

# Localization of integrin heterodimer $\alpha_9\beta_1$ on the surface of uterine endometrial stromal and epithelial cells in mice

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## ABSTRACT

Previously, we reported that endometrial stromal (ES) and endometrial epithelial (EE) cells did not attach to tenascin C, indicating the absence of active integrin  $\alpha_9\beta_1$  on the surface of mouse ES and EE cells. However, that study used recombinant tenascin C without fibronectin (FN) type III repeats interacting with integrin heterodimers. Therefore, we re-evaluated the presence of integrin  $\alpha_9\beta_1$  actively functioning on the surface of mouse ES and EE cells using full-length native tenascin C with FN type III repeats. The functionality of integrin  $\alpha_9\beta_1$  was confirmed using attachment and antibody inhibition assays. Both mouse ES and EE cells showed significantly increased adhesion to native tenascin C, and functional blocking of integrin  $\alpha_9\beta_1$  significantly inhibited adhesion to native tenascin C. These results demonstrate that the integrin  $\alpha_9$  and  $\beta_1$  subunits function as active heterodimers on the plasma membrane of mouse ES and EE cells, respectively.

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## Introduction

Tenascin C (herein referred to as native tenascin C) (Figure 1A), an extracellular matrix glycoprotein expressed in endometrial tissues (Harrington et al. 1999; Sedele et al. 2002; Tan et al. 2008), is composed of an assembly domain, epidermal growth factor (EGF)-like repeats, fibronectin (FN) type III repeats, alternatively spliced FN type III repeats, and a fibrinogen globe domain; a variety of cell-surface receptors and extracellular matrix (ECM) components can also interact with each of these domains. Specifically, transmission of signals derived from native tenascin C into the cytoplasm of cells via interaction with native tenascin C can be mediated by integrins, which are transmembrane cell-adhesion molecules formed as a heterodimer of  $\alpha$  and  $\beta$  subunits (Bokel and Brown 2002; Takada et al. 2007; Tucker and Chiquet-Ehrismann 2015; Park et al. 2019), while FN type III repeats have a domain that interacts with integrin heterodimers in the structure of native tenascin C (De Laporte et al. 2013; Adams et al. 2015; Yoshida et al. 2015).



In our previous study, we reported that uterine endometrial stromal (ES) and endometrial epithelial (EE) cells in mice had both integrin  $\alpha_9$  and  $\beta_1$  subunits on the cellular membrane but not attach to tenascin C,

indicating the absence of active integrin  $\alpha_9\beta_1$  interacting tenascin C on the surface of mouse ES and EE cells (Park et al. 2017). However, FN type III repeat-free recombinant tenascin C (Figure 1B) was used in the attachment assay of that study. Therefore, we re-examined the presence of active integrin  $\alpha_9\beta_1$  on the surface of mouse ES and EE cells using full-length native tenascin C containing FN type III repeats in functional analyses.

