

# Laeverin/aminopeptidase Q induces trophoblast invasion during human early placentation

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**BACKGROUND:** In primate placenta, extravillous trophoblast (EVT) invades maternal tissue in temporally- and spatially-regulated fashions. We previously identified a novel placenta-specific cell-surface aminopeptidase, laeverin/aminopeptidase Q, which is expressed on EVT-lineage cells in the fetal membrane. Laeverin possesses a peptide-binding site that is evolutionally unique to primates, suggesting possible involvement of laeverin in a primate-specific phenomenon during placentation. Thus, this study was designed to elucidate the molecular characteristics and physiological roles of laeverin in human EVT.

**METHODS:** Placental tissues of various developmental stages were subjected to immunostaining and western blotting. Effects of siRNA and a soluble form of recombinant laeverin on EVT cells isolated from primary villous explant cultures were examined using Matrigel invasion assays and cell proliferation assays.

**RESULTS:** Laeverin was specifically immunolocalized to HLA-G-positive EVT in placentas from early and term pregnancy. In primary villous explant cultures, laeverin expression was induced on the cell surface of the outgrowing EVT. In western blotting, laeverin protein was detected as two distinct bands at 130 and 160 kDa along with a broad band ranging from 200 to 270 kDa. De-glycosylation treatment showed that these native laeverin isoforms are N-linked glycoproteins sharing a common 115-kDa core protein. In invasion assays, the reduction of laeverin expression by siRNA suppressed migration of the isolated EVT, while the soluble form of recombinant laeverin enhanced its migration.

**CONCLUSIONS:** Laeverin is a specific cell-surface marker for human EVT and plays a regulatory role in EVT migration.

**Key words:** differentiation / extravillous trophoblast / glycosylation / isotype

## Introduction

During placentation, human trophoblast differentiates into villous trophoblast or extravillous trophoblast (EVT). EVT invades the maternal decidual tissues, infiltrates and reconstructs maternal spiral arteries in temporally- and spatially-regulated fashions. In the case of insufficient shallow invasion, resultant placental dysfunction leads to various obstetrical disorders such as pre-eclampsia (Pijnenborg *et al.*, 1983). Although numerous factors have been reported to control the invasive capacity of human EVT (Aplin, 1991; Damsky *et al.*, 1994; Bischof *et al.*, 1995; Lala and Chakraborty, 2003), it is

still difficult to explain the temporal and spatial regulation of EVT invasion.

To further investigate the mechanism that regulates human EVT invasion, we raised several monoclonal antibodies against human chorion laeve, which is a component of the fetal membrane, and isolated a novel protein that was specifically expressed on EVT-lineage cells in the chorion laeve. This novel protein was named laeverin (Fujiwara *et al.*, 2004). Laeverin was proved to be a novel membrane-bound aminopeptidase belonging to the M1 peptidase family and also termed aminopeptidase Q (Maruyama *et al.*, 2007). Aminopeptidases hydrolyze the N-terminal amino acid of proteins or peptide substrates.

Among these, the M1 family of zinc aminopeptidases (gluzincin) shares the consensus GXMEN (peptide-binding site) and HEXHXI8E (cutting site) motifs that are essential for enzymatic activity and consists of 11 enzymes in humans. In contrast to other M1 aminopeptidases, the first glycine (Gly) residue within the GXMEN motif is uniquely substituted with histidine (His) in primate laeverin (Maruyama et al., 2007). The substitution of His (379) with Gly caused a conformational alteration in this enzyme and induced significant changes in substrate specificity toward natural peptide hormones (Maruyama et al., 2009). The presence of this HXMEN motif that primates uniquely obtained during the evolutionary process implies possible involvement of laeverin in a primate-specific phenomenon.

Membrane-bound peptidases can regulate local concentration of biologically active peptides before they access their specific receptors on the cell surface. We have demonstrated that membrane-bound peptidases, carboxypeptidase-M and dipeptidyl peptidase-IV were expressed on human EVT (Sato et al., 2002; Nishioka et al., 2003) and proposed that these two peptidases suppress EVT migration by degrading invasion-promoting soluble factors (Fujiwara, 2007). Since laeverin can degrade several placenta-derived peptides including kisspeptin-10 (Maruyama et al., 2007), which suppresses trophoblast as well as cancer migration (Bilban et al., 2004; Navenot et al., 2005; Cho et al., 2009), laeverin could also be involved in the regulation of EVT migration.

