Integrins functioning in uterine endometrial stromal and epithelial cells in estrus

Hye Jin Park¹, Ji Eun Park¹, Hyun Lee¹, Seong Jae Kim¹, Jung Im Yun², Minseok Kim³, Kyu Hyun Park² and Seung Tae Lee^{1,4}

¹Department of Animal Life Science, Kangwon National University, Chuncheon, Korea, ²Division of Animal Resource Science, Kangwon National University, Chuncheon, Korea, ³Animal Nutrition and Physiology Team, National Institute of Animal Science, RDA, Wanju, Korea and ⁴Division of Applied Animal Science, Kangwon National University, Chuncheon, Korea

Correspondence should be addressed to S T Lee; Email: stlee76@kangwon.ac.kr

Abstract

Here, as a basic study in the construction of a non-cellular niche that supports artificial organization of three-dimensional endometrial tissue, we defined the types of integrin heterodimers that are expressed transcriptionally, translationally and functionally in endometrial stromal (ES) and endometrial epithelial (EE) cells isolated from the mouse uterus in estrus. Gene and protein expression of integrin subunits were analyzed at the transcriptional and translational level by real-time PCR and fluorescent immunoassay, respectively. Moreover, the functionality of integrin heterodimers was confirmed by attachment and antibody inhibition assays. *Itga2*, *Itga5*, *Itga6*, *Itga9*, *Itgav*, *Itgb1*, *Itgb3* and *Itgb5* in ES cells, and *Itga2*, *Itga5*, *Itga6*, *Itga7*, *Itga9*, *Itgav*, *Itgb1*, *Itgb3*, *Itgb4*, *Itgb5* and *Itga6* in EE cells showed significantly higher transcriptional levels than the other integrin subunits. Furthermore, translational expression of the total integrin α and β subunit genes that showed increased transcription was determined in ES and EE cells. ES cells showed significantly increased adhesion to collagen I, fibronectin and vitronectin, and functional blocking of integrin α_2 , α_5 or α_v significantly inhibited adhesion to these molecules. Moreover, EE cells showed significantly increased adhesion to collagen I, fibronectin, laminin and vitronectin, and functional blocking of integrin α_2 , α_5 , α_6 or α_v significantly inhibited adhesion to these molecules. Accordingly, we confirmed that integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, and integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and/or $\alpha_6\beta_4$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, actively function on the surface of ES and EE cells from mouse uterus in estrus phase, respectively.

Reproduction (2017) **153** 351–360

Introduction

Uterine endometrial stromal (ES) and endometrial epithelial (EE) cells undergo dynamic periodic alterations during the reproductive period (Chan *et al.* 2004, Schwab *et al.* 2005). This is particularly evident during the estrus cycle, when the endometrium undergoes histological and functional remodeling (Tang *et al.* 2005, Arai *et al.* 2013), resulting in an increase in the receptivity of the endometrium to embryos (Ponsuksili *et al.* 2012, Chadchan *et al.* 2016). Therefore, during estrus, the endometrial microenvironment plays an important role in successful embryo implantation and pregnancy maintenance.

Endometrial tissue is structured as a three-dimensional (3D) microenvironment *in vivo* that consists of extracellular matrix (ECM) components and a number of distinct cell populations (Jokimaa *et al.* 2002, Yamada *et al.* 2002). The specificity of cells that constitute endometrial tissue at each stage of the estrus cycle is regulated by the alteration of 3D

communication networks formed by the integration of cell-to-cell and cell-to-ECM contacts (Burghardt *et al.* 2002). To date, the endometrial microenvironment has been primarily constructed on two-dimensional culture plates rather than in 3D (Arnold *et al.* 2001), making it difficult to evaluate the effects of specific stimulations on endometrial tissue *in vitro*. Therefore, an *in vitro* mimic of the 3D endometrial microenvironment equivalent to endometrial tissue at each stage of the estrus cycle *in vivo* will be important for future studies.

The ECM, a non-cellular 3D macromolecular network composed of diverse fibrous ECM proteins, proteoglycans and glycoproteins (Kim *et al.* 2011, Theocharis *et al.* 2016) provides not only a physical scaffold to structure the 3D microenvironment but also signals a variety of cellular responses (Strauss 2013, Leppert *et al.* 2014). In particular, ECM-derived signals are transported to the cytoplasm through integrins that directly recognize components of the ECM (Rosso *et al.* 2004, Byron & Frame 2016), resulting in cytological alterations (Gronthos *et al.* 2001,

Vitillo *et al.* 2016). Therefore, the stimulation of ECM protein-derived signals through integrins makes it possible to accurately regulate the specificity of endometrial cells at each stage of the estrus cycle, which requires detailed information on the integrins that are functionally expressed on the membranes of cells that make up the endometrium.

As a step toward developing a defined non-cellular niche that uses integrin signaling to construct artificial and stereoscopic 3D endometrial tissues in mice, we examined the types of integrin heterodimers expressed on the membranes of ES and EE cells derived from the mouse uterus in estrus. Integrin subunits expressed in ES and EE cells were identified at the transcriptional and translational level, and the combinations of α and β integrin subunits were determined with functional assays.

Materials and methods

Animals

Six- or seven-week-old female ICR mice in estrus identified through vaginal cytological evaluation described previously (McLean *et al.* 2012) were used as uterus endometrium cell donors. All animal housing, 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 conducted according to the Animal Care and Use Guidelines of Kangwon National University.

Isolation of ES and EE cells from uterus

Uterine horns washed with Hank's balanced salt solution (HBSS; Invitrogen) supplemented with 2% (v/v) antibiotic–antimycotic solution (Welgene Inc., Daegu, Korea) were split longitudinally and the tissue fragmented into fine pieces by surgical scissors was digested using 1.5 mg/mL collagenase (Sigma-Aldrich) in HBSS at 37°C for 45 min. Then, the digested cells were filtered through 100 μ m nylon mesh (SPL, Pocheon, Korea). 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 remove debris included in cell clump. Subsequently, an adherent step separated attached ES and suspended EE cells by incubating the cell clumps on a 100 mm culture plate at 37°C for 10 min that was repeated twice. Subsequently, two types of cells were isolated and counted using a hemocytometer.

