

Original article

Versican V1 in human endometrial epithelial cells promotes BeWo spheroid adhesion *in vitro*

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Abstract

The endometrium extracellular matrix (ECM) is essential for embryo implantation. Versican, a large chondroitin sulfate proteoglycan that binds hyaluronan and forms large ECM aggregates, can influence fundamental physiological phenomena, such as cell proliferation, adhesion, and migration. The present study investigated the possible role of versican in human embryo implantation. Versican V1 expression and secretion in human endometrial epithelial cells (EECs) was most prominent in the mid-secretory phase. Versican expression in EECs significantly increased after treatment with estrogen and progesterone, but not by estrogen alone. We also established versican V1-overexpressing Ishikawa (endometrial cancer cell line) cells (ISKW-V1), versican V3-overexpressing (ISKW-V3), and control GFP-overexpressing (ISKW-GFP) Ishikawa cells. By the *in vitro* implantation model, the attachment ratio of BeWo (choriocarcinoma cell line) spheroids to the monolayer of ISKW-V1, but not of ISKW-V3, was found significantly enhanced compared with attachment to the ISKW-GFP monolayer. The conditioned medium derived from ISKW-V1 (V1-CM) also promoted the attachment of BeWo spheroids to the ISKW monolayer. However, this attachment-promoting effect was abolished when V1-CM was pretreated with chondroitinase ABC, which degrades chondroitin sulfate. Therefore, out of the ECM components, versican V1 may facilitate human embryo implantation.

Introduction

Improvements in *in vitro* fertilization, embryo transfer, and laboratory techniques have resulted in increased pregnancy rates, yet implantation rates remain disappointingly low (Ng, *et al.* 2018). The extracellular matrix (ECM) of the endometrium plays critical roles in the process of embryo implantation (Salgado, *et al.* 2009). Endometrial ECMs are predominantly composed of collagens, non-collagenous multiadhesive glycoproteins (e.g., fibronectin, laminin, and vitronectin), elastin, hyaluronan, and proteoglycans (Kresse and Schonherr 2001). Fibronectin, laminin, and collagen type IV defects in human endometrium are associated with endometrial dysfunction and implantation failure (Bilalis, *et al.* 1996). Hyaluronan is abundant in secretory fluid of the reproductive tract in humans (Chun, *et al.* 2016) and its synthesis increases during the implantation window (Afify, *et al.* 2006). Moreover, hyaluronan promotes bovine embryo development and implantation *in vitro* (Lane, *et al.* 2003, Stojkovic, *et al.* 2002). The beneficial effects of hyaluronan during human embryo implantation are also substantiated by a recent Cochrane review showing that clinical pregnancy rates are significantly higher when a hyaluronan-rich medium is used for embryo transfer (Bontekoe, *et al.* 2014). Proteoglycans are classified into four groups according to their localization as they can be intracellular, on the cell surface (transmembrane or GPI-anchored), pericellular, or extracellular (hyalectan or small leucine-rich proteoglycans; [Iozzo and Schaefer 2015]). Heparan sulfate proteoglycans are expressed on the surface of peri-implantation murine embryos and promote their initial attachment to the uterine epithelia (Carson, *et al.* 1994, San Martin, *et al.* 2004); they have also been shown to mediate attachment of JAR cells (human choriocarcinoma cell line) to monolayers of RL95-2 cells (human endometrial cancer cell line; (Rohde and Carson 1993), suggesting their supportive roles in human embryo implantation.

Versican belongs to the hyalectan family (named for their ability to bind hyaluronan; (Wight 2002), is a chondroitin sulfate proteoglycan, and was originally isolated from developing chicken limb buds (Kimata, *et al.* 1986) and human fibroblasts (Naso, *et al.* 1994). Twelve to fifteen chondroitin sulfate side chains are covalently bound to glycosaminoglycan (GAG)- α and GAG- β domains encoded by exons 7 and 8, respectively. Alternative splicing of exon 7 and/or exon 8 generates four isoforms, V0, V1, V2, and V3. Versican V0, the largest isoform, contains both GAG- α and GAG- β domains, whereas versican V1 and V2 lack GAG- α and GAG- β , respectively. Versican V3, the shortest isoform, does not contain either of the GAG domains and is consequently devoid of chondroitin sulfate side chains (Dours-Zimmermann and Zimmermann 1994, Zako, *et al.* 1995). All versican isoforms contain globular

domains at the N-terminus (G1) and C-terminus (G3). The G1 domain interacts with hyaluronan, the G3 domain interacts with integrin $\beta 1$, and the GAG domains interact with CD44 and selectins (Wu, *et al.* 2005). By interacting with these molecules, versican plays many pivotal roles in regulating variable cellular processes including adhesion, proliferation, apoptosis, migration, and invasion (Wight 2002, Wight, *et al.* 2014). Recently, Tani *et al.* (2016) demonstrated that versican is upregulated in the macroscopically-normal peritonea of women with peritoneal endometriosis compared to those without. *In vitro*, versican promotes the attachment of endometrial stromal cells (ESCs) to the monolayer of peritoneal cells as well as the invasion of ESCs through the basement membrane (Tani, *et al.* 2016). Based on these findings, we propose that versican may be involved in human embryo implantation.

Although it has been reported that versican is expressed in both murine (Salgado, *et al.* 2009) and human endometria (Yamaguchi, *et al.* 2006), the specific expression and function of versican isoforms in the human endometrium has not been fully elucidated. It is both technically and ethically problematic to examine the process of human embryo implantation *in vivo*. To overcome this formidable barrier, several experimental models have been developed to reproduce the process of human embryo implantation *in vitro*. Since the availability of human blastocysts is extremely limited and primary endometrial cells are susceptible to inter-individual variations, such as age, menstrual cycle phase, and genetic background, cell line *in vitro* models have been commonly used to investigate the process of human embryo implantation (Heneweer, *et al.* 2005, Uchida, *et al.* 2012). We utilized an *in vitro* embryo implantation model consisting of an endometrial cancer cell line (Ishikawa), which retains the characteristics of normal endometrial cells (Hannan, *et al.* 2010, Nishida 2002), and a choriocarcinoma cell line (BeWo), which shares similar characteristics with undifferentiated trophoblasts of blastocysts (Hamilton 1998). The purpose of this study was to investigate the function of versican during human embryo implantation by employing our *in vitro* implantation model.

Materials and Methods

Antibodies and reagents

Antibodies used in this study are listed in Supplementary Table 1. Goat anti-human versican polyclonal antibody (pAb; #AF3054), which recognizes an amino acid sequence in the GAG- β domain specific to the V0 and V1 isoforms, was purchased from R&D Systems (Minneapolis, MN). Mouse anti-human versican monoclonal antibody (mAb; clone 2B1, #270428) recognizing the amino acid sequence in the C-terminal G3 domain common to all isoforms (Isogai, *et al.* 1996, Sobue, *et al.* 1988) was purchased from Seikagaku Corp. (Tokyo, Japan). Rabbit anti-human versican pAb (#HPA004726), which recognizes the amino acid sequence in the N-terminal G1 domain common to all isoforms, was obtained from Sigma-Aldrich (St. Louis, MO). Normal goat immunoglobulin G (IgG) for negative control (#sc-2028) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (pAb; #sc-2054) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated rabbit anti-goat IgG (pAb; #F0250), TRITC-conjugated swine anti-rabbit IgG (pAb; #R0156), HRP-conjugated rabbit anti-goat IgG (pAb; #P0449), and HRP-conjugated goat anti-mouse IgG (pAb; #P0447) were purchased from Dako Denmark A/S (Glostrup, Denmark).

Tissue samples

Human endometrial tissue samples were obtained from normally cycling women aged 28–45 years old who had undergone therapeutic hysterectomies for the treatment of benign uterine diseases or cervical carcinomas *in situ*. None of the patients were treated with hormonal medication or contraceptive pills for at least 3 months prior to surgery. According to the Declaration of Helsinki, informed consent for the use of these tissues was obtained from each donor. The use of the samples was approved by the Ethics Committee of Kyoto University Hospital.