Based on this background, in the present study, we examined the comprehensive expression profiles of laeverin on EVT in the placental tissues at various developmental stages. We also investigated possible roles of laeverin in the proliferation, migration and differentiation of EVT using human primary EVT culture.

## Materials and Methods

### Antibodies and reagents

FITC-conjugated mouse anti-human cytokeratin 7 monoclonal antibody (mAb, clone LP5K and IgG2b) was purchased from Cymbus Biotechnology (Hants, UK). FITC- and phycoerythrin (PE)-conjugated mouse anti-human HLA-G (clone MEM-G/9, IgG1) mAbs were both obtained from Abcam (Cambridge, UK). This HLA-G antibody can only react with native HLA-G1 isotype that is specifically expressed on human EVT (Menier et al., 2003). FITC-conjugated and non-conjugated mouse IgG1 (clone DAK-GO1), IgG2a (clone DAK-GO5) and IgG2b (clone DAK-GO9) for negative controls were obtained from Dako (Glostrup, Denmark). Mouse anti-trinitrophenyl (TNP) mouse mAb (unrelated mAb, IgG1; Tsujimura et al., 1990) was used as blocking antibody for double staining. FITC-conjugated rabbit anti-mouse immunoglobulin pAb (Dako), FITC-conjugated swine anti-rabbit immunoglobulin pAb (Dako) and rhodamine-conjugated goat anti-mouse immunoglobulin pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as secondary antibodies for immunocytochemistry.

The soluble form of recombinant laeverin, which was produced by a Baculovirus system in our laboratory (Maruyama et al., 2007), was used as a positive control on western blot analysis and for proliferation and invasion assays as described below.

The mouse anti-human laeverin mAb (clone CHL-2) was raised against chorion laeve of fetal membrane as previously described (Fujiwara et al., 2004) and the rabbit anti-laeverin pAb was raised by immunizing rabbits with recombinant laeverin and purified from the serum using affinity chromatography with recombinant laeverin (Maruyama et al., 2009).

### Tissue samples and cell lines

Tissue samples at the implantation sites of human conceptus during early placentation were obtained from four women who underwent therapeutic hysterectomy for cervical intraepithelial neoplasia or uterine myoma during normal pregnancy at 9 ( $n = 2$ ), 10 ( $n = 1$ ) and 11 ( $n = 1$ ) weeks of gestation. These tissue samples were subjected to immunohistochemical study. Human placental tissues for chorionic villous explant cultures or immunohistochemical staining were aseptically obtained from legal abortions of normal pregnancies (4–12 weeks of gestation,  $n = 36$ ). There are considerable amounts of EVT that have invaded maternal tissues in the decidual tissues of the term placenta (feto-maternal interface). Therefore, we collected the placental samples at the basal plate including the boundary site between chorionic villous tissue and decidual tissue from patients who underwent elective Caesarean sections at 37–41 weeks of gestation ( $n = 6$ ) due to their history of Caesarean sections. These placental tissues were also used for western blot and RT-PCR analyses. The gestational age was calculated from the date of the last menstrual period and, if necessary, was adjusted according to ultrasonic measurements of the fetal crown-rump length. Informed consent for the use of these tissues was obtained from each donor. The usage of these samples was approved by the Ethical Committee of Kyoto University Hospital.

For negative control studies, human choriocarcinoma cell line, BeWo cells (Pattillo et al., 1968), which have no expression of laeverin by RT-PCR, were used.

### Human chorionic villous explant culture and isolation of EVT

EVT was isolated from human villous explant cultures as described (Sato et al., 2003, 2005). Briefly, small fragments (2 mm in diameter) of chorionic villi were put on each of 10 cm dishes coated with collagen type I (Iwaki, Chiba, Japan). After incubation for 4 h in RPMI 1640 (Sigma-Aldrich Co., Ayrshire, UK) supplemented with 10% fetal calf serum (FCS, Life Technologies, Inc., Grand Island, NY, USA) at 37°C under 20% O<sub>2</sub> conditions to allow the explants to adhere, RPMI 1640 with 10% FCS was gently added and the dishes were incubated for an additional 48 h. After observing cells that had grown out from the adherent villous tips, the specimens were washed gently with phosphate-buffered saline (PBS), dispersed with 0.05% trypsin (Difco, Detroit, MI, USA)/0.05% EDTA (Nakalai, Kyoto, Japan) solution, passed through a 40- $\mu$ m-pore-sized Nylon Cell Strainer (Becton Dickinson, Bedford, MA, USA) to remove chorionic villous parts, and replated on collagen type I-coated 6-well plates (Iwaki) in RPMI 1640 plus 10% FCS at 37°C under 20% O<sub>2</sub> conditions. After 4-h incubation, the cells that remained attached were defined as 'isolated EVT' and used for further experiments as described below.