Real-time polymerase chain reaction

According to the manufacturer's instructions, the Dynabeads mRNA Direct Kit (Ambion) was used for extracting total mRNA from the cells and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan). Then, the quantification of the specific gene expression was conducted using a THUNDERBIRD SYBR qPCR Mix (Toyobo) under the 7500 Real-time PCR system (Applied Biosystems), and melting curve data was analyzed for identifying PCR specificity. The mRNA level was presented as $2^{-\Delta C_t}$, where

Table 1 Oligonucleotide primers and PCR cycling conditions.

Genes	GenBank number	Primer sequence		Size (bp)	Temp ^a
		Sense (5' > 3')	Anti-sense (5' > 3')		
<i>Actb</i>	X03672	TACCACAGGCATTGTGATGG	TCTTTGATGTCACGCACGATT	200	60
<i>Itga1</i>	NM_001033228	TGGCCAACCCAAAGCAAGAA	AGGGCCACATGCCAGAAAT	200	60
<i>Itga2</i>	NM_008396	TGTGCACCCAGAGCACTT	TGTTCACTTGAAGGCCCGGA	181	60
<i>Itga3</i>	NM_013565	AGCAACCTGCAGATGCGAGC	CTCATGCGCATCTTCCCCAG	158	60
<i>Itga4</i>	NM_010576	AGCAAAAAGGCATAGCGGGG	AACGCTGGCTTCTTCCAC	160	60
<i>Itga5</i>	NM_010577	AGGCTGCGCTGTGAGTTTG	TGCCGAGGCAGGATCTGGTA	178	60
<i>Itga6</i>	X63251	AGGTTGCAAGTGACGGTGTT	GTATCGGGGAATGCTGTCT	185	60
<i>Itga7</i>	NM_008398	GCTTCCAGACATTGCCGTG	TCCATCCACATCCAGGCCAC	182	60
<i>Itga8</i>	NM_001001309	GCATTCTTGACGTGGGCTGG	ATCCTCTGGGGAGGCAGCAG	154	60
<i>Itga9</i>	NM_133721	GGGGCAGGTCACCGTCTACC	AGCCACATCTGGGAACCCGT	156	60
<i>Itga10</i>	BC115770	GCTGTCTCCAGACATCACAGG	GTGGGGAGGCATCACATCCA	186	60
<i>Itga11</i>	NM_176922	TCCGGTAACCCAGGGCAACT	GCTTCCACACTCGTGCGACC	172	60
<i>Itgav</i>	NM_008402	AAGGCGCAGAATCAAGGGGA	CCAGCCTTCATCGGGTTTCC	194	60
<i>Itgal</i>	NM_008400	GGAAAGCCTGGTGGGCTCAGT	AGCTCAGCACACACCCGA	180	60
<i>Itgam</i>	NM_008401	CTTTGCAATTGAGGGCACGC	GAAAGCTCCACCTGCCAGT	150	60
<i>Itgad</i>	NM_001029872	TGTGGAGAAGCCCGTCGTGT	AGTGGCAGGCGCACAGTCAT	157	60
<i>Itgax</i>	NM_021334	GCTAGGGGACGTGAATGGGG	GGAGGGGATCTGGGATGCTG	165	60
<i>Itgae</i>	NM_008399	ACACAAGCCAAAGCCCTTCT	CAGGCTCTTGACTCTGGGTG	186	60
<i>Itgb1</i>	NM_010578	CTGGTCCCGACATCATCCCA	CCGTGCTCCCACTTGGCATTC	167	60
<i>Itgb2</i>	NM_008404	GGTGGCTCAGATCGGGGTTT	TGCACCTGTTGCATTGGCAG	165	60
<i>Itgb3</i>	NM_016780	CCCCACCACAGGCAATCAAA	CCCTCTGGGGCATCTCGATT	166	60
<i>Itgb4</i>	BC080751	GGCCAGTGGCTCTCTCAGCA	GTGGTCAGCAAGCTCGTGGG	151	60
<i>Itgb5</i>	NM_010580	AGGGCGTCTCTATGCTCAGGC	AGACACAACGGCTCGGTCA	161	60
<i>Itgb6</i>	NM_021359	GTCCAAGGTGGCTGTGCCTG	TGCGGGAGACAGGGTTTCA	199	60
<i>Itgb7</i>	NM_013566	AAGGAGGGCTCTGCAAGTGGG	TACAGTTGGCTGCCAGGGGA	182	60
<i>Itgb8</i>	BC125343	GCCTCAAGGTGCGCTCTCAA	AGGCTGCCCAAGAACCAAG	181	60

^aTemp, Temperature.

Ct = threshold cycle for target amplification, $\Delta Ct = Ct_{\text{target gene}}$ (specific genes for each sample) – $Ct_{\text{internal reference}}$ (β -actin for each sample). Design of primer sequences by Primer3 software (Whitehead Institute/MIT Center for Genome Research) was performed with information of cDNA sequences obtained from GenBank for mouse, and Table 1 shows general information and sequences of primers.

Immunocytochemistry

The cells fixed with 4% (v/v) paraformaldehyde (Junsei, Tokyo, Japan) for 10 min were washed with Dulbecco's phosphate-buffered saline (DPBS; Welgene), and the fixed cells were stained for 16 h at 4°C with fluorescence-conjugated anti-integrin antibodies diluted in DPBS. Table 2 describes the detailed information and dilution rate of the used antibodies. After rinsing with DPBS, the stained cells counterstained with mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) were monitored under fluorescence microscope (BX53, Olympus).

Fluorescence immunoassay

The fixed cells were produced by incubating living cells in 4% (v/v) paraformaldehyde for 10 min. After washing twice with DPBS, they were stained for 2 h at room temperature with fluorescence-conjugated anti-integrin antibodies diluted in DPBS. Table 2 describes the detailed information and dilution rate of the used antibodies. Subsequently, the stained cells were washed with DPBS and fluorescence intensity was measured using SoftMax Pro 6.2.2. (Molecular Devices Cooperation, Sunnyvale, CA, USA) after adding 100 μ L DPBS to the stained cells.