Immunohistochemical staining

Endometrial samples were histologically staged according to the criteria of Noyes (Noyes, *et al.* 1975) and were classified as early proliferative (n = 2; cycle days 5–7), mid-proliferative (n = 2; cycle days 8–10), late proliferative (n = 2; cycle days 11–14), early secretory (n = 6; cycle days 15–19), mid-secretory (n = 6; cycle days 20–24), and late secretory (n = 6; cycle days 25–28) phases. Immunohistochemistry was performed as described previously (Tani, *et al.* 2016). Briefly, formalin-fixed, paraffin-embedded tissue sections prepared from whole endometria were

immunostained with the streptavidin-biotin complex peroxidase method with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Sections were subjected to an antigen-retrieval technique with the Histofine SAB-PO Kit (Nichirei Biosciences, Tokyo, Japan) and treated with goat anti-human versican pAb specific for the versican V0 and V1 isoforms or normal goat IgG as negative control. Immunostaining intensities of versican in endometrial epithelial cells (EECs) and ESCs were separately assessed by an independent blinded observer using a semi-quantitative scoring system as follows, 0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong; 5, very strong.

Isolation of EECs and ESCs

EECs and ESCs were isolated from endometrial tissues as described previously (Kosaka, *et al.* 2003). Small pieces of minced endometrial tissue (< 1 mm³) were digested enzymatically with 0.2% collagenase (Sigma-Aldrich) and 0.005% deoxyribonuclease (DNase; Sigma-Aldrich). ESCs were then purified using differential gravitational sedimentation (Kariya, *et al.* 1991) and seeded onto a 10-cm tissue culture dish (Corning Inc., Corning, NY). The larger clumps containing EECs were digested again with collagenase and DNase and were then allowed to attach onto a 10-cm cell culture dish for 1 hour at 37 °C to remove adherent ESCs. Non-adherent clumps were collected and treated with 0.05% trypsin (Gibco, Waltham, MA) and 0.02% EDTA (Nakalai, Kyoto, Japan) to obtain isolated EECs. EEC preparation purity was greater than 95%, as they were positive for CD9 (endometrial epithelial cell marker) and negative for both CD13 (endometrial stromal cell marker) and CD45 (leukocyte marker; (Kosaka, *et al.* 2003).

Immunocytochemistry

EECs were isolated from proliferative-phase endometria (n = 5; cycle days 7–13) as described above. Isolated EECs (5 × 10⁵ cells) in 2 mL of phenol red-free MEM plus 10% fetal bovine serum (FCS) that had been pretreated with dextran-coated charcoal (Sigma-Aldrich) were plated on a 35-mm cell culture plate (Greiner Bio-One, Frickenhausen, Germany). After a 24 or 72-hour incubation period, immunocytochemical staining was performed on cultured EECs treated with goat anti-human versican pAb or normal goat IgG using standard protocols. FITC-conjugated rabbit anti-goat secondary antibody was used for detection.

Treatment of isolated EECs with ovarian steroids

EECs (5×10^5 cells in 2 mL of phenol red-free MEM plus 10% FCS pretreated with dextran-coated charcoal) isolated from proliferative-phase endometria ($n = 5$; cycle days 7–13) were plated on a 35-mm round-bottom cell culture plate and incubated for 24 hours. Estradiol (E_2 ; Sigma-Aldrich) at a final concentration of 10 nM with or without progesterone (P_4 ; Sigma-Aldrich) at a final concentration of 1 μ M was added to the medium. The same amount of 99% ethanol (EtOH), of which the final concentration was 0.1% (v/v), was added as control. After 48 hours of incubation, EECs were immunostained with goat anti-human versican pAb followed by the addition of FITC-conjugated secondary antibody as described above. Intensity of the immunoreactive FITC signal was calculated as an integrated density using Image J v1.47 (NIH, Bethesda, MD). The immunostained specimen was divided into five areas (central, upper, lower, right, and left) all at a magnification of 100 $\times$ and the sum of the integrated densities from the five areas was defined as the versican expression level of the specimen. The result was presented as relative expression levels compared with the control (EtOH).

RT-PCR and real-time PCR

Total RNA was extracted from whole endometrial tissue using TRIzol[®] reagent (Thermo Fisher Scientific, Waltham, MA) and then reverse-transcribed with random primers using a First Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ). Each PCR product was electrophoresed on a 1.0% agarose gel and visualized by ethidium bromide staining. Real-time PCR was performed using the Power SYBR[®] Green PCR Master Mix (Life Technologies, Carlsbad, CA) and the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers used are shown in Table 1.

Western blotting

EEC proteins were obtained from 10 different patients (5 from proliferative endometria and 5 from secretory endometria; the EEC sample from “cycle day 3”, defined as menstrual phase (Noyes, *et al.* 1950), was histologically compatible with one of the proliferative phase). Versican protein expression was evaluated by western blotting as previously described (Wasa, *et al.* 2012). Briefly, EECs were treated with chondroitinase ABC (0.1 U/50 μ g protein) at 37 °C for 45 minutes to cleave the chondroitin sulfate side chains covalently bound to the core proteins. The proteins (15 μ g) were then resolved by 5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride

(PVDF) membranes (Millipore, Billerica, MA). The membranes were then blocked in Block Ace (Dainippon Pharmaceutics, Osaka, Japan) and incubated with goat anti-human versican pAb or mouse anti-GAPDH mAb. ECL Select Western Blotting System (GE Healthcare) was used for detection.

Spheroid formation of BeWo cells

BeWo, a cell line established from human choriocarcinoma (Pattillo and Gey 1968), was obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). BeWo cells were incubated in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Spheroid mass formation of BeWo cells was induced as described previously with slight modifications (Kosaka, *et al.* 2003). BeWo cell suspensions (5×10^2 cells in 50 µL of 1% FCS medium) were transferred to a PrimeSurface® (Sumitomo Bakelite, Tokyo, Japan) and incubated for 24 hours. This procedure enabled us to obtain a single, uniform-sized (~250 µm), multicellular spheroid from each well.

Construction of human versican V1 and V3-overexpressing Ishikawa cells

Ishikawa, a cell line established from human endometrial cancer, was generously provided by Dr. Masato Nishida (Kasumigaura Medical Center, Tsuchiura, Japan). Human versican was stably overexpressed in Ishikawa cells using the Trap-In system, a gene expression system combining promoter trapping and site-specific gene integration methods as previously reported (Shinomura, *et al.* 2008).

First, the Ishikawa cells were infected with the retroviral promoter trap vector (rvPtrap). The rvPtrap vector contains a gene encoding a fusion protein of β-galactosidase and hygromycin phosphotransferase (βgyg), a splice acceptor sequence of mouse immunoglobulin (SA), and a chemically synthesized FLP recombinase target sequence (FRT). Since βgyg does not possess its own promoter, rvPtrap should theoretically be integrated into an active gene in the Ishikawa cell to form hygromycin-resistant colonies. The colonies expressing the highest level of β-galactosidase were selected as Trap-In platform Ishikawa cells (ISKW).

Next, the plasmid containing the full coding sequence of human versican V1 [pInSRT-hM(V1)], versican V3 [pInSRT-hM(V3)], or GFP as control (pInSRT-GFP) was co-transfected along with the FLP recombinase-expressing pOG44 into ISKW using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

All three vectors were originally constructed (Supplementary Table 2). In this way, we established three stable clones expressing versican V1, two stable clones expressing versican V3, and five stable clones expressing GFP by dilution cloning in medium containing 1 µg/mL puromycin (Gibco). After verifying overexpression of the intended molecules by western blotting or immunocytochemistry, we arbitrarily selected a single clone from the versican V1-overexpressing, versican V3-overexpressing, and GFP-overexpressing clones and designated them ISKW-V1, ISKW-V3, and ISKW-GFP, respectively.

Preparation of conditioned medium derived from ISKW-V1 and ISKW-GFP cells

To prepare conditioned medium, 1×10^6 cells of ISKW-V1 or ISKW-GFP in 1 mL of RPMI 1640 supplemented with 1% FCS were plated in 24-well plates (Corning Inc.). After 24 hours of incubation, the culture medium from ISKW-V1 or ISKW-GFP was retrieved and centrifuged at $400 \times g$ for 10 minutes. The supernatant was collected as ISKW-V1 conditioned medium (V1-CM) or ISKW-GFP conditioned medium (GFP-CM) and stored at -80 °C until further use.