### Immunostaining

Frozen sections were prepared from tissue samples taken from the implantation sites and the placental tissues as described previously (Sato et al., 2002, 2003). Villous explant cultures were performed in CELLSTAR<sup>®</sup> Dish, diameter of 35 × 10 mm, Four Rings (Greiner Bio-One, Frickenhausen, Germany) that had been pre-coated with Collagen I, rat tail (BD Biosciences, Bedford, MA, USA). After observing outgrown cells from the adherent villous tips, the slides were washed gently with PBS and fixed with 1% paraformaldehyde at 4°C for 10 min followed by acetone at -20°C for 10 min.

The frozen sections or villous explant culture samples were double stained using anti-laeverin mAb (clone CHL-2) that was produced in our laboratory (Fujiwara et al., 2004) along with anti-HLA-G mAb or anti-cytokeratin 7 mAb as previously described (Sato et al., 2003).

Briefly, the slides were incubated with anti-laeverin mAb (5 µg/ml, diluted in culture medium) or negative control mAb (5 µg/ml) for 40 min. Specimens were then stained with rhodamine-conjugated secondary antibody (diluted 1:40) for 40 min. After incubation with anti-TNP mAb (unrelated mAb, 50 µg/ml) to block non-specific binding (Tsujiura *et al.*, 1990), the slides were reacted with FITC-conjugated anti-cytokeratin 7 mAb (diluted 1:40) or FITC-conjugated anti-HLA-G mAb (diluted 1:40) for 40 min.

To confirm the specificity of the rabbit anti-laeverin pAb that had been raised by immunizing rabbits with recombinant laeverin and purified with affinity chromatography, the placental sections were double immunostained together with the mouse anti-laeverin mAb. The slides were sequentially incubated for 40 min with rabbit anti-laeverin pAb (10 µg/ml), FITC-conjugated swine anti-rabbit immunoglobulin pAb, mouse anti-laeverin mAb (5 µg/ml) and rhodamine-conjugated goat anti-mouse immunoglobulin pAb.

The slides were mounted with an anti-fade agent (Perma Fluor Aqueous Mounting Medium; Immunon, Pittsburgh, PA, USA) and examined under a confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany).

### Flow cytometry

Flow cytometry was carried out as previously described (Sato *et al.*, 2002). Isolated EVT cells were suspended in Hanks' balanced salt solution containing 0.1% BSA and 0.1% NaN<sub>3</sub>. The cells were incubated with anti-laeverin rabbit pAb (1 µg/µl, 5 µl) or control pAb (1 µg/µl, 5 µl) followed by FITC-conjugated swine anti-rabbit immunoglobulin. For double staining, the cells were further labeled with PE-conjugated anti-HLA-G mAb (undiluted, 5 µl). Cell surface labeling was analyzed by FITC and PE-fluorescence detection using FACScalibur (BD Biosciences, Tokyo, Japan).

### RNA isolation and RT-PCR analysis

Total RNA was extracted from isolated EVT with Trizol (Invitrogen, Carlsbad, CA, USA) and was reverse transcribed using a First Strand cDNA Synthesis Kit (Pharmacia, Inc., Piscataway, NJ, USA). Thirty-five cycles of PCR were performed with oligonucleotides from human laeverin cDNA as primers (Fujiwara *et al.*, 2004; sense primer 5'-CATTGGCC AGGTATGTTTTCCC-3'; positions 849–870; antisense primer 5'-TGCC CCTTCTGTTCTGTTGAC-3'; positions 1076–1097, Genbank accession number: NM\_173800) or with human S26 primers (Vincent *et al.*, 1993; sense primer 5'-GGTCCGTGCCTCCAAGATGA-3'; positions 8–27; antisense primer 5'-TAAATCGGGGTGGGGGTGT-3'; positions 308–327, Genbank accession number: BC002604). Positive control (S26) and negative control (without cDNA) were included for all amplifications. PCR products were sequenced to confirm their identity.

