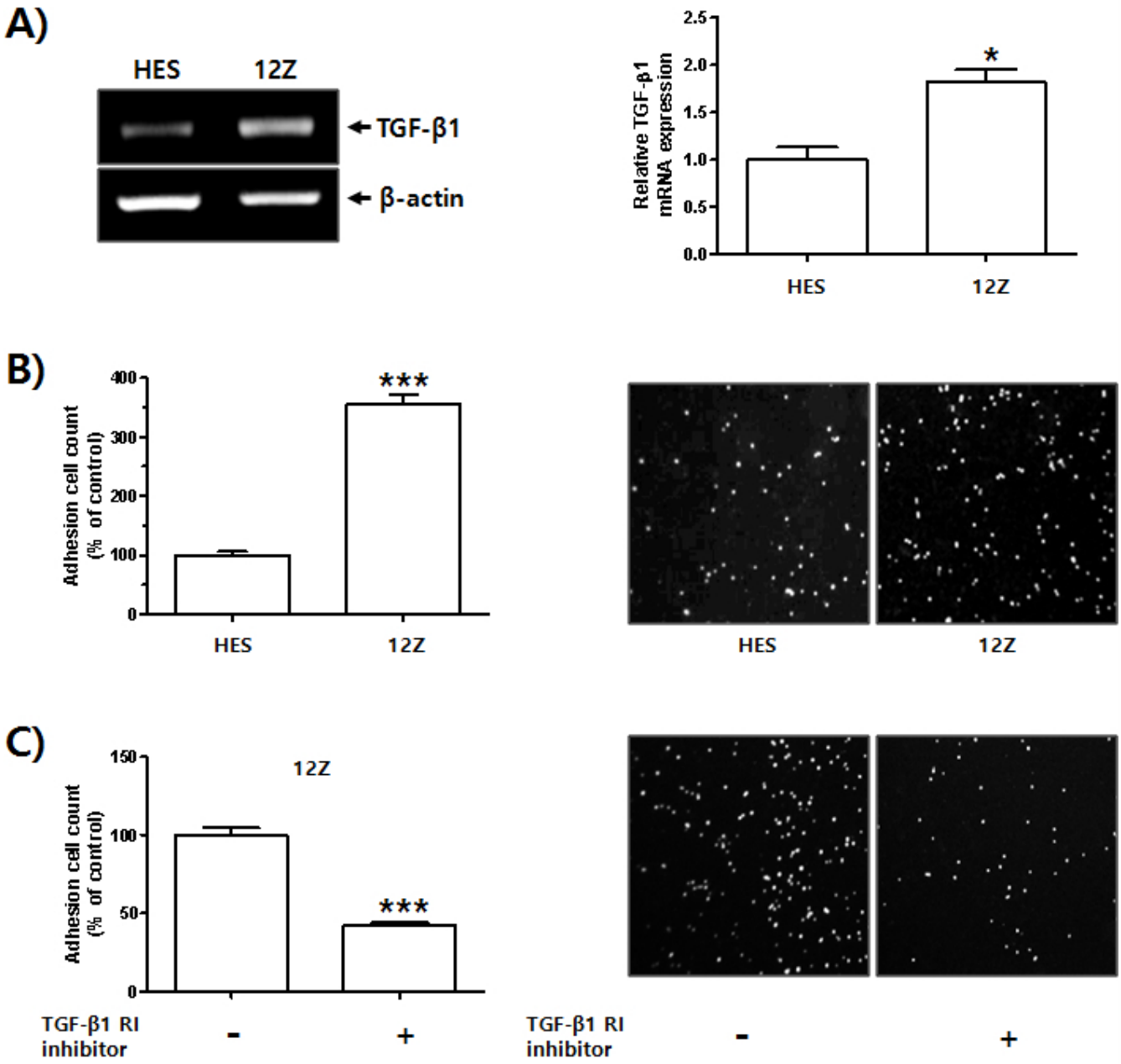


Fig. 1

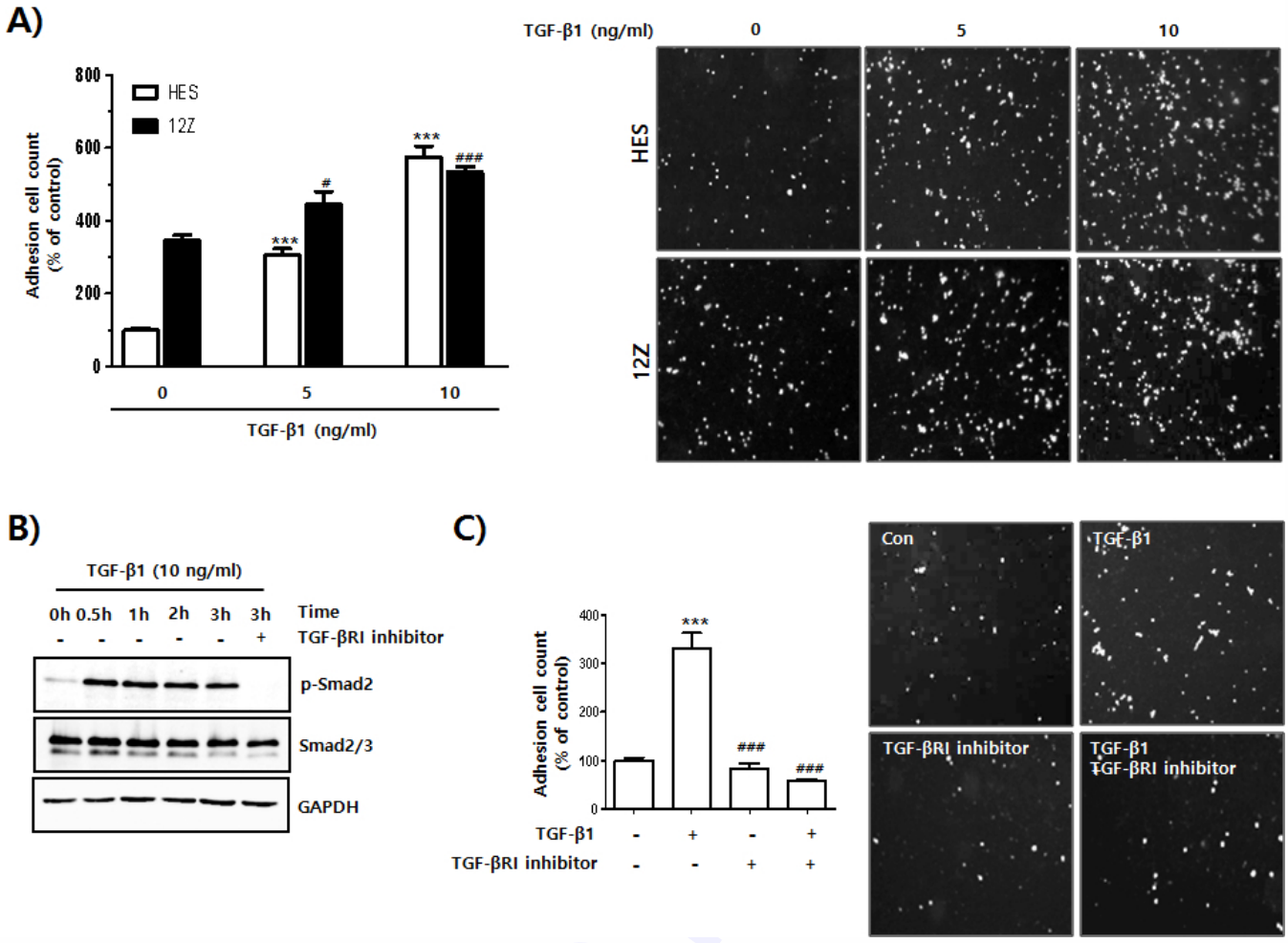


Fig. 2

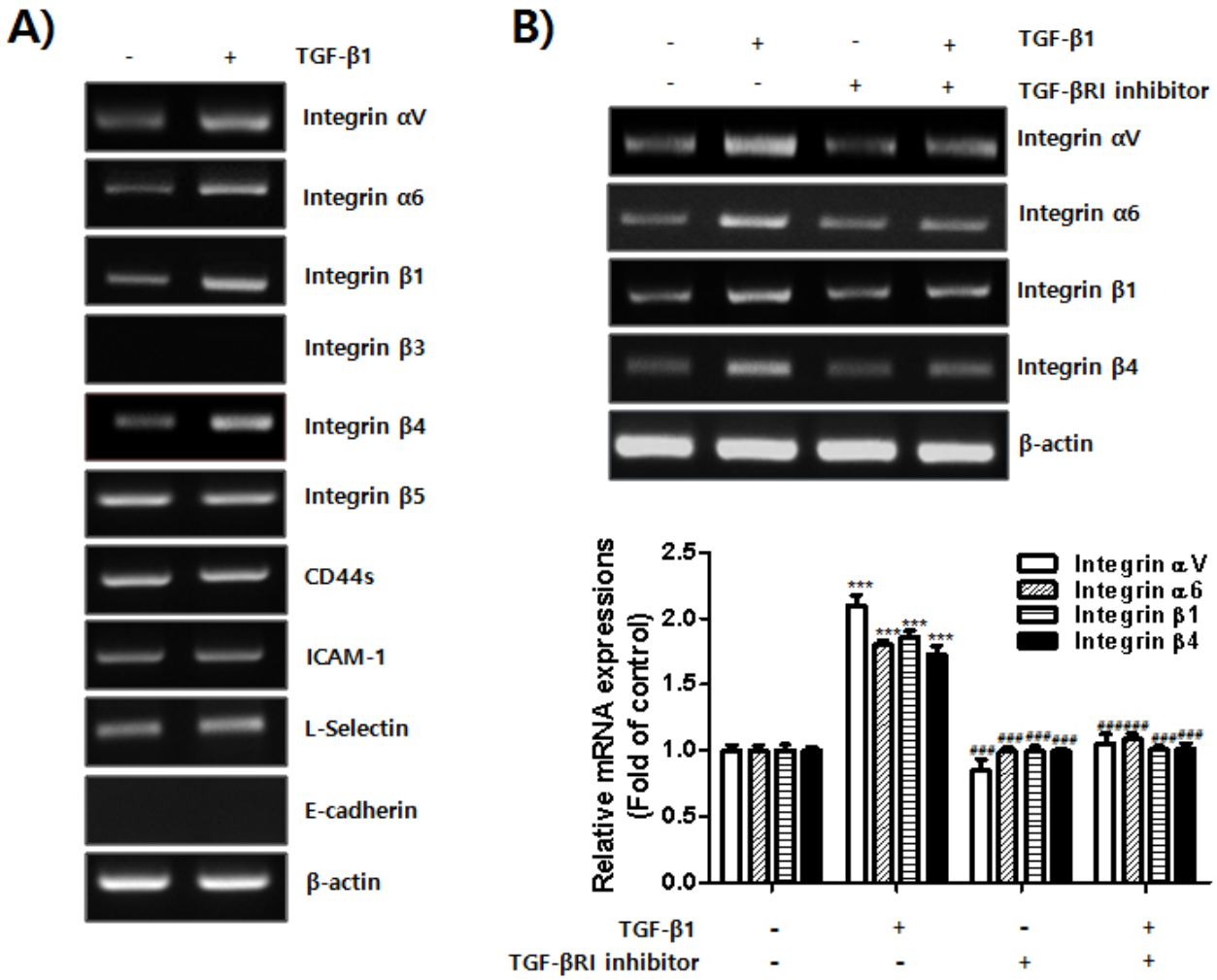


Fig. 3