Attachment assay

Three sets of attachment assays were carried out. In the first set, ISKW-GFP, ISKW-V1, or ISKW-V3 cells (1×10^6 cells in 1 mL RPMI 1640 supplemented with 1% FCS) were seeded onto 24-well plates (Corning Inc.) and incubated for 24 hours to obtain a confluent monolayer. In the second set, ISKW monolayers were cultured in the presence of V1-CM or GFP-CM with 1% FCS. Chondroitinase ABC (0.1 units/mL) derived from *Proteus vulgaris* (Sigma-Aldrich), which cleave the core protein-linked chondroitin sulfate side chains, was added to each well and incubated for 2 hours. Chondroitinase ABC has been shown to degrade not only chondroitin sulfate but also hyaluronan (Grondahl, *et al.* 2011). In the third set, to rule out the possible influence of hyaluronan digestion by chondroitinase ABC, hyaluronidase (10 units/mL) derived from *Streptomyces* (Sigma-Aldrich), which can specifically degrade hyaluronan, was added to ISKW confluent monolayers cultured in the presence of V1-CM or GFP-CM.

Ten to fifteen BeWo spheroids were carefully placed into the center of each well and allowed to attach to the monolayer for 1 hour. The culture plate was then shaken at 180 rpm for 1 minute using a Multi Shaker MMS (Tokyo Rikakikai, Tokyo, Japan) to remove non-attached BeWo spheroids. The number of BeWo spheroids still attached to

the monolayer was manually counted under a phase-contrast microscope (Olympus, Tokyo, Japan). A spheroid attachment ratio was calculated as the number of attached spheroids divided by the total number of spheroids plated into the well. Three parallel attachment assays were performed for each experimental condition and the mean spheroid attachment ratio was defined as the spheroid attachment ratio for the experimental condition.

Statistical analysis

All quantitative experiments were performed at least three times. Data are presented as the mean $\pm$ standard deviation. Statistical analysis was performed using StatMate v3.14 (ATOMS, Oita, Japan) and comparisons were performed using unpaired Student's *t*-tests and one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison tests as appropriate. For all analyses, *p*-values < 0.05 were considered statistically significant.

Results

mRNA and protein expression of versican in the human endometrium

First, mRNA was extracted from whole human endometria of various menstrual cycle phases and subjected to RT-PCR using isoform-specific primers, revealing that versican isoforms were detectable throughout the menstrual cycle.

Second, whole endometria of various menstrual cycle phases were immunostained using anti-human versican pAb specific for the V0 and V1 isoforms (Figure 2A). In both luminal and glandular EECs, versican expression was higher during the secretory phases than during the proliferative phases, with the most prominent expression at the mid-secretory phase. Intense versican expression was also observed in the secretory products (Figure 2A [m]). In contrast, versican expression in ESCs appeared relatively constant throughout the menstrual cycle. Semi-quantitative scoring confirmed that the immunointensity scores of EECs were significantly higher in secretory phases than in proliferative phases (Figure 2B [a]), whereas those of ESCs were relatively constant throughout the menstrual cycle (Figure 2B [b]).

Finally, proteins extracted from isolated EECs were subjected to western blotting using the same antibody used for immunohistochemistry. Ten EEC samples from different patients (5 from proliferative endometria and 5 from secretory endometria) were used. Several proteins with different signal intensities ranging from 440 to 500 kDa, which are likely to correspond to the V1 isoform, were detected (Figure 3A). The signal intensities of these proteins were significantly higher in secretory-phase EECs than in proliferative-phase EECs (Figure 3B).

Secretion of versican from cultured EECs

EECs isolated from proliferative-phase endometria were seeded on a tissue culture plate. After 24 hours (day 1) or 72 hours (day 3) of culturing, EECs were fixed with paraformaldehyde and immunostained using anti-versican pAb specific for the V0 and V1 isoforms. Theoretically, the antibody cannot pass through the paraformaldehyde-fixed cytoplasmic membrane; therefore, immunocytochemistry assays can only detect versican that was secreted from the EECs and was located on the cell surface or in the extracellular space. As shown in Figure 4A, versican deposition in the extracellular space markedly increased after 72 hours of incubation.

Estradiol and progesterone upregulate versican secretion from EECs

EECs isolated from proliferative-phase endometrial tissues ($n = 5$) were treated for 48 hours with ethanol (EtOH) as control, estradiol alone (E_2), or estradiol and progesterone ($E_2 + P_4$). The amount of versican secreted from cultured EECs was evaluated by immunocytochemistry and as shown in Figure 4B, versican expression levels were significantly higher in the $E_2 + P_4$ group than in the EtOH or E_2 groups.

Characterization of ISKW-V1 and ISKW-V3

The plasmid containing the full coding sequence of human versican V1, versican V3, or control GFP was transfected into Ishikawa cells to generate the V1, V3, and GFP-overexpressing cells; note that versican V3 is devoid of chondroitin sulfate side chains, which occur in versican V1. Western blotting showed that immunoreactive versican V1 was detected in the cell lysates (Figure 5A) as well as the conditioned medium (Figure 5B) derived from ISKW-V1. On the other hand, versican V3 was only detectable in the cell lysates derived from ISKW-V3 (Figures 5C, D). Immunocytochemistry revealed that a large amount of versican V1 and versican V3 was secreted from ISKW-V1 and ISKW-V3, respectively (Figure 5E, F). Notably, versican V1 expression was not only detected on the cell surface but also in the extracellular space, whereas versican V3 was only detectable on the cell surface, which is in agreement with the data in Figure 5B and D.

Versican V1, but not versican V3, promotes BeWo spheroid attachment to ISKW monolayers

Attached and non-attached BeWo spheroids to the ISKW monolayers were easily distinguishable under a phase-contrast microscope (Figure 6A). The number of BeWo spheroids directly attached to the ISKW-V1 monolayer was significantly larger than those attached to the control ISKW-GFP monolayer (Figure 6B). In contrast, the number of BeWo spheroids directly attached to the ISKW-V3 monolayer was not significantly different from those attached to the control ISKW-GFP monolayer (Figure 6C).

Chondroitin sulfate side chains are required for enhancement of BeWo spheroid attachment to ISKW monolayers

The addition of V1-CM derived from ISKW-V1 cells significantly enhanced the number of BeWo spheroids attached to the ISKW monolayer (Figure 7A). This attachment-promoting effect of V1-CM was completely abrogated when it

263 was pretreated with chondroitinase ABC (Figure 7B). On the other hand, pretreatment with hyaluronidase did not
264 affect the attachment-promoting effect of V1-CM (Figure 7C).

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Discussion

In this study, we investigated *in vivo* expression and *in vitro* function of versican in the human endometrium to test the hypothesis that endometrial versican is involved in human embryo implantation. All endometrial tissue samples were obtained from patients undergoing hysterectomy for benign diseases including adenomyosis, uterine fibroids, or cervical carcinomas *in situ*. The endometrium was histologically examined to exclude the possibility of any endometrial lesions.

In our RT-PCR analysis, all versican isoforms were detectable in the human endometrium. On the other hand, Yamaguchi *et al.* (2006) reported that versican V2 mRNA was not expressed in the endometrium by RT-PCR analysis using the same primers as in our study. The versican V2 isoform is mostly found in the central nervous system but has been found in other tissues as well (Zimmermann and Dours-Zimmermann 2008). As PCR amplification efficiency and linearity are different among different PCR primer sets, it would not be appropriate to compare the amount of PCR product among the four versican isoforms; however, it is possible that expression levels of versican V2 in the human endometrium is relatively lower than that of the other three isoforms. In immunohistochemistry, the antibody specific for versican V0 and V1 isoforms was used and versican was found expressed in both EECs and ESCs throughout the menstrual cycle, but not in the postmenopausal endometrium (data not shown). Western blotting using the same antibody as for immunohistochemistry revealed that EECs of various menstrual cycle phases yielded multiple proteins of uneven signal intensities ranging from 440 to 500 kDa; this range corresponds to the multiple glycosylation variants of the V1 isoform (Mazzucato, *et al.* 2002, Perissinotto, *et al.* 2000), indicating that the immunoreactive signals observed in immunohistochemistry represent the V1 isoform. Versican V0 was not detected through western blotting, leading us to speculate that this isoform is mainly expressed in ESCs. Although we could not achieve detailed comparisons of versican expression patterns during each secretory phase due to limited amounts of sample, we were able to conclude that versican V1 expression and secretion in EECs peak during the mid-secretory phase, corresponding to the embryo implantation window (Cha, *et al.* 2012).