### Western blotting

Placental tissues were lysed in RIPA (radioimmunoprecipitation assay) buffer (50 mM, pH 7.4, Tris-HCl, 1% NP-40, 5 mM EDTA, 1% deoxycholic acid and 100 mM NaCl) containing 0.2 µg/ml phenylmethylsulfonyl fluoride hydrochloride (Wako Pure Chemicals, Osaka, Japan) and 10 µg/ml leupeptin (Peptide Institute, Osaka, Japan) and then centrifuged at 15 000g for 20 min. Each supernatant including 50 µg of protein was electrophoresed in 6% SDS-polyacrylamide gel containing 10% glycerol and transferred to Immobilon PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) in CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 11.0, Nacalai Tesque, Kyoto, Japan) with 10% methanol. The PVDF membrane was treated with Block Ace (Dainihon Pharmaceuticals, Osaka, Japan), rabbit pAb against laeverin (1 µg/ml) or human β-actin (ab8227, Abcam Co., Tokyo, Japan), and then horseradish peroxidase (HRP)-conjugated goat antibody against rabbit immunoglobulins

(DAKO). HRP was visualized using an ECL Western Blotting System (GE Healthcare, Piscataway, NJ, USA).

### De-glycosylation of laeverin

Placental laeverin and recombinant laeverin were de-glycosylated as described previously (Maruyama *et al.*, 2007). Briefly, after heat denaturing (100°C, 5 min), placental tissue lysates and recombinant laeverin in RIPA buffer was incubated with peptide: N-glycosidase F (30 µg/ml, New England Biolabs, Beverly, MA, USA) at 37°C for 1 h. The treated samples were electrophoresed in 6.0% SDS-polyacrylamide gel containing 10% glycerol, transferred to Immobilon-P PVDF membrane and subjected to western blot analysis using anti-laeverin pAb.

### Silencing of endogenous laeverin with siRNA

Two siRNAs for human laeverin, siRNA/laeverin-1 (LVRN-HSSI37155; sense: UUC AAG AAA CAA GAA AGC AUC CGG G; antisense: CCC GGA UGC UUU CUU GUU UCU UGA A) and siRNA/laeverin-2 (LVRN-HSSI37157; sense: AAC UCU GAU CCU CUG GUG UUC CUC C; antisense: GGA GGA ACA CCA GAG GAU CAG AGU U) were obtained from Invitrogen. Validated siRNAs for control (siRNA/CTR; sense: UUC UCC GAA CGU GUC ACG UdT dT; antisense: ACG UGA CAC GUU CGG AGA AdT dT) were purchased from Qiagen (Valencia, CA, USA). Each siRNA at a final concentration of 50 nM was transfected into primary villous explant culture or isolated EVT using TransIT-TKO<sup>®</sup> (Takara Bio, Inc., Otsu, Japan). After 48-h incubation, the expression levels of laeverin mRNA and protein in each sample was compared by RT-PCR and flow cytometry or immunocytochemistry, respectively, as described above.

### Invasion assay

Invasion assays were carried out as previously described (Sato *et al.*, 2003, 2005). The isolated EVT or BeWo cells (1 × 10<sup>5</sup> cells/200 µl of RPMI 1640 plus 10% FCS) were cultured in cell culture inserts (6.4 mm in diameter; Beckton Dickinson Labware, Bedford, MA, USA) containing polyethylene terephthalate membranes with 8 µm-diameter pores. The membrane of the culture insert was pre-coated with Matrigel (200 µg/ml, Collaborative Research Co., Bedford, MA, USA). Culture inserts were placed in each well of a 24-well tissue culture plate (Beckton Dickinson Labware) and the lower wells were filled with 800 µl of RPMI 1640 plus 10% FCS.