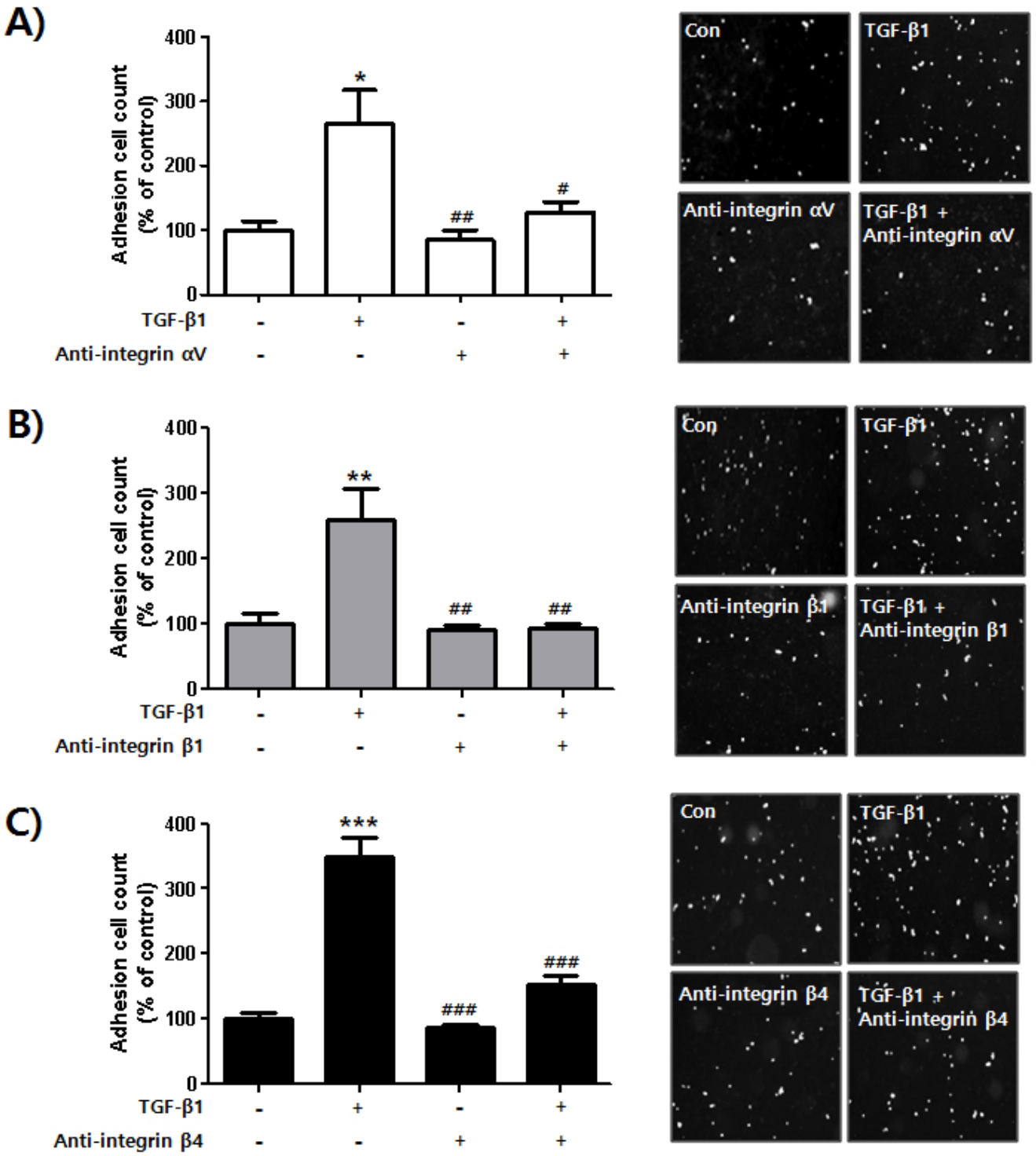

A)
Adhesion cell count (% of control)
400
300
200
100
0
*
##
#
TGF-β1 - + - +
Anti-integrin αV - - + +
Con
TGF-β1
Anti-integrin αV
TGF-β1 + Anti-integrin αV
B)
Adhesion cell count (% of control)
400
300
200
100
0
**
##
##
TGF-β1 - + - +
Anti-integrin β1 - - + +
Con
TGF-β1
Anti-integrin β1
TGF-β1 + Anti-integrin β1
C)
Adhesion cell count (% of control)
400
300
200
100
0
***
###
###
TGF-β1 - + - +
Anti-integrin β4 - - + +
Con
TGF-β1
Anti-integrin β4
TGF-β1 + Anti-integrin β4