Attachment assay

In order to prepare ECM substrate for cell adhesion, 96-well tissue culture plates were, respectively, coated with following concentrations of the purified ECM proteins: 0, 5 and 10 μ g/mL collagen I (Sigma-Aldrich) interacting with integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$ (White *et al.* 2004, Znoyko *et al.* 2006, Heino 2007); 0, 40 and 80 μ g/mL fibronectin (Millipore) interacting with integrin $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_8\beta_1$ (Sanchez-Aparicio *et al.* 1994, Muller *et al.* 1995, Su *et al.* 2016, Veqa & Schwarzbauer 2016); 0, 200 and 400 μ g/mL laminin (Sigma-Aldrich) interacting with integrin $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_7\beta_1$ (Kikkawa *et al.* 2000, Nishiuchi *et al.* 2006); 0, 20 and 40 μ g/mL tenascin C (R&D systems) interacting with integrin $\alpha_9\beta_1$ (Yokosaki *et al.* 1994, Fiorilli *et al.* 2008); and 0, 5 and 10 μ g/mL vitronectin (R&D Systems) interacting with integrin $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Bodary & McLean 1990, Wayner *et al.* 1991, Delannet *et al.* 1994, Horton 1997) overnight at 4°C. Subsequently, 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 DPBS. Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) and 1% (v/v) antibiotic-antimycotic solution was used as the culture medium to resuspend 5×10^4 cells. The resuspended cells were plated on to each well. After incubating at 37°C for 2 h, the removal of non-adherent cells were conducted by washing sufficiently each well and adherent cells were fixed in 4% (v/v) paraformaldehyde 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 distilled water, the amount of adherent cells were quantified at 570 nm using a microplate reader (Epoch Microplate Spectrophotometer; BioTek Instruments Inc.) after

Table 2 Antibodies used in experiments.

Antibody name	Catalog number	Company	Application	Dilution rate
FITC-conjugated American hamster anti-mouse integrin α_2	103503	BioLegend	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated rabbit anti-mouse integrin α_5	orb222105	Biorbyt	FI ^a	1:100
PE-conjugated American hamster anti-mouse integrin α_5	12-0493	eBioscience	ICC ^c	1:50
Alexa Fluor488-conjugated rat anti-human/mouse integrin α_6	313608	BioLegend	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated mouse anti-mouse integrin α_7	LS-C179570	LSBio	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated rabbit anti-mouse integrin α_9	orb188618	Biorbyt	FI ^a	1:100
PE-conjugated goat anti-mouse integrin α_9	FAB3827P	R&D Systems	ICC ^c	1:50
FITC-conjugated rabbit anti-mouse integrin α_v	orb7231	Biorbyt	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated rat anti-mouse integrin β_1	FAB2405F	R&D Systems	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated American hamster anti-mouse integrin β_3	104305	BioLegend	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated rat anti-mouse integrin β_4	FAB4054F	R&D Systems	FI ^a	1:100
			ICC ^c	1:50
FITC-conjugated mouse anti-mouse integrin β_5	11-0497	eBioscience	FI ^a	1:100
			ICC ^c	1:50
LEAF purified American hamster anti-mouse integrin α_2	103507	BioLegend	AIA ^b	1:10
LEAF purified rat anti-mouse integrin α_5	103807	BioLegend	AIA ^b	1:10
Rat anti-mouse integrin α_6	MAB1378	Millipore	AIA ^b	1:10
LEAF purified rat anti-mouse integrin α_v	104107	BioLegend	AIA ^b	1:10

^aFI, fluorescence immunoassay, ^bAIA, antibody inhibition assay, ^cICC, immunocytochemistry.

adding 50 μ L of 0.2% (v/v) triton X-100 (Biopure, Cambridge, MA, USA) diluted with distilled water.

Antibody inhibition assay

Each well of 96-well tissue culture plate was coated with 5 μ g/mL collagen I, 40 μ g/mL fibronectin, 200 μ g/mL laminin or 5 μ g/mL vitronectin overnight at 4°C, and the coated wells was blocked with 1% (w/v) BSA for 1 h at 4°C. Subsequently, function of integrins was inhibited by incubating 5×10^4 cells in DMEM/F12 culture medium including anti-integrin α_2 (HM α 2), anti-integrin α_5 (5H10-27 (MFR5)), anti-integrin α_6 (NKI-GoH3) or anti-integrin α_V (RMV-7) blocking antibody for 2 h at 37°C, and the detailed information regarding the used antibodies is described in Table 2. Next, the functionally blocked cells was plated on 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 was stained with 0.1% (w/v) crystal violet in 20% (v/v) methanol for 5 min. Finally, the wells washed twice with distilled water were supplemented with 50 μ L 0.2% (v/v) triton X-100

diluted with distilled water and the amount of dye was measured at 570 nm using a microplate reader.

Statistical analysis

The Statistical Analysis System (SAS) program was used for analyzing statistically all the numerical data shown in each experiment. Comparison among treatment groups were performed by the least-square or DUNCAN method, when significance of the main effects through variance (ANOVA) analysis was detected in the SAS package. Moreover, significant differences among treatments were determined when *P* value was less than 0.05.