## Materials and methods

### Animals

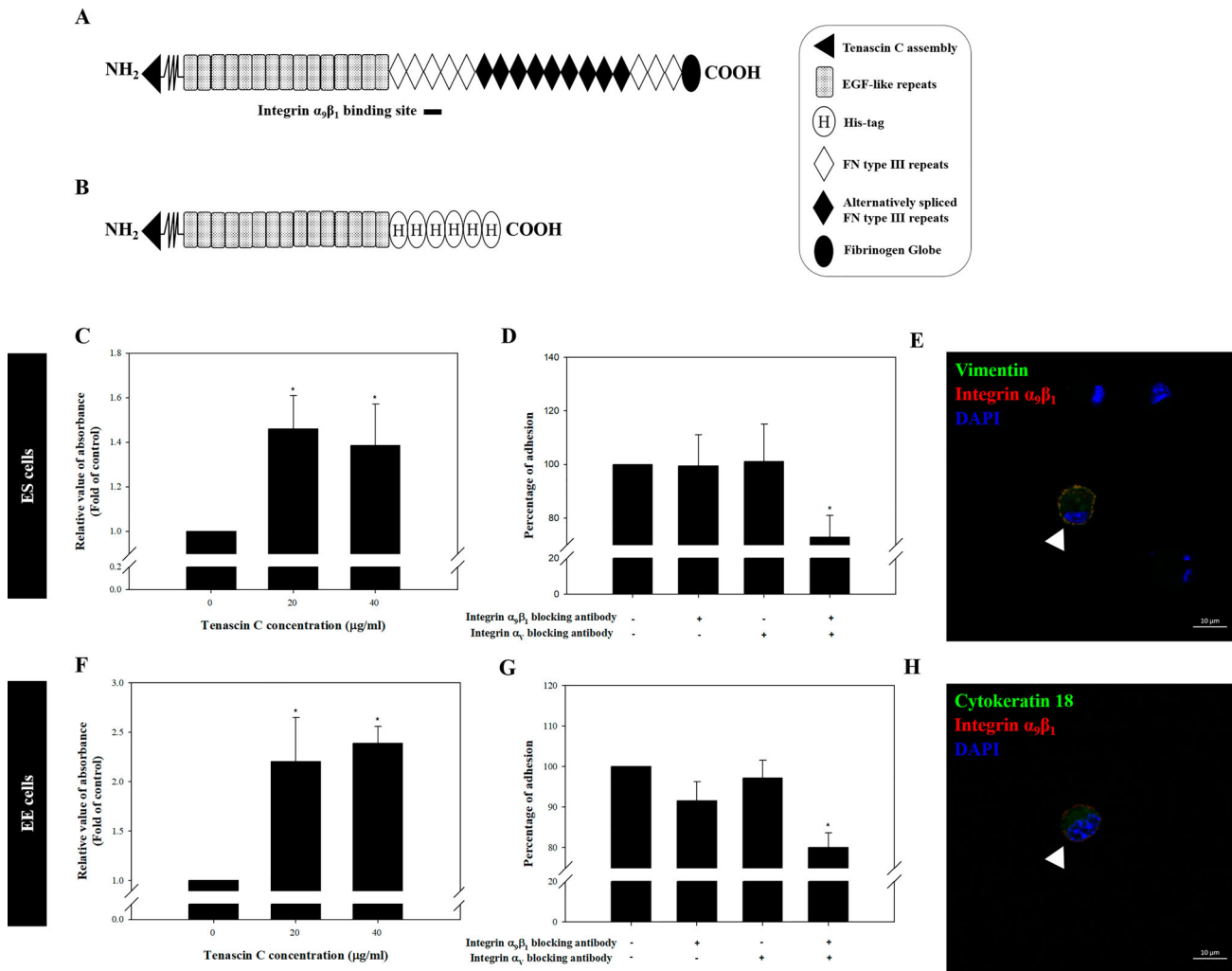
Six- or seven-week-old female ICR mice (weight 24–30 g,  $n=6$ ) were purchased from DBL (Eumseong, Korea) and used as uterus endometrium cell donors. Estrus cycle was identified through vaginal cytological evaluation described previously (McLean et al. 2012). All of the animal house, handling, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-150327-3) and performed in accordance with the National Institutes of Health Guidelines for the Care of Use of Laboratory Animals.