Many EEC secretory proteins regulated by ovarian steroids have been implicated in the process of embryo implantation (Bell, *et al.* 1986, Seppala, *et al.* 1994). We demonstrated that immunoreactive signals for versican V1 in EECs were not affected by estradiol treatment alone, but significantly increased after treatment with estradiol and progesterone together. In mice, uterine versican is regulated by ovarian steroids (Salgado, *et al.* 2009) and accumulates

in the endometrium during the peri-implantation period (San Martin, *et al.* 2003), suggesting a supporting role during embryo implantation. Moreover, bioinformatic analysis revealed that versican mRNA levels in goats were significantly higher in receptive endometria than in pre-receptive ones, suggesting that versican is possibly involved in the development of endometrial receptivity (Zhang, *et al.* 2015) and that versican is a novel player in human embryo-endometrium interactions (Altmae, *et al.* 2012). These findings indicate that versican V1 may be an important ECM component that mediates human embryo implantation.

Consequently, the possible roles of versican during human embryo implantation were investigated. The attachment-enhancing effect of BeWo spheroids was observed for versican V1, but not V3. Such isoform specificity is likely to be attributable to the structural differences between versican V1 and V3, i.e., the V3 isoform lacks glycosaminoglycan domains and chondroitin sulfate side chains. Western blotting and immunocytochemistry analyses demonstrated that secreted versican V1 is released into the surrounding environment in its soluble form whereas secreted versican V3 is more likely to remain anchored to the cell surface. A similar phenomenon was described for brevican, another member of the chondroitin sulfate proteoglycan family in rats. Brevican has two isoforms translated from alternatively processed transcripts of the same gene. One isoform is secreted into the extracellular space, whereas the other remains attached to the cell membrane via glycosylphosphatidylinositol (GPI) anchors (Seidenbecher, *et al.* 1995).

To examine the importance of versican V1-linked chondroitin sulfate side chains, versican V1-rich conditioned medium was pretreated with chondroitinase ABC. The attachment-enhancing effect of V1-CM was largely abrogated after this pretreatment, indicating that chondroitin sulfate side chains are required for the attachment-enhancing effect of versican V1. This is the first study to demonstrate the importance of versican chondroitin sulfate side chains in mediating cell-cell attachment. However, chondroitinase ABC was shown to degrade not only chondroitin sulfate but also hyaluronan (Grondahl, *et al.* 2011). There is accumulating evidence that hyaluronan, a major ECM component that interacts with versican, promotes embryo implantation (Chun, *et al.* 2016). In our study, pretreatment with *Streptomyces* hyaluronidase did not affect the attachment-enhancing effect of versican V1. We suspect the possible reason for this inconsistency is inherent in the culture conditions (1% FCS) of our experimental system, which contained almost no hyaluronan. This is supported by the finding that hyaluronidase treatment of GFP-CM did not affect the attachment of BeWo spheroids.

The molecular mechanisms behind versican promotion of BeWo spheroid attachment onto the Ishikawa monolayers were not elucidated in this study. Perlecan, a heparan sulfate proteoglycan (HSPG), surrounds blastocysts after hatching and has been suggested to play a role in human blastocyst attachment. Perlecan acts as a bridge between the trophoblast and luminal epithelial cells by binding certain adhesion molecules expressed on the luminal epithelium and trophectoderm (Kimber and Spanswick 2000). In this respect, we suspect that possible interactions between versican and specific adhesion molecules may contribute to the observed promotion of BeWo attachment to Ishikawa cells. It has been demonstrated that versican interacts with CD44 through its GAG chains as well as with integrin $\beta 1$ through its C-terminal domain (Wu, *et al.* 2002). Moreover, CD44 and integrin $\beta 1$ are both expressed on the surface of pre-implantation human embryos and endometrial epithelium (Kimber 2000). We confirmed that CD44 and integrin $\beta 1$ are expressed on the surface of Ishikawa and BeWo cells, respectively (data not shown). Further studies are required to elucidate the mechanisms behind the attachment-enhancing effects of versican V1.

There are several limitations to our *in vitro* model. First, we used human carcinoma-derived endometrial cells, which are luminal epithelial-like cells. In this regard, the observed cellular potential may not fully recapitulate that of actual EECs. Ishikawa cells, however, have been reported as a suitable alternative model for investigating human embryo implantation (Cheng, *et al.* 2017, Uchida, *et al.* 2012) and thus for analyzing the function of versican V1. Ishikawa cells retain mixed characteristics of glandular and luminal epithelia (Mo, *et al.* 2006) and express not only functional steroid receptors, such as estrogen and progesterone (Lessey, *et al.* 1996), but also produce many of the enzymes and structural proteins that are found in normal endometria (Castelbaum, *et al.* 1997). Ishikawa cells have also been shown to grow as a monolayer with uniform size and cylindrical cell shape and they form tight as well as adherent junctions (Wang, *et al.* 2012). Furthermore, Ishikawa cells possess a capacity for trophoblast apical adhesiveness *in vitro* (Heneweer, *et al.* 2005). Based on these similarities between Ishikawa cells and EECs, we consider Ishikawa cells as an appropriate model for understanding the function of versican in human EECs. The second limitation is that our *in vitro* monoculture system lacks the paracrine interactions that normally influence cell function and behavior *in vivo*. Indeed, rodent epithelial/stromal transplantation studies show that stimulation of endometrial epithelial cell mitogenesis by estrogen is mediated through stromal cells (Cooke, *et al.* 1997). Due to these limitations, the data obtained from our model should be regarded as hypothesis-generating and future research should be aimed at developing more sophisticated human implantation models using primary endometrial cells.

In summary, we have demonstrated for the first time that versican expression in EECs is regulated by ovarian steroids and is most prominent at the mid-secretory phase, which corresponds to the embryo implantation window. In the *in vitro* embryo implantation model, versican V1 enhanced the attachment of BeWo spheroids to the monolayer of Ishikawa cells. From these findings, we propose that versican V1 is an ECM component that facilitates human embryo implantation. *In vivo* experiments, such as those using conditional knockout mice or transgenic mice with tissue-specific expression, are required to substantiate the supportive role of versican during embryo implantation.

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.

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Figure legends

Figure 1 mRNA expression pattern of versican in human endometrium. (A) RT-PCR analysis shows that all versican isoforms (V0–V3) are detected in human endometrial tissue throughout the menstrual cycle. (B) Expression of versican (a) V0, (b) V1, (c) V2, and (d) V3 across different phases of the menstrual cycle was analyzed by quantitative RT-PCR (proliferative, $n = 4$; early secretory, $n = 4$; mid-secretory, $n = 4$; late secretory, $n = 4$). One-way analysis of variance (ANOVA) was used for statistical analysis.

Figure 2 Versican protein expression in human endometrium. (A) Endometrial tissues of various menstrual cycle phases were immunostained with anti-versican polyclonal antibody specific for the V0 and V1 isoforms. (a) Early proliferative phase, (b) mid-proliferative phase, (c) late proliferative phase, (d) early secretory phase, (e) mid-secretory phase, (f) late secretory phase; (g)–(l) are higher magnification views of the area indicated in (a)–(f), respectively. (m) Glandular epithelium in the functional layer of mid-secretory endometrium. (n) Negative control for (m) in which the anti-versican antibody was replaced by isotype-matched control antibody. Versican expression was detected in both endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs) throughout the menstrual cycle. The intensity of EEC versican expression was higher in the secretory phases than in the proliferative phases. Note that intense versican expression was also detected in intra-glandular products secreted from EECs and are indicated by arrowheads in (m). Scale bars = 100 μm in (a)–(f); 50 μm in (g)–(n). (B) Signal intensities of versican V1 were assessed in the immunohistochemical images of whole endometria of various menstrual cycle phases (representative images shown in Figure 2A). Immunointensity scores (0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong; 5, very strong) were separately given to (a) EECs and (b) ESCs. Note that immunointensity scores of (a) EECs were significantly higher in the secretory phases than in the proliferative phases, whereas those of (b) ESCs were constant throughout the menstrual cycle. In all panels, error bars represent the standard deviation. * $p < 0.05$; ** $p < 0.01$; N.S., not significant.