Recombinant laeverin (0, 0.1 and 1 µg/ml) was added to the upper chamber. Alternatively, the isolated EVT cells that had been transfected with laeverin or control siRNA were inoculated into the upper chamber. After 24-h incubation at 37°C under 20% O<sub>2</sub> conditions, cells that reached the lower surface were fixed with methanol and stained with hematoxylin. The membrane was divided into five areas, i.e. central, upper, lower, right and left areas, and the number of invaded cells in each area was manually counted under a light microscope at a magnification of ×200. The sum of the cell numbers obtained from the five areas was regarded as the invaded cell number for the membrane. Three parallel invasion assays were performed for each experimental condition and the average was defined as 'invaded cell number' under the experimental condition. The result was expressed as the percentage of the invaded cell number in the control. These experiments were repeated at least four times and the differences were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's F-test for multiple comparisons.

## Cell proliferation assay

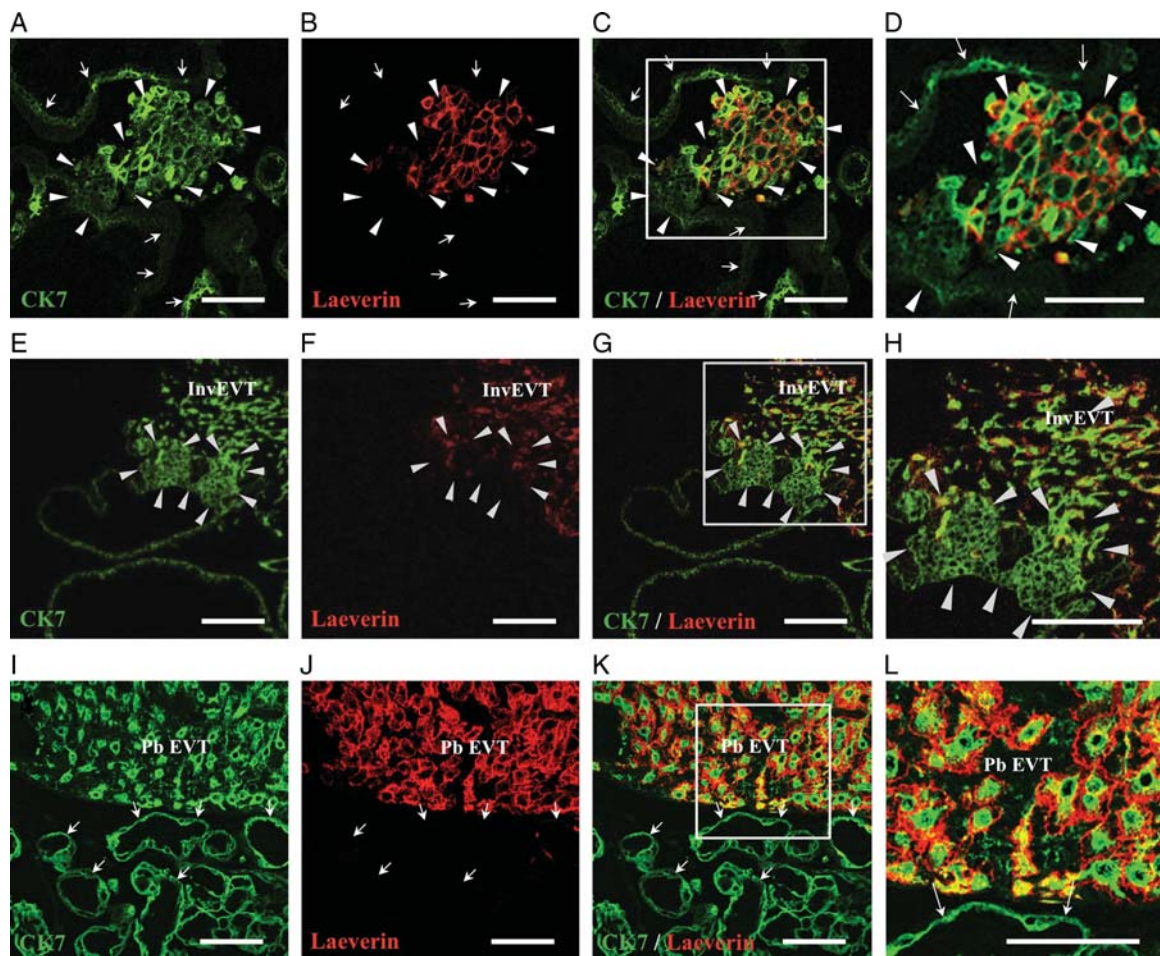
The isolated EVT or BeWo cells ( $5 \times 10^4$  cells/100  $\mu$ l of RPMI 1640 plus 10% FCS) were inoculated in each well of a 96-well plate coated with collagen type I. Recombinant laeverin (0, 0.1 and 1  $\mu$ g/ml) was added to the culture medium. Alternatively, the isolated EVT cells that had been transfected with laeverin or control siRNA were cultured in the well. After 24-h incubation at 37°C under 20% O<sub>2</sub> conditions, viable cells were assessed using the Premix WST-1 Cell Proliferation Assay System (Takara, Kusatsu, Japan). Three parallel proliferation assays were performed for each experimental condition and the average was defined as 'viable cell number' under the experimental condition. The result was expressed as the percentage of the viable cell number in the control. These experiments were repeated at least three times and the differences were analyzed by one-way ANOVA followed by Scheffe's F-test for multiple comparisons.