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**Figure 1.** Structure of the two types of tenascin C used in experiment and functional identification of integrin heterodimer  $\alpha_9\beta_1$  interacting with native tenascin C on the plasma membrane of endometrial stromal (ES) and epithelial (EE) cells derived from mouse uterine tissues. (A and B) Structure of native and recombinant human tenascin C. Native tenascin C is an oligomeric glycoprotein composed of an assembly domain, epidermal growth factor (EGF)-like repeats, fibronectin (FN) type III repeats, alternatively spliced FN type II repeats, and a fibrinogen globe domain (A). The FN type III repeats domains have sites binding to integrin heterodimer  $\alpha_9\beta_1$ . Recombinant tenascin C used in the previous study does not have any integrin heterodimer  $\alpha_9\beta_1$  binding sites because it is synthesized from N-terminal to EGF-like repeats (B). (C and F) Attachment assay of mouse ES (C) or EE (F) cells on native tenascin C. A 96-well tissue culture plate was coated with 0, 20 or 40  $\mu\text{g/ml}$  native tenascin C, and then  $5 \times 10^4$  EE or ES cells resuspended in DMEM/F12-based culture medium were plated to each well. After incubation for 2 h at  $37^\circ\text{C}$ , the adherent cells were stained with crystal violet and the adherent level was quantified using a microplate reader. The percentage of maximum adhesion is represented as the optical density of cells plated on tenascin C-free plates. Both ES and EE cells cultured on native tenascin C-coated culture plates showed significantly higher levels of adhesion than those on tenascin C-free culture plates. But, increasing concentration of native tenascin C on the culture plates did not induce a significant improvement of ES or EE cell adhesion level. (D and G) Antibody inhibition assay of the integrin heterodimer  $\alpha_9\beta_1$  supposed to function on the surface of ES (D) or EE (G) cells. Mouse ES and EE cells incubated in the absence or presence of anti-integrin  $\alpha_9\beta_1$  and/or anti-integrin  $\alpha_v$  blocking antibodies were plated on 20  $\mu\text{g/ml}$  native tenascin C-coated wells, and incubated 8 h at  $37^\circ\text{C}$ . After staining adherent cells with crystal violet, the quantification of adhesion level was conducted using a microplate reader. As a parameter of functional blocking by antibodies, the percentage of maximum adhesion, which is represented by the optical density of cells plated on native tenascin C-coated well in the absence of any blocking antibodies, was determined. As the results, both ES and EE cells treated with integrin  $\alpha_9\beta_1$  or  $\alpha_v$  subunit blocking antibody showed no significant difference in the adhesion level compared with those without blocking antibodies. However, compared with functional no-blocking of integrin subunits, functional co-blocking of integrin  $\alpha_9\beta_1$  and  $\alpha_v$  subunits in ES or EE cells significantly decreased the levels of adhesion to native tenascin C. All data shown are means  $\pm$  standard deviation (SD) ( $n = 3$  mice in attachment assay and  $n = 3$  mice in antibody inhibition assay) of three independent experiments.  $*p < 0.05$ . (E and H) Localization of integrin  $\alpha_9\beta_1$  on the surface of ES or EE cells. The presence of integrin  $\alpha_9\beta_1$  on the surface of ES or EE cells was identified by immunocytochemistry based on an antibody detecting integrin  $\alpha_9\beta_1$  heterodimers. Integrin  $\alpha_9$  and  $\beta_1$  subunit proteins (E and H; red) were localized as an active heterodimeric form on the surface of ES cells expressing vimentin (E; green) or EE cells expressing cytokeratin 18 (H; green). All figures are representative immunocytochemistry images of integrin  $\alpha_9\beta_1$  expressed on the surface of ES or EE cells. Nuclear counterstaining was conducted using DAPI.  $n = 3$ . Scale bar is 10  $\mu\text{m}$ .

### **Isolation of ES and EE cells from uterus**

Ovary and cervix-free uterine horns were washed with Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) supplemented with 2% (v/v) antibiotic-antimycotic solution (Welgene Inc., Daegu, Korea). Subsequently, the washed uterine horns were split longitudinally, fragmented into fine pieces by surgical scissors, and digested by incubation in HBSS containing 1.5 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO) for 45 min at 37°C. The digested cells were filtered through 100 µm nylon strainer (SPL, Pocheon, Korea) and washed with Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Invitrogen) containing 10% (v/v) heat inactivated fetal bovine serum (FBS; Welgene) and 1% (v/v) antibiotic-antimycotic solution (herein referred to as DMEM/F12 culture medium). A sedimentation step collected cell clumps in the tube bottom after separating the filtrated cells under unit gravity by incubating in a 15 mL tube at room temperature for 15 min. This procedure was repeated three times to eliminate debris included in cell clump. Subsequently, an adherent step separated attached ES and suspended EE cells by incubating the cells clump on a 100 mm culture plate at 37°C for 10 min that was repeated twice. Then two types of cells were isolated and enumerated using a hemocytometer, respectively.