Results

Characterization of the integrin subunits expressed on the membranes of ES and EE cells derived from the mouse uterine endometrium in estrus

To determine the types of integrin heterodimers expressed on the membranes of ES and EE cells in the uterine endometrium in estrus, we investigated the

Figure 1 Transcriptional levels of α and β integrin subunit genes in endometrial stromal (ES) and endometrial epithelial (EE) cells of mouse uterine tissue. Endometrial cells were retrieved enzymatically from uterus derived from imprinting control region (ICR) mice, and the isolation of ES and EE cells was conducted using a sedimentation-adherence method. mRNA levels of α and β integrin subunit genes in the isolated ES or EE cells were quantitatively monitored by real-time PCR. Among the total 17 α and 8 β integrin subunit genes, 5 α (α_2 , α_5 , α_6 , α_9 , and α_V) (A) and 3 β (β_1 , β_3 , and β_5) (B) subunits in ES cells, and 4 α (α_2 , α_5 , α_6 and α_V) (C) and 5 β (β_1 , β_3 , β_4 , β_5 , and β_6) (D) subunits in EE cells showed significantly increased transcription compared with the other integrin subunit genes. Moreover, 2 α (α_7 and α_9) subunits in EE cells showed stronger transcription than 9 α (α_1 , α_3 , α_4 , α_{10} , α_{11} , α_{12} , α_{13} , α_{14} , and α_{15}) subunits showing the minimum transcription, with no significant difference in the transcriptional level from integrin α_2 subunit (C). All data shown are mean $\pm$ standard deviation (s.d.) of three independent experiments. $a-dP < 0.05$. ND = not detected.

transcriptional and translational expression of integrin subunits. Transcriptional analysis of the genes encoding 17 α and 8 β integrin subunits revealed that ES cells showed significantly higher expression of integrin α_2 , α_5 , α_6 , α_9 and α_V (Fig. 1A), and integrin β_1 , β_3 and β_5 (Fig. 1B) subunit genes. EE cells showed significantly higher expression of integrin α_2 , α_5 , α_6 and α_V (Fig. 1C), and integrin β_1 , β_3 , β_4 , β_5 and β_6 (Fig. 1D) subunit genes. The minimum level of expression was detected for the integrin subunit genes α_1 , α_3 , α_4 , α_7 , α_8 , α_{10} , α_{11} , α_D , α_E , α_L , α_M and α_X (Fig. 1A), and β_2 , β_4 , β_6 , β_7 and β_8 (Fig. 1B) in ES cells, and for α_1 , α_3 , α_4 , α_7 , α_9 , α_{10} , α_{11} , α_E , α_L , α_M and α_X (Fig. 1C), and β_2 and β_7 (Fig. 1D) in EE cells. Moreover, in EE cells, the expression of the integrin α_7 and α_9 subunit genes with no significant difference from integrin α_2 subunit gene was higher than the other integrin subunit genes with minimum expression (Fig. 1C). No transcription of the subunits α_8 , α_D (Fig. 1C) or β_8 (Fig. 1D) was detected in EE cells.

We also examined translational regulation of the α and β integrin subunits that showed increased transcription. In ES cells, the expression of integrin α_2 , α_5 , α_6 , α_9 , α_V , β_1 ,

β_3 and β_5 subunit proteins was observed on the surface of cells (Fig. 2A, B, C, D, E, F, G and H), and the integrin subunit proteins α_5 (Fig. 3A) and β_3 (Fig. 3B) had the strongest expression among the five α (α_2 , α_5 , α_6 , α_9 and α_V) and three β (β_1 , β_3 and β_5) integrin subunits. In addition, among the α (α_2 , α_6 , α_9 and α_V) and β (β_1 and β_5) subunits with significantly weaker expression than α_5 and β_3 , significantly stronger expression was detected in integrin α_9 and α_V subunit proteins (Fig. 3A) compared with α_2 and α_6 , and no significant difference in the expression of β_1 and β_5 subunits was observed (Fig. 3B). In EE cells, the localization of integrin α_2 , α_5 , α_6 , α_7 , α_9 , α_V , β_1 , β_3 , β_4 and β_5 subunit proteins was identified on the surface of cells (Fig. 2I, J, K, L, M, N, O, P, Q and R). The strongest expression among the six α (α_2 , α_5 , α_6 , α_7 , α_9 and α_V) and four β (β_1 , β_3 , β_4 and β_5) integrin subunits was detected for α_5 (Fig. 3C) and β_3 (Fig. 3D), whereas significantly lower expression was observed for the other integrin α (α_2 , α_6 , α_7 , α_9 and α_V) and β (β_1 , β_4 and β_5) subunits. The expression of α integrin subunits did not show any significant differences among the α_2 , α_6 , α_7 , α_9 and α_V subunits (Fig. 3C), and the integrin β_1 subunit

Figure 2 Translational expression of α and β integrin subunit genes highly expressed in ES and EE cells of mouse uterine tissue. Using a sedimentation-adherence method, ES and EE cells were isolated from endometrial cells retrieved enzymatically from uterus derived from ICR mice. Translational expression of α and β integrin subunit genes in the sorted ES and EE cells were identified by immunocytochemistry. As a result, integrin α_2 , α_5 , α_6 , α_9 , α_V , β_1 , β_3 and β_5 subunit proteins (A, B, C, D, E, F, G and H) were localized on the surface of ES cells, and the surface of EE cells showed the expression of integrin α_2 , α_5 , α_6 , α_7 , α_9 , α_V , β_1 , β_3 , β_4 and β_5 subunit proteins (I, J, K, L, M, N, O, P, Q and R). All figures are representative immunocytochemistry images of integrin subunit proteins expressed on the surface of ES and EE cells, respectively. $n=3$. Scale bars represent 20 μm .

Figure 3 Translational levels of α and β integrin subunit genes highly expressed in ES and EE cells of mouse uterine tissue. Using a sedimentation-adherence method, ES and EE cells were isolated from endometrial cells retrieved enzymatically from uterus derived from ICR mice. Translational levels of α and β integrin subunit genes in the sorted ES and EE cells were quantified by fluorescence immunoassays. Among the total of five α and three β integrin subunits in ES cells, significantly strong expression of three α (α_5 , α_9 and α_V) (A) and β_3 (B) subunits was detected, whereas two α (α_2 and α_6) (A) and two β (β_1 and β_5) (B) subunits were weakly expressed. Among the total of six α and four β integrin subunits in EE cells, α_5 and β_3 subunits were strongly expressed, whereas significantly weak expression of five α (α_2 , α_6 , α_7 , α_9 and α_V) (C) and three β (β_1 , β_4 and β_5) (D) subunits was detected. All data shown are mean $\pm$ S.D. of three independent experiments. *, *** $P < 0.05$.

showed the highest significant expression among the β_1 , β_4 and β_5 subunits (Fig. 3D). These results indicate that ES cells present α_2 , α_5 , α_6 , α_9 and α_V , and β_1 , β_3 and β_5 integrin subunits, and EE cells present α_2 , α_5 , α_6 , α_7 , α_9 and α_V , and β_1 , β_3 , β_4 and β_5 integrin subunits on the cell surface.