## Results

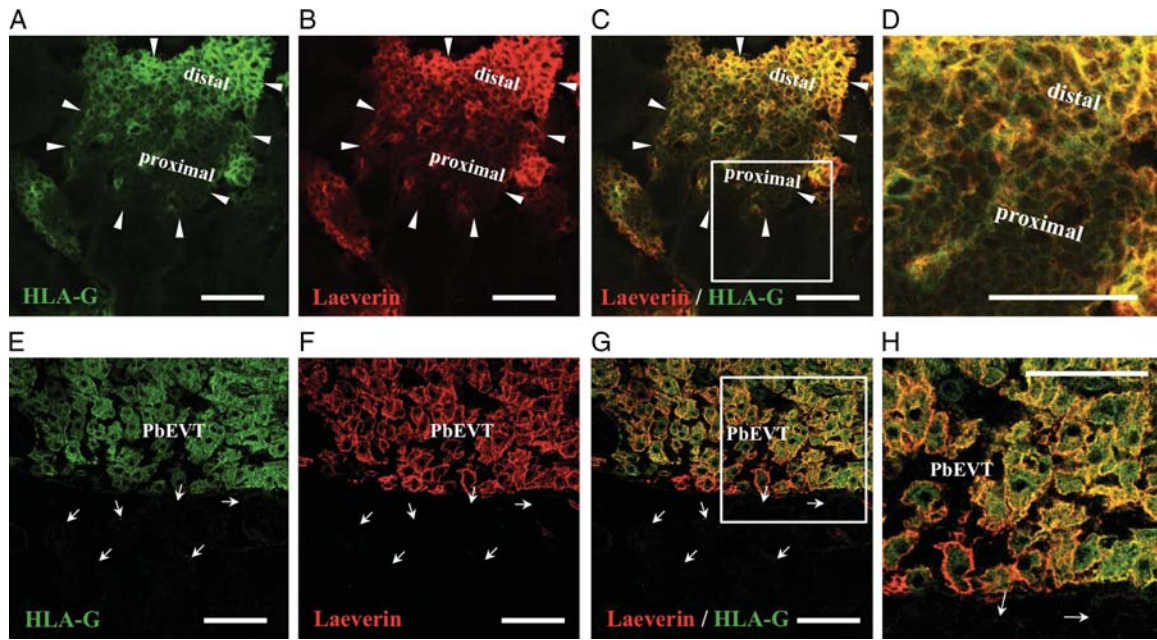
### Immunolocalization of laeverin in human placental tissues at various stages

In chorionic tissues at 4 weeks of gestation, immunoreactive laeverin was detected on EVT located in the distal parts of cell column (Fig. 1A–D). Similar expression profiles of laeverin in the cell column were observed at 11 weeks of gestation (Fig. 1E–H). Laeverin was also detected on invading EVT in the maternal decidua of early pregnancy (Fig. 1E–H) and on EVT in the placental bed of term pregnancy (Fig. 1I–L).