Fig. 4

Transforming growth factor β1 enhances adhesion of endometrial cells to mesothelium by regulating expression of integrins

Hee-Jung Choi *et al.*

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_2/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_2/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 μg) 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′-TCCTTCTGCATCCTGTCGGCA-3′.

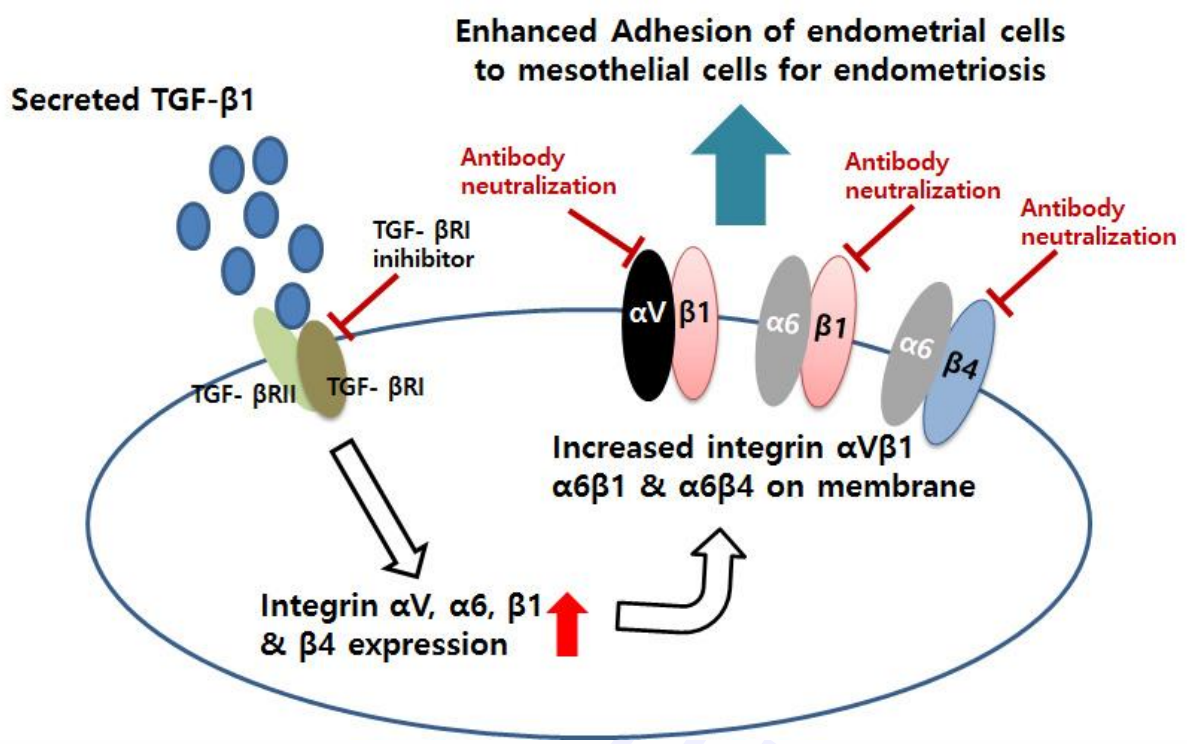
Western blot analysis

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_3VO_4)

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).

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 ± SD. Minimum significance level was set at a *P* value of 0.05. At least 3 independent replications were performed for each experiment.



Supplematary Fig. 1. Schematic representation describing adhesion of endometrial cells to mesothelium by TGF- β1-mediated adhesion molecules expression.

This study show that the secreted TGF β1 is involved in the increases of endometrial cell adhesion to mesothelium by enhancing expression of adhesion molecules including integrin αV, α6, β1, and β4.

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