### **Attachment assay**

96 well tissue culture plates were coated with the following concentrations of purified ECM proteins overnight at 4°C: 0, 20 and 40 µg/mL native tenascin C (RayBiotech, Peachtree Corners, GA). Then, for inhibiting non-specific binding of cells, each well was blocked with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) at 4°C for 1 h and washed three times with Dulbecco's phosphate-buffered saline (DPBS, Welgene). ES or EE cells were derived from three mice for three independent experiments, and  $5 \times 10^4$  cells re-suspended in DMEM/F12 culture medium were plated to the each well. After incubating at 37°C for 2 h, the removal of non-adherent cells were conducted by washing sufficiently the each well and adherent cells were fixed in 4% (v/v) paraformaldehyde (Junsei, Tokyo, Japan) at room temperature for 10 min. Then, the fixed adherent cells were stained with 0.1% (w/v) crystal violet (Sigma-Aldrich) in 20% (v/v) methanol (Sigma-Aldrich) for 5 min. After washing twice with DPBS, the amount of adherent cells were quantified at 570 nm using a microplate reader (Epoch Microplate Spectrophotometer; BioTek Instruments Inc., Winooski, VT) after adding 50 µL of 0.2% (v/v) triton X-100 (Biopure, Cambridge, MA) diluted with distilled water.

### **Antibody inhibition assay**

Each well of 96-well tissue culture plates were coated with 20 µg/mL native tenascin C overnight at 4°C, and the coated wells were blocked with 1% (w/v) BSA for 1 h at 4°C. Subsequently, for inhibition of integrin function,  $5 \times 10^4$  of ES or EE cells derive from three mice for three independent experiments were resuspended in DMEM/F12 culture medium containing mouse anti-human integrin  $\alpha_9\beta_1$  (dilution rate = 1:10; cat no. MAB2978Z; Chemicon International, Burlington, MA) and/or LEAF™ Purified rat anti-mouse integrin  $\alpha_v$  (dilution rate = 1:10; cat no. 104108; BioLegend, San Diego, CA) blocking antibody for 2 h at 37°C. Then, the functionally blocked cells were plated in the each well and incubated at 37°C for 8 h. The non-adherent cells were removed by washing extensively with DPBS, the adherent cells were fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature, and the fixed adherent cells were stained with 0.1% (w/v) crystal violet in 20% (v/v) methanol for 5 min. Finally, the wells were washed twice with DPBS and supplemented with 50 µL 0.2% (v/v) triton X-100 diluted with distilled water. The amount of dye was measured at 570 nm using a microplate reader.

### **Immunocytochemistry**

After fixing cells in 4% (v/v) paraformaldehyde for 10 min, the fixed cells were rinsed with DPBS and subsequent stained with fluorescence-unconjugated integrin  $\alpha_9\beta_1$  primary antibody (dilution rate = 1:50; Chemicon International) diluted in DPBS for 16 h at 4°C. Then, the localization of integrin  $\alpha_9\beta_1$  primary antibody was identified by incubating Alexa Fluor 594-conjugated secondary antibody (dilution rate = 1:50; Invitrogen) diluted in DPBS for 2 h at room temperature. Additionally, the stained cells were double stained with FITC-conjugated vimentin (an ES cell marker; dilution rate = 1:50; Abcam, Cambridge, UK) or FITC-conjugated cytokeratin 18 (an EE cell marker; dilution rate = 1:50; Abcam) antibody diluted in DPBS containing 0.01% (v/v) triton X-100 for 16 h at 4°C. The double stained cells were rinsed with DPBS and counterstained with mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, INC., Burlingame, CA). Moreover, the localization of each protein was monitored under a confocal laser scanning microscope (LSM880; ZEISS, Jena, Germany).

### **Statistical analysis**

The SAS program was used for statistical analysis of the numerical data shown in each experiment. Comparisons

among treatment groups were performed by the least-squares or DUNCAN method, and the significance of the main effects was determined by analysis of variance (ANOVA) in the SAS package. In all of the analyses,  $p < 0.05$  was taken to indicate statistical significance.