Figure 3 Cyclic changes in versican expression in human endometrium. (A) Lysates of isolated EECs were subjected to western blotting using the same anti-versican antibody as in Figure 2A, which is specific for versican V0 and V1. The lower panel shows GAPDH as control. Versican V1 was detected as multiple protein signals ranging from 440 to 500 kDa. (B) Protein signal intensities for versican V1 were significantly higher in secretory phases than in

proliferative phases. Error bars represent standard deviation. $*p < 0.05$.

Figure 4 Effects of ovarian steroid hormones on versican expression in isolated EECs. (A) EECs were isolated from the endometrial tissue of cycle day 12 and cultured for 24 hours (day 1) or 72 hours (day 3). Note the intense versican deposition in the extracellular space on day 3, indicating that versican secretion from EECs increased in a time-dependent manner. (a, c) and (b, d) represent immunofluorescence and phase-contrast images, respectively. Scale bars = 100 μ m. (B) Isolated EECs were treated with ethanol (EtOH), 10 nM estradiol (E₂), or 10 nM E₂ plus 1 μ M progesterone (P₄) and then immunostained. The final concentration of EtOH was 0.1% (v/v). Versican expression levels in EECs were measured by mean fluorescent intensity. The sum of the integrated densities from five areas (central, upper, lower, right, and left) in the immunostained specimen is defined as the “versican expression level”. The versican expression level was significantly higher after treatment with E₂ and P₄ (E₂ + P₄) than after treatment with EtOH or E₂ alone (n = 5). Error bars represent standard deviation. $*p < 0.05$.

Figure 5 Characterization of versican V1- and V3-overexpressing Ishikawa cells. (A) Cell lysates extracted from original Ishikawa cells (Ishikawa; lane 1), platform Ishikawa cells (ISKW; lane 2), GFP-overexpressing Ishikawa cells (ISKW-GFP; lane 3), and versican V1-overexpressing Ishikawa cells (ISKW-V1; lane 4) were subjected to western blotting. ISKW possesses a specific sequence into which foreign DNA can be inserted by a recombinase. An intense protein signal at 440 kDa corresponding to versican V1 is only detectable in ISKW-V1. (B) Cell lysates from ISKW-V1 (lane 1), conditioned medium derived from ISKW-V1 (V1-CM; lane 2), and conditioned medium derived from ISKW-GFP (GFP-CM; lane 3) were subjected to western blotting using the same anti-versican antibody as in (A). Note that the protein signal at 440kDa is detected in both ISKW-V1 cell lysates (lane 1) and V1-CM (lane 2). (C) Cell lysates extracted from original Ishikawa cells (Ishikawa; lane 1), platform Ishikawa cells (ISKW; lane 2), GFP-overexpressing Ishikawa cells (ISKW-GFP; lane 3), and versican V3-overexpressing Ishikawa cells (ISKW-V3; lane 4) were subjected to western blotting using an anti-versican monoclonal antibody raised against the G3 domain common in all versican isoforms. An intense protein signal at 76 kDa corresponding to versican V3 was only detected in ISKW-V3. (D) Cell lysates from ISKW-V3 (lane1), conditioned medium derived from ISKW-V3 (V3-CM; lane2), and CM derived from ISKW-GFP (GFP-CM; lane 3) were subjected to western blotting using the same anti-versican

antibody as in (C). Note that the protein signal at 76 kDa is only detectable in the ISKW-V3 cell lysates (lane 1) but not in V3-CM (lane 2). (E) ISKW-V1 cells cultured for 48 hours were subjected to immunocytochemistry using the same anti-versican antibody as in (A). Note that intense immunoreactive versican is detected not only on the cell surface but also in the extracellular space (circumscribed with the dotted line). Images (a) and (b) represent immunofluorescence and phase-contrast images, respectively. Scale bars = 100 μ m. (F) ISKW-V3 cells cultured for 48 hours were subjected to immunocytochemistry using anti-versican polyclonal antibody raised against the G1 domain common in all versican isoforms. Note that intense immunoreactive versican is detected exclusively on the cell surface and not in the extracellular space (circumscribed with the dotted line). Images (a) and (b) represent immunofluorescence and phase-contrast images, respectively. Scale bars = 100 μ m.

Figure 6 Differential effects of versican V1 and V3 on BeWo spheroid attachment. (A) Phase-contrast images of BeWo spheroids after 1-hour incubation on a monolayer of (a) GFP-overexpressing Ishikawa cells (ISKW-GFP), (b) versican V1-overexpressing Ishikawa cells (ISKW-V1), and (c) versican V3-overexpressing Ishikawa cells (ISKW-V3). Note that all spheroids were attached to the (b) ISKW-V1 monolayer, whereas some spheroids were floating (arrowheads) over the (a) ISKW-GFP and (c) ISKW-V3 monolayers. Scale bars = 200 μ m. (B) Attachment ratios to the ISKW-V1 monolayer ($n = 4$) were significantly higher compared with that of the control ISKW-GFP monolayer ($n = 4$). (C) Attachment ratios to the ISKW-V3 monolayer ($n = 6$) were not significantly different than those of the control ($n = 6$). All values are relative to the control, ISKW-GFP. In all panels, error bars represent standard deviation. $*p < 0.05$; N.S., not significant.

Figure 7 Versican V1 promotion of BeWo spheroid attachment requires the presence of chondroitin sulfate side chains. (A) BeWo spheroids were incubated on ISKW monolayers in the presence of GFP-CM or V1-CM with or without chondroitinase ABC (ChABC) pretreatment. The attachment ratio significantly increased in the presence of V1-CM ($n = 7$) compared with that of GFP-CM ($n = 7$). Note that the attachment-promoting effect of V1-CM was completely abrogated by ChABC pretreatment ($n = 7$). All values are relative to the control, GFP-CM, without ChABC treatment. (B) BeWo spheroids were incubated on ISKW monolayers in the presence of V1-CM with or without hyaluronidase (Hase) pretreatment. Note that Hase pretreatment did not affect the attachment ratio that was increased by untreated

V1-CM (n = 4). In all panels, error bars represent standard deviation. * $p < 0.05$; N.S., not significant.

Table 1. Primers for RT-PCR and real-time PCR

Gene	Sequence	Reference
RT-PCR		
Versican V0	F-TCAACATCTCATGTTCTCC R-TTCTTCACTGTGGTATAGGTCTA	Cattaruzza, <i>et al.</i> 2002
Versican V1	F-GGCTTTGACCAGTGCGATTAC R-TTCTTCACTGTGGTATAGGTCTA	Cattaruzza, <i>et al.</i> 2002
Versican V2	F-TCAACATCTCATGTTCTCC R-CCAGCCATAGTCACATGTCTC	Cattaruzza, <i>et al.</i> 2002
Versican V3	F-GGCTTTGACCAGTGCGATTAC R-CCAGCCATAGTCACATGTCTC	Cattaruzza, <i>et al.</i> 2002
S26	F-GGTCCGTGCCTCCAAGATGA R-TAAATCGGGTGGGGGTGTT	Vincent, <i>et al.</i> 1993
Real-time PCR		
Versican V0	F-GCACAAAATTCACCCTGACATT R-CTTCTTTAGATTCTGAATCTATTGGATGAC	Takahashi, <i>et al.</i> 2012
Versican V1	F-CCCAGTGTGGAGGTGGTCTAC R-CTCAAATCACTCATTGACGTT	Takahashi, <i>et al.</i> 2012
Versican V2	F-TCAGAGAAAATAAGACAGGACCTGATC R-CATACGTAGGAAGTTTCAGTAGGATAACA	Takahashi, <i>et al.</i> 2012
Versican V3	F-CCCTCCCCCTGATAGCAGAT R-GGCACGGGTTCATTTTGC	Takahashi, <i>et al.</i> 2012
GAPDH	F-GAGTCAACGGATTTGGTCGTATTGG R-GCCATGGGTGGAATCATATTGGAAC	Chigusa, <i>et al.</i> 2013