Double immunostaining confirmed that laeverin was co-localized with HLA-G, which is a well-known marker for human EVT (McMaster



**Figure 1** Immunohistochemical localization of laeverin in human placental tissues at various developmental stages. Placental tissues at 4 weeks (A–D), 11 weeks (E–H) and 38 weeks (I–L) of gestation were double immunostained with anti-laeverin (red) and anti-cytokeratin 7 (CK7, green) monoclonal antibodies. D, H and L are magnified images of the boxed areas indicated in C, G and K, respectively. (A–D) At 4 weeks of gestation, immunoreactive laeverin was detected on EVT in the cell column (arrowheads), whereas villous trophoblast (arrows) and villous stromal cells did not express laeverin. Note that laeverin expression was only observed at the distal site of the cell column. (E–H) At 11 weeks of gestation, laeverin was similarly expressed at the distal site of the cell column (arrowheads). The invading EVT (InvEVT) in the maternal decidua tissues was also positive for laeverin staining. (I–L) At 38 weeks of gestation, laeverin was expressed on EVT in the placental bed (PbEVT), while chorionic villi (arrows) were negative for laeverin staining. Bars indicate 100  $\mu$ m.



**Figure 2** Co-localization of laeverin with HLA-G. Placental tissues of 6 weeks (A–D) and 38 weeks (E–H) of gestations were double stained with anti-laeverin (red) and anti-HLA-G (green) monoclonal antibodies. D and H are magnified images of the boxed area indicated in C and G, respectively. The section used for E–H is adjacent to that used for Fig. 11–L. (A–D) In the cell column, the expression profile of laeverin was virtually the same as that of HLA-G, which is a well-known marker for human EVT, in the cell column (arrowheads). Note that the intensity of laeverin as well as HLA-G expression was higher in the distal site of the cell column than in its proximal site. (E–H) At 38 weeks of gestation, laeverin was also co-localized with HLA-G on the invaded EVT residing in the placental bed. Neither molecule was detected in chorionic villi (arrows). Bars indicate 100  $\mu\text{m}$ .

*et al.*, 1995), both in the cell column of early pregnancy (Fig. 2A–D) and on EVT in the placental bed of term pregnancy (Fig. 2E–H). In the cell column, the expression of laeverin was induced from the same portion as HLA-G (Fig. 2A–D).

### Molecular biological characterization of laeverin

RT–PCR analysis detected mRNA transcripts for laeverin in the placental samples obtained at 4, 9 and 38 weeks of gestation as well as in the isolated EVT (Fig. 3A). Its expression was undetectable in human choriocarcinoma cell line, BeWo cells (Fig. 3A).

Double immunostaining with anti-laeverin rabbit polyclonal antibody and mouse monoclonal antibody (clone CHL-2) produced identical staining profiles, indicating that anti-laeverin rabbit polyclonal antibody that had been raised against and affinity purified with recombinant laeverin (Maruyama *et al.*, 2007) reacts specifically with the native form of human laeverin (Fig. 3B). As previously reported, laeverin purified from term placental tissues using affinity chromatography with CHL-2 antibody exhibited a 160 kDa main and a 130 kDa minor protein band on silver staining (Fujiwara *et al.*, 2004). In the present study, western blot analysis using the polyclonal antibody revealed an additional broad protein band around 200–270 kDa (Fig. 3C). On the other hand, recombinant laeverin was detected as a single protein band at 120 kDa as reported previously (Maruyama *et al.*, 2007). After placental lysates were treated with N-glycosidase, laeverin appeared as a single molecular band at 115 kDa (Fig. 3C). The

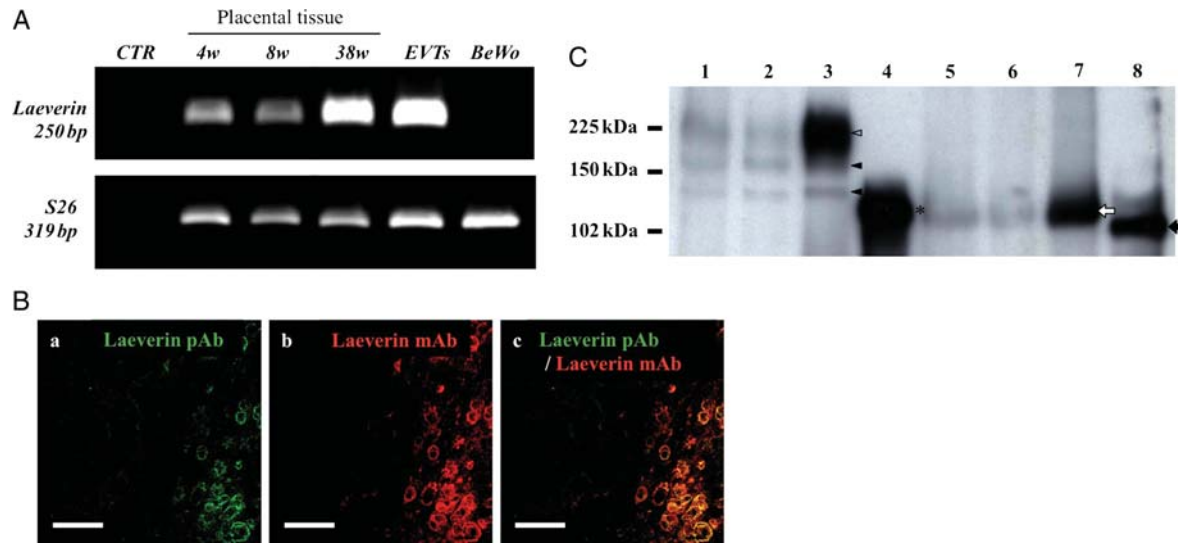
molecular weight of de-glycosylated recombinant laeverin was shifted to 106 kDa as described previously (Maruyama *et al.*, 2007).