Functional identification of integrin heterodimers expressed on the membranes of ES and EE cells derived from mouse uterine endometrium in estrus

Based on the identification of the α and β integrin subunits expressed on the membranes of ES and EE cells derived from mouse uterine endometrium in estrus, we surmised that ES cells possess $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_9\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, and EE cells possess $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$, $\alpha_9\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ as active forms of integrin heterodimers, as described previously (Lessey 1988, Reddy & Mangale 2003). The presence of these integrin heterodimers was investigated by estimating levels of adherent ES and EE cells cultured on purified ECM proteins that interact specifically with each integrin

heterodimer. Post-culture adherent levels of ES and EE cells treated with antibodies specifically blocking the function of each integrin were determined.

Compared with purified ECM protein-free culture plates, ES cells showed significantly improved adhesion to collagen I- (Fig. 4A), fibronectin- (Fig. 4B) and vitronectin-coated (Fig. 4E) culture plates, and EE cells to collagen I- (Fig. 4F), fibronectin- (Fig. 4G), laminin- (Fig. 4H) and vitronectin-coated (Fig. 4I) culture plates. Moreover, no significant differences in adhesion levels were observed in ES cells cultured on laminin- (Fig. 4C) or EE cells cultured on tenascin C-coated (Fig. 4J) culture plates. Rather, ES cells cultured on tenascin C-coated culture plates showed a significant decrease in the level of adhesion, compared to those cultured on purified ECM protein-free culture plates (Fig. 4D). These results suggest the presence of the collagen I-specific integrin $\alpha_2\beta_1$, fibronectin-specific integrin $\alpha_5\beta_1$ and vitronectin-specific integrin $\alpha_V\beta_1$, $\alpha_V\beta_3$ or $\alpha_V\beta_5$ on the cell membrane of mouse ES cells, with the presence of individual integrin subunits α_6 and α_9 . Simultaneously, we speculate the presence of the collagen I-specific integrin

Figure 4 Identification of integrin heterodimers that interact with fibronectin, laminin and vitronectin on the membrane of ES and EE cells derived from the mouse uterus. A 96-well tissue culture plate was coated with collagen I, fibronectin, laminin, tenascin C, vitronectin, and ES or EE cells resuspended in culture medium were plated in each well. Subsequently, adherent cells were stained with crystal violet, and the level of adhesion was quantified using a microplate reader. The percentage of maximum adhesion is represented as the optical density of cells plated on extracellular matrix (ECM) protein-free plates. Both mouse ES and EE cells cultured on collagen I- (A, F), fibronectin- (B, G) and vitronectin-coated (E, J) culture plates showed significantly higher levels of adhesion than those on ECM protein-free culture plates. However, no significant difference or significant decrease in adhesion level was detected in ES cells cultured on laminin- (C) and tenascin C-coated (D) culture plates, respectively. In addition, EE cells cultured on laminin- (H) or tenascin C-coated (I) culture plates showed significant improvement, or no significant difference, in adhesion levels compared to those on ECM protein-free culture plates. All data shown are mean $\pm$ s.d. of three independent experiments. *, ** $P < 0.05$.

$\alpha_2\beta_1$; fibronectin-specific integrin $\alpha_5\beta_1$; laminin-specific integrins $\alpha_6\beta_1$, $\alpha_6\beta_4$ and/or $\alpha_7\beta_1$; and vitronectin-specific integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ on the cell membrane of mouse EE cells, with the presence of the individual integrin subunit α_9 .

These specific integrin function-blocked mouse ES or EE cells were incubated on 5 μ g/mL collagen I, 40 μ g/mL fibronectin, 200 μ g/mL laminin or 5 μ g/mL vitronectin as the minimum concentration among those seen in

the ECM showing significantly improved adhesion of ES or EE cells (Fig. 5). Significantly lower adhesion was detected in ES cells with blockage of the integrin subunit α_2 (Fig. 5A), α_5 (Fig. 5B) or α_v (Fig. 5C), and in EE cells with blockage of the integrin subunit α_2 (Fig. 5D), α_5 (Fig. 5E), α_6 (Fig. 5F) or α_v (Fig. 5G), compared with cells without blockage of those integrin heterodimers. From these results, we could confirm that the mouse endometrial ES cells derived from the uterus in estrus

Figure 5 Functional analysis of integrin heterodimers on the membrane of ES and EE cells derived from the mouse uterus. Mouse ES and EE cells incubated in the absence or presence of anti-integrin α_2 , anti-integrin α_5 , anti-integrin α_6 or anti-integrin α_v blocking antibodies were plated on 5 μ g/mL collagen I-, 40 μ g/mL fibronectin-, 200 μ g/mL laminin- and 5 μ g/mL vitronectin-coated wells, and incubated for 2 h at 37°C. After staining adherent cells with crystal violet, the level of adherence was quantified using a microplate reader. As the parameter of functional blocking by antibodies, the percentage of maximum adhesion, represented by the optical density of cells plated on each ECM protein-coated well in the absence of blocking antibodies was determined. Mouse ES cells treated with integrin α_2 (A), α_5 (B) and α_v (C) subunit blocking antibodies showed significantly lower rates of attachment to collagen I, fibronectin and vitronectin compared with those without blocking antibodies, respectively. Moreover, compared with those not treated with blocking antibodies, functional blocking of integrin α_2 (D), α_5 (E), α_6 (F) and α_v (G) subunits in EE cells significantly decreased the rates of attachment to collagen I, fibronectin, laminin and vitronectin, respectively. All data shown are mean $\pm$ s.d. of three independent experiments. * $P < 0.05$.

simultaneously express the functional integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ on the cell membrane, and integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and/or $\alpha_6\beta_4$, and $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ on the membrane of mouse uterine endometrial EE cells.