Figure 1

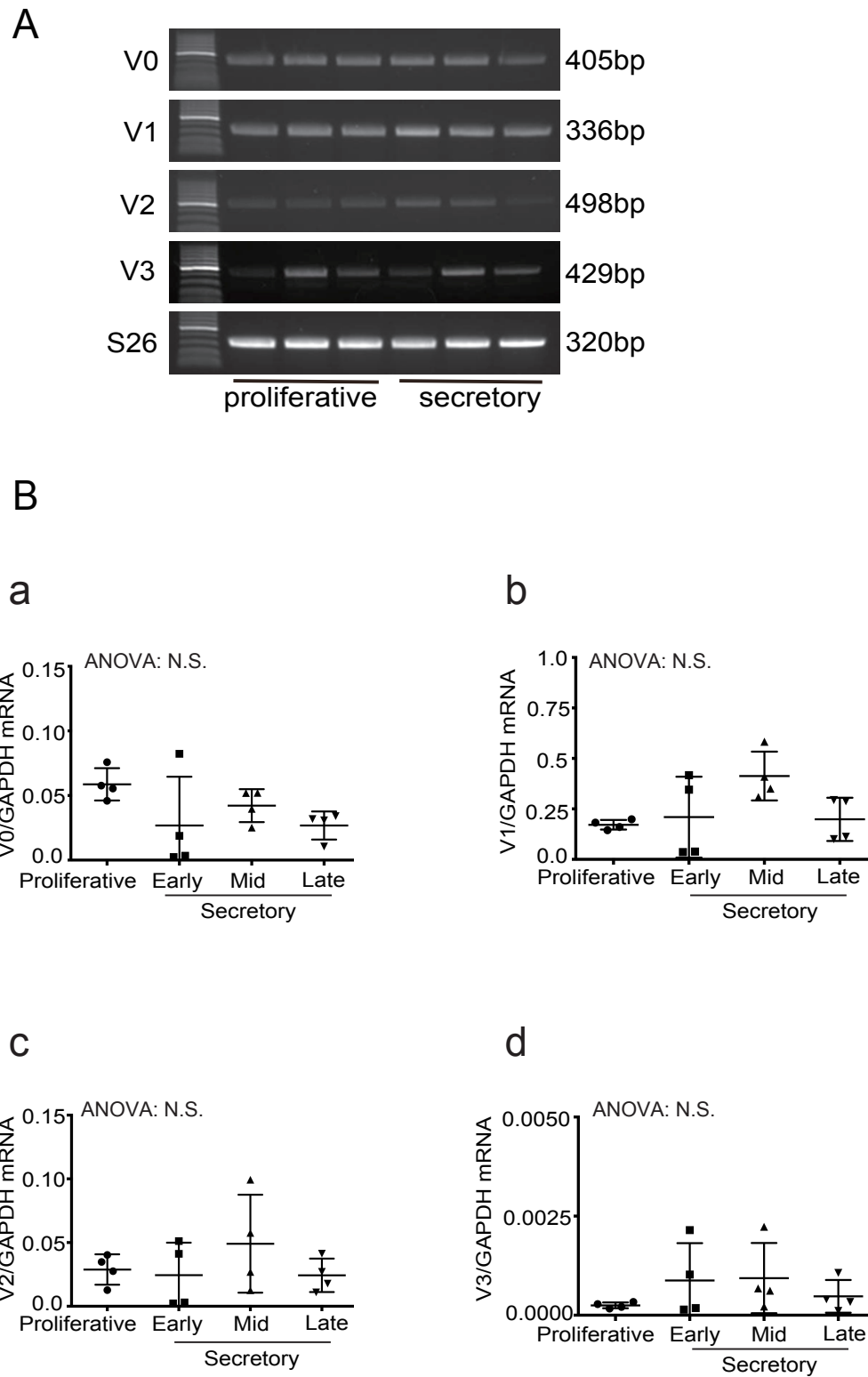
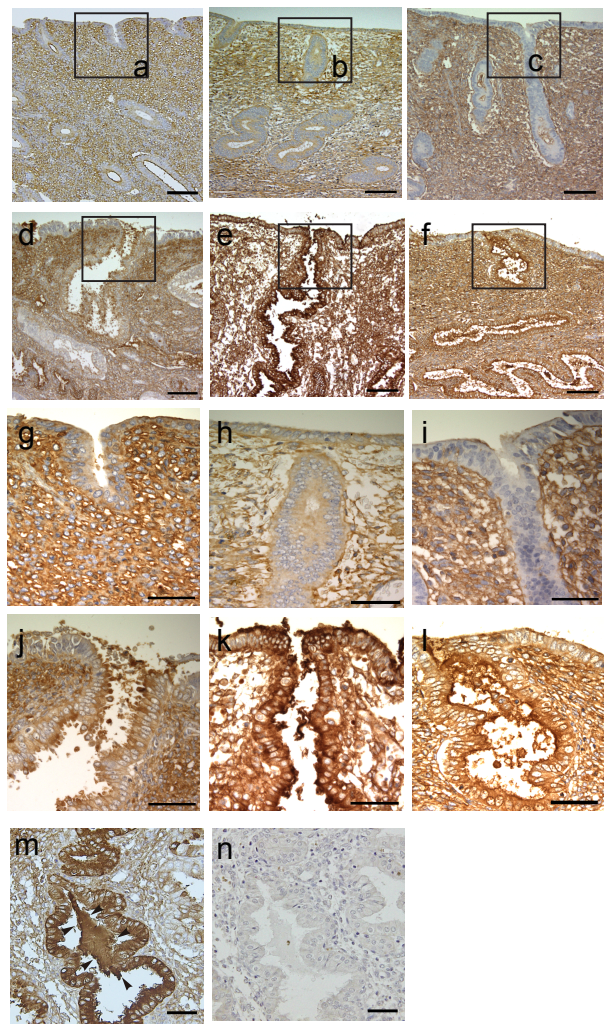