## Results and discussion

To identify the presence of integrin  $\alpha_9\beta_1$  on the surface of mouse ES and EE cells, we estimated the levels of adherent mouse ES and EE cells cultured on native tenascin C interacting specially with integrin  $\alpha_9\beta_1$ , and treated with antibodies to block the function of integrin  $\alpha_9\beta_1$  in native tenascin C. Compared with cells cultured on plates without native tenascin C, both ES (Figure 1C) and EE cells (Figure 1F) showed significantly improved adhesion to plates coated with native tenascin C. Moreover, an increase in native tenascin C concentration did not induce any significant increase in the adherence of cells to native tenascin C (Figure 1C and F). These results demonstrate that both ES and EE cells express integrin  $\alpha_9\beta_1$  on the cell surface. Previous studies have shown that native tenascin C has a functional domain interacting with integrin  $\alpha_v\beta_1$  (Kato et al. 2013; Grahovac and Wells 2014), which is found on the surface of ES and EE cells (Park et al. 2017). Therefore, combinatorial blockades of integrin  $\alpha_9\beta_1$  and  $\alpha_v\beta_1$  function were conducted in ES or EE cells. The cells were then incubated on 20  $\mu\text{g}/\text{mL}$  native tenascin C, which was the minimum concentration that showed significantly improved adhesion of ES (Figure 1C) and EE cells (Figure 1F). The results indicated no significant decrease in adherence of integrin- $\alpha_9\beta_1$ - or integrin- $\alpha_v\beta_1$ -blocked ES (Figure 1D) and EE cells (Figure 1G) to native tenascin C compared with ES and EE cells without the blockade of both integrin  $\alpha_9\beta_1$  and  $\alpha_v\beta_1$ . However, ES (Figure 1D) and EE cells (Figure 1G) with the co-blockade of integrin  $\alpha_9\beta_1$  and  $\alpha_v\beta_1$  showed significant decreases in adherence to native tenascin C compared with those without the co-blockade. These results confirm that integrin heterodimer  $\alpha_9\beta_1$  is localized as an active form on the surface of ES and EE cells. In addition, images of immunocytochemistry demonstrated the presence of integrin heterodimer  $\alpha_9\beta_1$  on the surfaces of ES (Figure 1E) and EE cells (Figure 1H).

Our study demonstrates that integrin  $\alpha_9\beta_1$  is present as an active heterodimer on the surface of ES and EE cells derived from uterine tissues in estrus, and not as an inactive subunit as reported previously (Park et al. 2017). These findings indicate that signals derived from integrin  $\alpha_9\beta_1$  interacting predominantly with native tenascin C (Yokosaki et al. 1994; Yokosaki et al. 1998; Kon and Uede 2018) may play an important role in

triggering the process of embryo implantation. Moreover, the importance of integrin  $\alpha_9\beta_1$  expressed on the surface of cells related to embryo implantation has been emphasized by previous studies showing that native tenascin C expression is observed in the uterine epithelium (Julian et al. 1994; Michie and Head 1994) and the stroma surrounding the endometrial glands, or beneath the uterine luminal epithelial cells during the implantation period (Noda et al. 2000). Therefore, the absence or shortage of integrin  $\alpha_9\beta_1$  on the surface of ES and EE cells may cause numerous difficulties related to the adhesion of blastocysts to uterine epithelial or endometrial cells, the penetration of embryos into endometrial tissues, and the thickening of endometrial tissues. In the future, the role of signals derived from integrin  $\alpha_9\beta_1$  activated by native tenascin C in ES and EE cells of the endometrium should be clarified. Simultaneously, the incorporation of integrin- $\alpha_9\beta_1$ -activating proteins into the bioscaffold may be essential for the development of acellular niches used for fabricating artificial endometrial tissue. Moreover, these achievements will greatly contribute to diagnosing and overcoming infertility, discovering causes of infertility, and expecting infertile potential of fertile women.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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