Discussion

Knowledge of the ECM-derived signals that mediate intracytoplasmic transduction through cell surface receptors that belong to the integrin family will be essential in the construction of an artificial microenvironment that accurately reflects the *in vitro* organization of the endometrium, including in terms of ES and EE cells. Identification of the integrins expressed in ES and EE cells will be particularly important. Here, we report the types of integrin heterodimers expressed on the surface of ES and EE cells derived from the mouse endometrium during estrus. Transcriptional analysis of 17 α and 8 β integrin subunits, followed by confirmation of their expression at the translational level, attachment to ECM proteins and inhibition with blocking antibodies revealed the presence of integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ as heterodimers, and α_6 and α_9 as individual subunits on the ES cell membrane. EE cells expressed the integrin heterodimers $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and/or $\alpha_6\beta_4$, and $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, and the individual subunits α_7 and α_9 on the cell membrane. These results suggest that the collagen I-specific integrin $\alpha_2\beta_1$, fibronectin-specific integrin $\alpha_5\beta_1$ and vitronectin-specific integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in ES cells, and the collagen I-specific integrin $\alpha_2\beta_1$, fibronectin-specific integrin $\alpha_5\beta_1$, laminin-specific integrins $\alpha_6\beta_1$ or $\alpha_6\beta_4$ and vitronectin-specific integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in EE cells may be important for the transmission of extracellular signals that organize endometrial tissue. Moreover, collagen I, fibronectin, laminin and vitronectin analogs will be important for the construction of niches customized to the organization of endometrial tissue. In addition, we speculate that the integrin subunits α_6 and α_9 in ES cells, or α_7 and α_9 in EE cells, may play an important role in the cytological changes of ES or EE cells generated during the estrus cycle.

Generally, functional integrins are organized as heterodimers of α and β subunits (Multhaupt *et al.* 2016, Pan *et al.* 2016). These heterodimeric transmembrane receptors are activated by direct interaction with a binding motif embedded in ECM proteins (Briszti *et al.* 2012, Seguin *et al.* 2015) and they induce a variety of biological responses (Hynes 2009, Schaefer & Reinhardt 2016). Therefore, despite the transcriptional and translational expression of the α_6 and α_9 integrin subunit genes in ES cells (Figs 1, 2 and 3), the lack of a significant increase in the adhesion of ES cells to laminin (Fig. 4C) or tenascin C (Fig. 4D) was observed. These results indicate that α_6 or α_9 are localized in the inactive form as an individual subunit, and not as the

active heterodimer, on the surface of the cell membrane. Furthermore, EE cells with the α_6 and α_7 subunits that recognize laminin exhibited a significant decrease in adhesion resulting from blockade of the α_6 subunit (Fig. 5F), indicating that α_6 is present as a heterodimer and α_7 as an individual subunit. There was also no significant difference in the adhesion level of EE cells to tenascin C (Fig. 4I), indicating that α_9 is present as an individual subunit on the cell membrane. These results are strongly supported by previous studies reporting the absence of laminin in ES tissue (Faber *et al.* 1986) and tenascin C in both ES (Julian *et al.* 1994, Michie & Head 1994) and EE tissue (Michie & Head 1994).

Interestingly, integrins $\alpha_6\beta_1$ and $\alpha_6\beta_4$, heterodimers that interact specifically with laminin were only observed on the surface of EE cells in estrus, and α_6 , identified as an individual subunit in the inactive form, are localized on the membrane of ES cells in estrus. This suggests that laminin-specific integrin heterodimers may play an important role in endometrial receptivity for implantation, which is supported by reports on the expression of laminin in the trophectoderm (Klaffky *et al.* 2006) and failed pregnancy in the laminin-deficient mouse (Zhang *et al.* 2000). Furthermore, the expression of laminin-specific integrin heterodimers in mouse EE cells has also been shown in humans (Koks *et al.* 2000, Park *et al.* 2000). Accordingly, at the clinical level, the presence of functional integrin heterodimers with the α_6 subunit may be important for the diagnosis of healthy uterine epithelium.

The uterine endometrium experiences physical and physiological alterations during implantation (Kao *et al.* 2002). The presence of inactive integrin subunits prior to implantation of the embryo can induce dramatic changes in the uterine endometrium through post-adhesion activation of embryos. In this study, specific integrin subunits (α_6 and α_9 in ES cells, and α_7 and α_9 in EE cells) were present in an inactive form on the cell membrane, indicating that signals derived from laminin (interacting with heterodimers with integrin α_6 or α_7 subunits) and tenascin C (interacting with heterodimers with integrin α_9 subunits) may be important in the remodeling of ES and EE tissue following embryo contact with EE cells. This is supported by the endometrial identification of laminin and tenascin C during pregnancy (Michie & Head 1994, Kaloglu & Onarlioglu 2010). Accordingly, research on the expression of inactive integrin subunits in specific cells may be useful in the generation of specific microenvironments.

In conclusion, the co-expression of integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, and integrin $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and/or $\alpha_6\beta_4$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, on the surface of ES and EE cells derived from mouse endometrium in estrus, respectively, was confirmed. Integrin α_6 and α_9 or α_7 and α_9 were also identified as individual subunits on the membranes of ES or EE cells. Identification of the integrin heterodimers or subunits expressed on ES

and EE cells will be useful for determining the type of ECM analogs that specifically activate each integrin heterodimer to generate synthetic niches to represent endometrial tissue *in vivo*. Moreover, the generation of synthetic endometrial tissue based on this knowledge will contribute to our understanding of the mechanism of implantation, and aid in the study of uterine receptivity and endometrial hormonal responses.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by a grant of the Korea Health Technology R&D Project (HI12C1404(A121515)), Ministry of Health and Welfare, Republic of Korea.