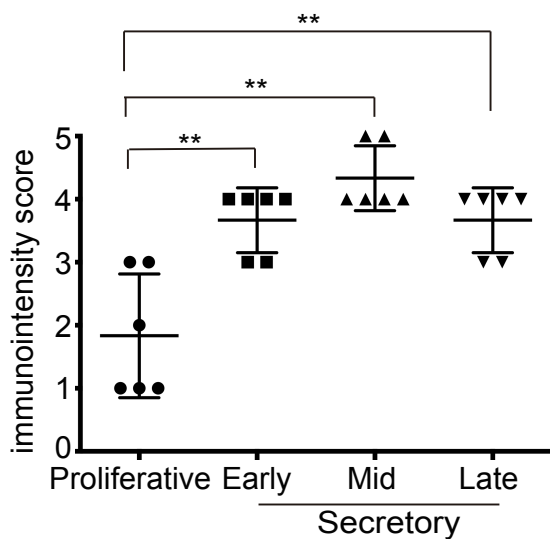
Figure 2

A



B

a



b

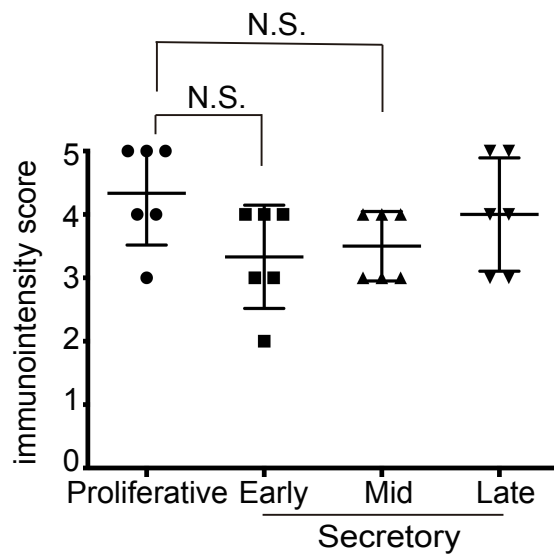
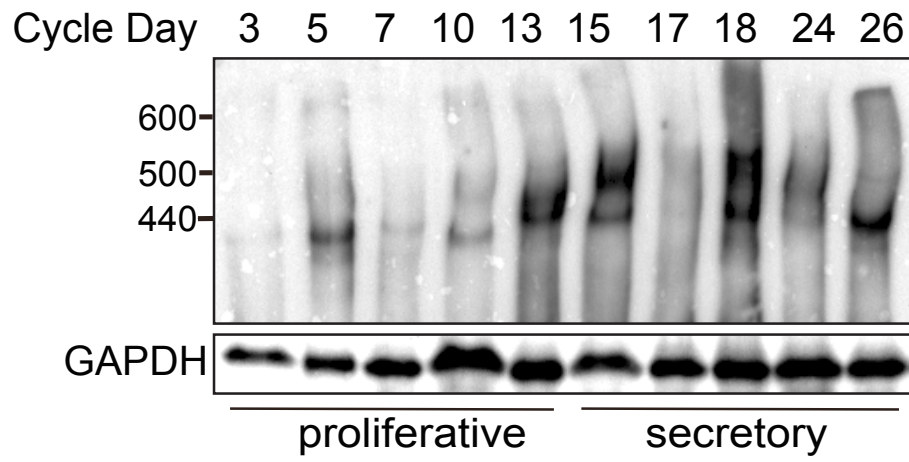


Figure 3

A



B

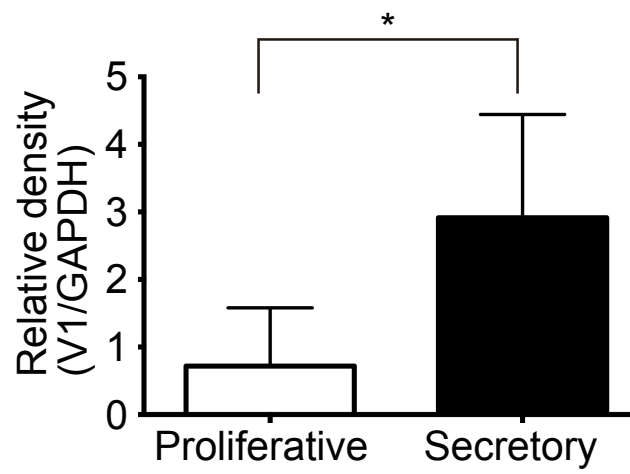
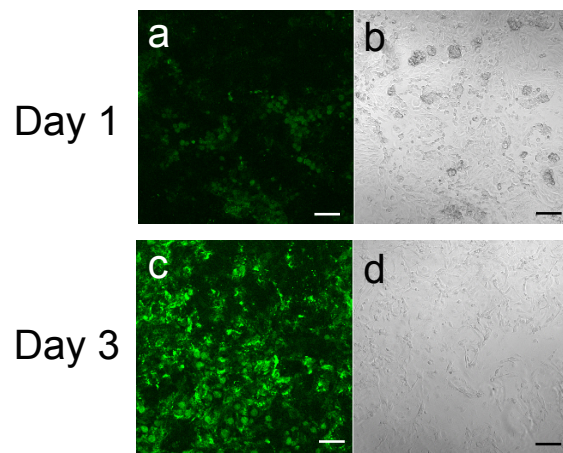


Figure 4

A



B

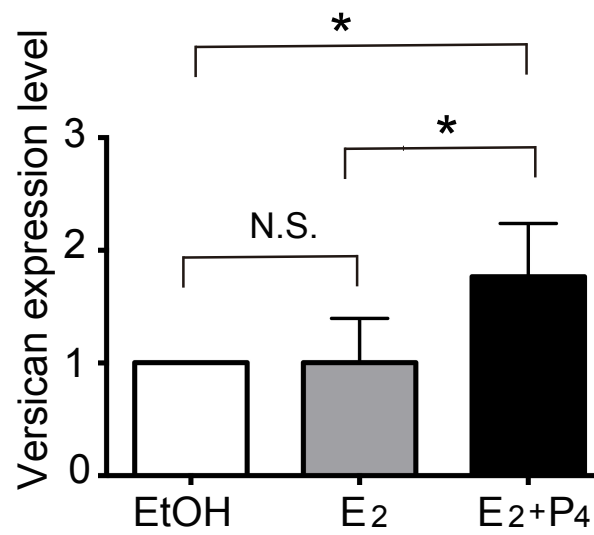
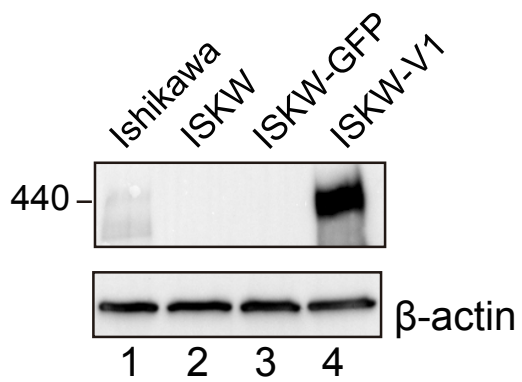
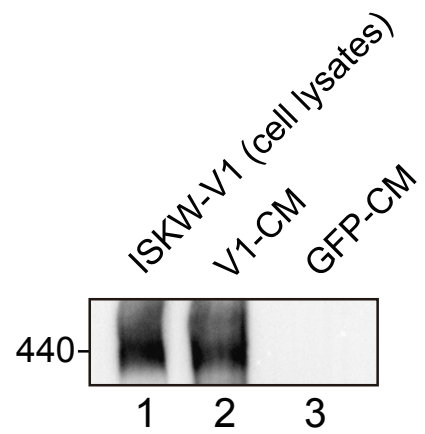


Figure 5

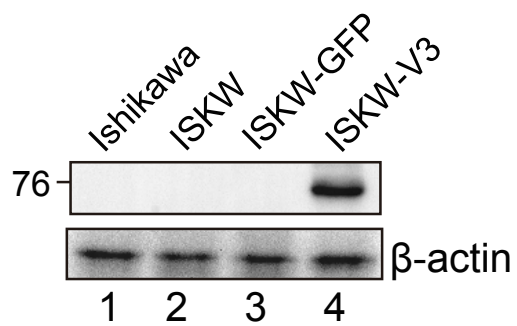
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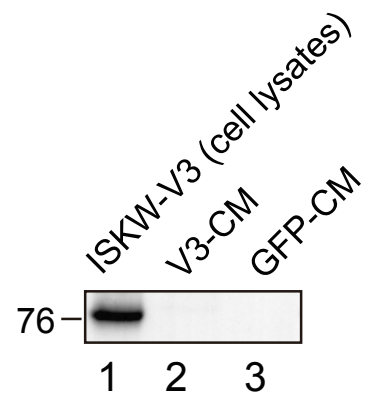
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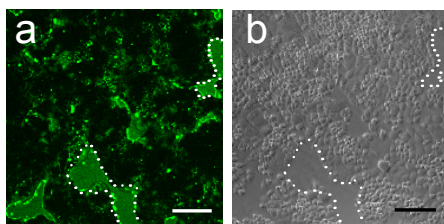
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D



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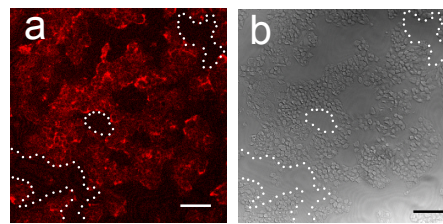


Figure 6

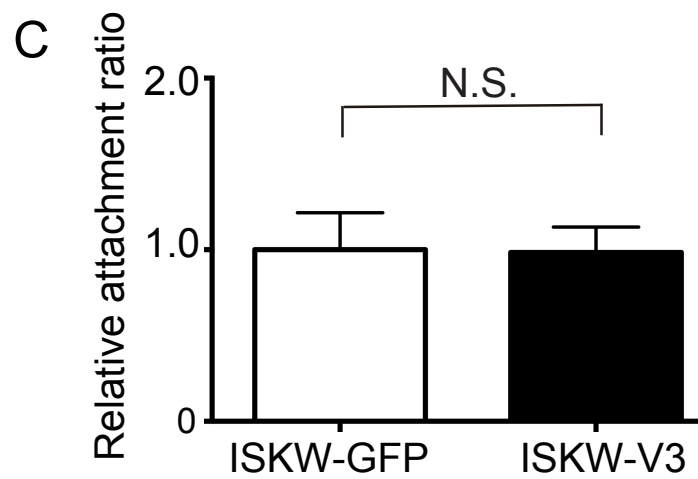
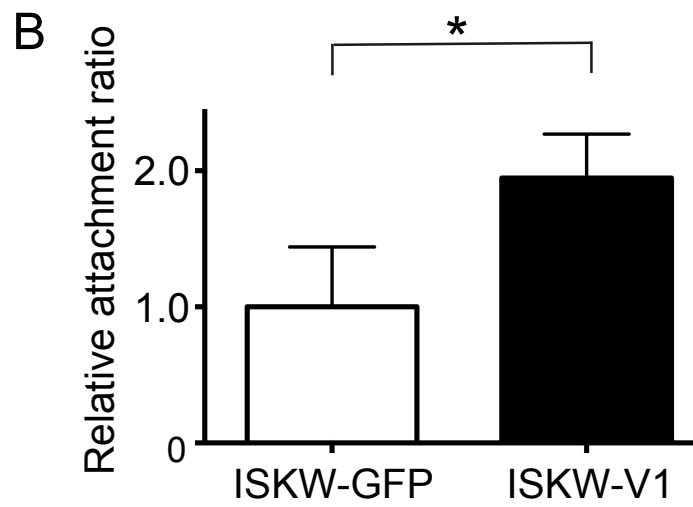
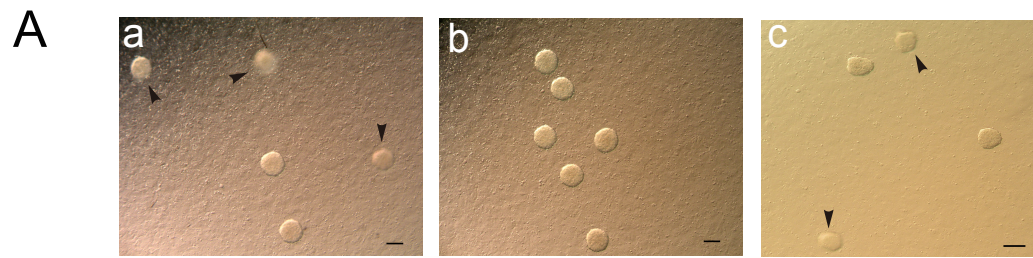
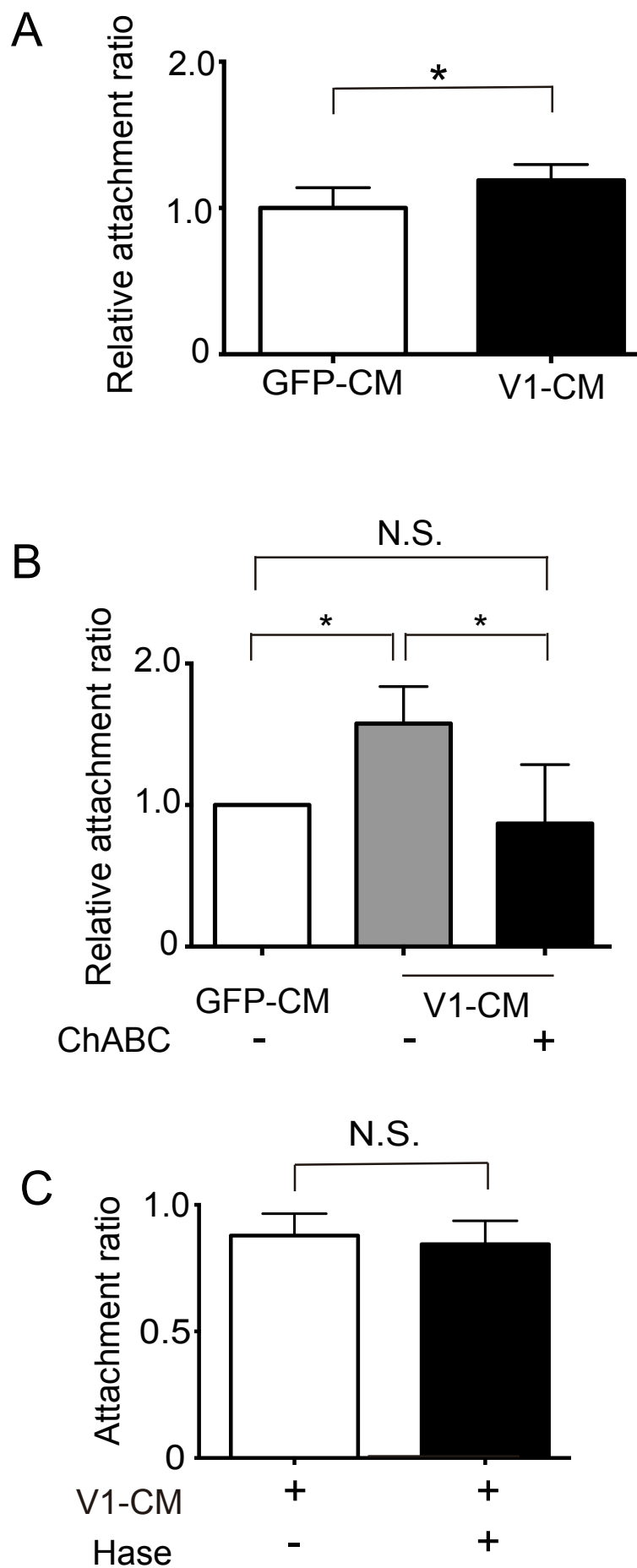


Figure 7



Supplementary Table 1. List of antibodies

Target Peptide/Protein	Antigen Sequence	Name of antibody (Clone) Isoform specificity	Manufacture (Catalog No.)	Species Monoclonal /Polyclonal	Dilution used	RRID
versican	N/A	Anti-versican V0/V1	R&D Systems (#AF3054)	Goat Polyclonal	5 µg/ml (IF, IHC) 1:1000 (WB)	AB_2214378
versican	PDRCKMN PCLNG	Anti-versican (Clone 2B1) All isoforms	Seikagaku Corp.	Mouse Monoclonal	1:2500 (WB)	N/A
versican	N/A	Anti-versican All isoforms	Sigma (#HPA004726)	Rabbit Polyclonal	10 µg/ml (IF)	AB_889072
CD9	N/A	Anti-CD9 (Clone ALB6)	Cosmo Bio.	Mouse Monoclonal	100 µg/ml, 10 µl (FCM)	N/A
CD13	N/A	Anti-CD13	Cosmo Bio.	Mouse Monoclonal	100 µg/ml, 10 µl (FCM)	N/A
CD45	N/A	Anti-CD45 (Clone T29/33)	Cosmo Bio.	Mouse Monoclonal	100 µg/ml, 10 µl (FCM)	N/A
GAPDH	N/A	Anti-GAPDH	Abcam (#ab9484)	Mouse Monoclonal	1:1000 (WB)	AB_307274
β-actin	N/A	Anti-β-actin	Abcam (#ab8227)	Rabbit Polyclonal	1:5000 (WB)	AB_2305186

N/A, not applicable. IF, immunofluorescence. IHC, immunohistochemistry. WB, western blotting. FCM, flow cytometry

Supplementary Table 2. Plasmids used in this study

Vector	Product	GenBank accession no.	Length of sequence
pInSRT-hM(V1)	Versican splice variant V1	LC417347	12143bp
pInSRT-hM(V3)	Versican splice variant V3	LC417348	7667bp
pInSRT-GFP	Green fluorescent protein	LC417349	5506bp