References

- Arai M, Yoshioka S, Tasaki Y & Okuda K 2013 Remodeling of bovine endometrium throughout the estrous cycle. *Animal Reproduction Science* **142** 1–9. (doi:10.1016/j.anireprosci.2013.08.003)
- Arnold JT, Kaufman DG, Seppala M & Lessey BA 2001 Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. *Human Reproduction* **16** 836–845. (doi:10.1093/humrep/16.5.836)
- Bodary SC & McLean JW 1990 The integrin beta 1 subunit associated with the vitronectin receptor alpha v subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. *Journal of Biological Chemistry* **265** 5938–5941.
- Brizzi MF, Tarone G & Defilippi P 2012 Extracellular matrix, integrins, and growth factor as tailors of the stem cell niche. *Current Opinion in Cell Biology* **24** 645–651. (doi:10.1016/j.ceb.2012.07.001)
- Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE & Bazer FW 2002 Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells, Tissues and Organs* **172** 202–217. (doi:10.1159/000066969)
- Byron A & Frame MC 2016 Adhesion protein networks reveal functions proximal and distal to cell-matrix contacts. *Current Opinion in Cell Biology* **39** 93–100. (doi:10.1016/j.ceb.2016.02.013)
- Chadchan SB, Kumar V, Mauray VK, Soni UK & Jha RK 2016 Endoglin (CD105) coordinates the process of endometrial receptivity for embryo implantation. *Molecular and Cellular Endocrinology* **425** 69–83. (doi:10.1016/j.mce.2016.01.014)
- Chan RW, Schwab KE & Gargett CE 2004 Clonogenicity of human endometrial epithelial and stromal cells. *Biology of Reproduction* **70** 1738–1750. (doi:10.1095/biolreprod.103.024109)
- Delannet M, Martin F, Bossy B, Cheresch DA, Reichardt LF & Duband JL 1994 Specific roles of the alpha V beta1, alpha V beta 3 and alpha V beta 5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development* **120** 2687–2702.
- Faber M, Wewer UM, Berthelsen JG, Liotta LA & Albrechtsen R 1986 Laminin production by human endometrial stromal cells relates to the cyclic and pathologic state of the endometrium. *American Journal of Pathology* **124** 384–391.
- Fiorilli P, Partridge D, Stanisewska I, Wang JY, Grabacka M, So K, Marcinkiewicz C, Reiss K, Khalili K & Croul SE 2008 Integrins mediate adhesion of medulloblastoma cells to tenascin and activate pathways associated with survival and proliferation. *Laboratory Investigation* **88** 1143–1156. (doi:10.1038/labinvest.2008.89)
- Gronthos S, Simmons PJ, Graves SE & Robey PG 2001 Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. *Bone* **28** 174–181. (doi:10.1016/S8756-3282(00)00424-5)
- Heino J 2007 The collagen family members as cell adhesion proteins. *BioEssays* **29** 1001–1010. (doi:10.1002/bies.20636)
- Horton MA 1997 The $\alpha\beta 3$ integrin 'vitronectin receptor'. *International Journal of Biochemistry and Cell Biology* **29** 721–725. (doi:10.1016/S1357-2725(96)00155-0)
- Hynes RO 2009 The extracellular matrix: not just pretty fibrils. *Science* **326** 1216–1219. (doi:10.1126/science.1176009)
- Jokimaa V, Oksjoki S, Kujari H, Vuorio E & Anttila L 2002 Altered expression of genes involved in the production and degradation of endometrial extracellular matrix in patients with unexplained infertility and recurrent miscarriages. *Molecular Human Reproduction* **12** 1111–1116. (doi:10.1093/molehr/8.12.1111)
- Julian J, Chiquet-Ehrismann R, Erickson HP & Carson DD 1994 Tenascin is induced at implantation sites in the mouse uterus and interferes with epithelial cell adhesion. *Development* **120** 661–671.
- Kaloglu C & Onarlioglu B 2010 Extracellular matrix remodelling in rat endometrium during early pregnancy: the role of fibronectin and laminin. *Tissue and Cell* **42** 301–306. (doi:10.1016/j.tice.2010.07.004)
- Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA & Giudice LC 2002 Global gene profiling in human endometrium during the window of implantation. *Endocrinology* **143** 2119–2138. (doi:10.1210/endo.143.6.8885)
- Kikkawa Y, Sanzen N, Fujiwara H, Sonnenberg A & Sekiguchi K 2000 Integrin binding specificity of laminin-10/11: laminin-10/11 are recognized by alpha3beta1, alpha6beta1 and alpha6beta4 integrins. *Journal of Cell Science* **113** 869–876.
- Kim SH, Turnbull J & Guimond S 2011 Extracellular matrix and cell signaling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *Journal of Endocrinology* **209** 139–151. (doi:10.1530/JOE-10-0377)
- Klaffky EJ, Gonzales IM & Sutherland AE 2006 Trophoblast cells exhibit differential responses to laminin isoforms. *Developmental Biology* **292** 277–289. (doi:10.1016/j.ydbio.2005.12.033)
- Koks CA, Groothuis PG, Dunselman GA, de Goeij AF & Evers JL 2000 Adhesion of menstrual endometrium to extracellular matrix: the possible role of integrin alpha(6)beta(1) and laminin interaction. *Molecular Human Reproduction* **6** 170–177. (doi:10.1093/molehr/6.2.170)
- Leppert PC, Jayes FL & Segars JH 2014 The extracellular matrix contributes to mechanotransduction in uterine fibroids. *Obstetrics and Gynecology International* **2014** 783289. (doi:10.1155/2014/783289)
- Lessey BA 1988 Endometrial integrins and the establishment of uterine receptivity. *Human Reproduction* **13** 247–258.
- McLean AC, Valenzuela N, Fai S & Bennett SA 2012 Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *Journal of Visualized Experiments* **15** e4389. (doi:10.3791/4389)
- Michie HJ & Head JR 1994 Tenascin in pregnant and non-pregnant rat uterus: unique spatio-temporal expression during decidualization. *Biology of Reproduction* **50** 1277–1286. (doi:10.1095/biolreprod50.6.1277)
- Muller U, Bossy B, Venstrom K & Reichardt LF 1995 Integrin alpha8 beta1 promotes attachment, cell spreading, and neurite outgrowth on fibronectin. *Molecular Biology of the Cell* **6** 433–448.
- Mulhaupt HA, Leitinger B, Gullberg D & Couchman JR 2016 Extracellular matrix component signaling in cancer. *Advanced Drug Delivery Reviews* **97** 28–40. (doi:10.1016/j.addr.2015.10.013)
- Nishiuchi R, Takagi J, Hayashi M, Ido H, Yagi Y, Sanzen N, Tsuji T, Yamada M & Sekiguchi K 2006 Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins. *Matrix Biology* **25** 189–197. (doi:10.1016/j.matbio.2005.12.001)
- Pan L, Zhao Y, Yuan Z & Qin G 2016 Research advances on structure and biological functions of integrins. *Springerplus* **5** 1094. (doi:10.1186/s40064-016-2502-0)
- Park KR, Inoue T, Ueda M, Hirano T, Higuchi T, Maeda M, Konishi I, Fujiwara H & Fujii S 2000 CD9 is expressed on human endometrial epithelial cells in association with integrins alpha(6), alpha(3) and beta(1). *Molecular Human Reproduction* **6** 252–257. (doi:10.1093/molehr/6.3.252)

- Ponsuksili S, Murani E, Schwerin M, Schellander K, Tesfaye D & Wimmers K 2012 Gene expression and DNA-methylation of bovine pretransfer endometrium depending on its receptivity after in vitro-produced embryo transfer. *PLoS ONE* **7** e42402. (doi:10.1371/journal.pone.0042402)
- Reddy KV & Mangale SS 2003 Integrin receptors: the dynamic modulators of endometrial function. *Tissue and Cell* **35** 260–273. (doi:10.1016/S0040-8166(03)00039-9)
- Rosso F, Giordano A, Barbarisi M & Barbarisi A 2004 From cell-ECM interactions to tissue engineering. *Journal of Cellular Physiology* **199** 174–180. (doi:10.1002/jcp.10471)
- Sanchez-Aparicio P, Dominguez-Jimenez C & Garcia-Pardo A 1994 Activation of the alpha4 beta1 integrin through the beta1 subunit induces recognition of the RGDS sequencing in fibronectin. *Journal of Cell Biology* **126** 271–279.
- Schaefer L & Reinhardt DP 2016 Special issue: extracellular matrix: therapeutic tools and targets in cancer treatment. *Advanced Drug Delivery Reviews* **97** 1–3. (doi:10.1016/j.addr.2016.01.001)
- Schwab KE, Chan RW & Gargett CE 2005 Putative stem cell activity of human endometrial epithelial and stromal cells during the menstrual cycle. *Fertility and Sterility* **84** 1124–1130. (doi:10.1016/j.fertnstert.2005.02.056)
- Seguin L, Desgrosellier JS, Weis SM & Cheresh DA 2015 Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance. *Trends in Cell Biology* **25** 234–240. (doi:10.1016/j.tcb.2014.12.006)
- Strauss JF 2013 Extracellular matrix dynamics and fetal membrane rupture. *Reproduction Sciences* **20** 140–153. (doi:10.1177/1933719111424454)
- Su Y, Xia W, Li J, Walz T, Humphries MJ, Vestweber D, Cabanas C, Lu C & Springer TA 2016 Relating conformation to function in integrin $\alpha 5 \beta 1$. *PNAS* **113** E3872–E3881. (doi:10.1073/pnas.1605074113v)
- Tang M, Xu Y, Julian J, Carson D & Tabibzadeh S 2005 Lefty is expressed in mouse endometrium in estrous cycle and peri-implantation period. *Human Reproduction* **20** 872–880. (doi:10.1093/humrep/deh717)
- Theocharis AD, Skandalis SS, Gialeli C & Karamanos NK 2016 Extracellular matrix structure. *Advanced Drug Delivery Reviews* **97** 4–27. (doi:10.1016/j.addr.2015.11.001)
- Vega ME & Schwarzbauer JE 2016 Collaboration of fibronectin matrix with other extracellular signals in morphogenesis and differentiation. *Current Opinion in Cell Biology* **42** 1–6. (doi:10.1016/j.ccb.2016.03.014)
- Vitillo L, Baxter M, Iskender B, Whiting P & Kimber SJ 2016 Integrin-associated focal adhesion kinase protects human embryonic stem cells from apoptosis, detachment, and differentiation. *Stem Cell Reports* **7** 167–176. (doi:10.1016/j.stemcr.2016.07.006)
- Yamada O, Todoroki J, Takahashi T & Hashizume K 2002 The dynamic expression of extracellular matrix in the bovine endometrium at implantation. *Journal of Veterinary Medical Science* **64** 207–214. (doi:10.1292/jvms.64.207)
- Yokosaki Y, Palmer EL, Prieto AL, Crossin KL, Bourdon MA, Pytela R & Sheppard D 1994 The integrin alpha9 beta1 mediates cell attachment to a non-RGD site in the third fibronectin type III repeat of tenascin. *Journal of Biological Chemistry* **269** 26691–26696.
- Wayner EA, Orlando RA & Cheresh DA 1991 Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *Journal of Cell Biology* **113** 919–929.
- White DJ, Puranen S, Johnson MS & Heino J 2004 The collagen receptor subfamily of the integrins. *International Journal of Biochemistry and Cell Biology* **36** 1405–1410. (doi:10.1016/j.biocel.2003.08.016)
- Zhang C, Duan E, Cao Y, Jiang G & Zeng G 2000 Effect of 32/67 kDa laminin-binding protein antibody on mouse embryo implantation. *Journal of Reproduction and Fertility* **119** 137–142. (doi:10.1530/jrf.0.1190137)
- Znoyko I, Trojanowska M & Reuben A 2006 Collagen binding alpha2beta1 and alpha1beta1 integrins play contrasting roles in regulation of Ets-1 expression in human liver myofibroblasts. *Molecular and Cellular Biochemistry* **282** 89–99. (doi:10.1007/s11010-006-1400-0)

Received 20 September 2016

First decision 6 October 2016

Revised manuscript received 23 November 2016

Accepted 19 December 2016