### Laeverin expression in primary villous explant culture and isolated EVT

During primary villous explant culture, EVT outgrew from the attached villous tips. Outgrowing EVT consisted of two subpopulations, i.e. column-like cell sheet-forming EVT at the proximal site and migrating EVT at the distal site of the tips. HLA-G expression was constantly detected on all the outgrowing EVT (Fig. 4B–D). By contrast, laeverin expression was observed on the limited population of HLA-G-bearing EVT in the cell sheet region, while its expression was constantly detected on the migrating EVT (Fig. 4A, C and D). To confirm this observation, the isolated EVT cells were double immunostained with HLA-G and laeverin antibodies and subjected to flow cytometric analysis (Fig. 4E). Reliability of the isolation process of primary EVT cells was demonstrated by the fact that nearly 95% of the isolated cells were HLA-G positive. Among these HLA-G-positive EVT cells, only ~40% were positive for laeverin. In western blot analysis, a 160-kDa protein band observed in the placental tissue was hardly detectable in the isolated EVT cells (Fig. 4F).

### Effects of silencing endogenous laeverin with siRNA on EVT invasion

In primary villous explant culture treated with siRNA/laeverin-1 and siRNA/laeverin-2, the reduction of laeverin expression was



**Figure 3** Molecular biological characterization of laeverin. **(A)** RT-PCR analysis. Laeverin mRNA was clearly detected in placental tissues obtained from 4, 9 and 38 weeks of gestation as well as in the isolated EVT (6 weeks of gestation), while its expression was undetectable in human chorioncarcinoma cell line, BeWo cells. **(B)** Double immunostaining of placental tissue obtained from 38 weeks of gestation using anti-laeverin rabbit polyclonal antibody (pAb, green, a) and anti-laeverin mouse monoclonal antibody (mAb, red, b). Merged image (c) showed identical staining profiles for pAb and mAb, suggesting that anti-laeverin pAb specifically reacted with the native form of human laeverin. **(C)** Western blot analysis of placental tissues at 4, 9 and 38 weeks of gestations (lanes 1–3), and recombinant laeverin (5 ng, lane 4) using anti-laeverin pAb. Laeverin was detected as two specific bands at 130 and 160 kDa (black arrowheads) along with a broad band of 200–270 kDa (white arrowhead) in the placental tissues. Recombinant laeverin was detected as a single protein band at 120 kDa (asterisk). After the lysates derived from placental tissues at 4, 9 and 38 weeks of gestation were treated with N-glycosidase (lanes 5–7), laeverin was detected as a common single band at 115 kDa (white arrow). The molecular weight of de-glycosylated recombinant laeverin was shifted to 106 kDa (lane 8, black arrow).

predominantly observed on the migrating EVT from the distal site of the cell sheet and migrating EVT directly from the anchored villous tips (Fig. 5A). RT-PCR analysis showed marked reduction of laeverin mRNA expression level in the isolated EVT cells after treatment with laeverin-specific siRNAs (Fig. 5B). Flow cytometric analysis confirmed that laeverin protein expression on the cell surface of the isolated EVT was also reduced by the treatment (Fig. 5C). Under these conditions, invasion of the isolated EVT was significantly suppressed (Fig. 5D), while the cell proliferation was not affected (Fig. 5E).

### Effects of recombinant laeverin on EVT invasion

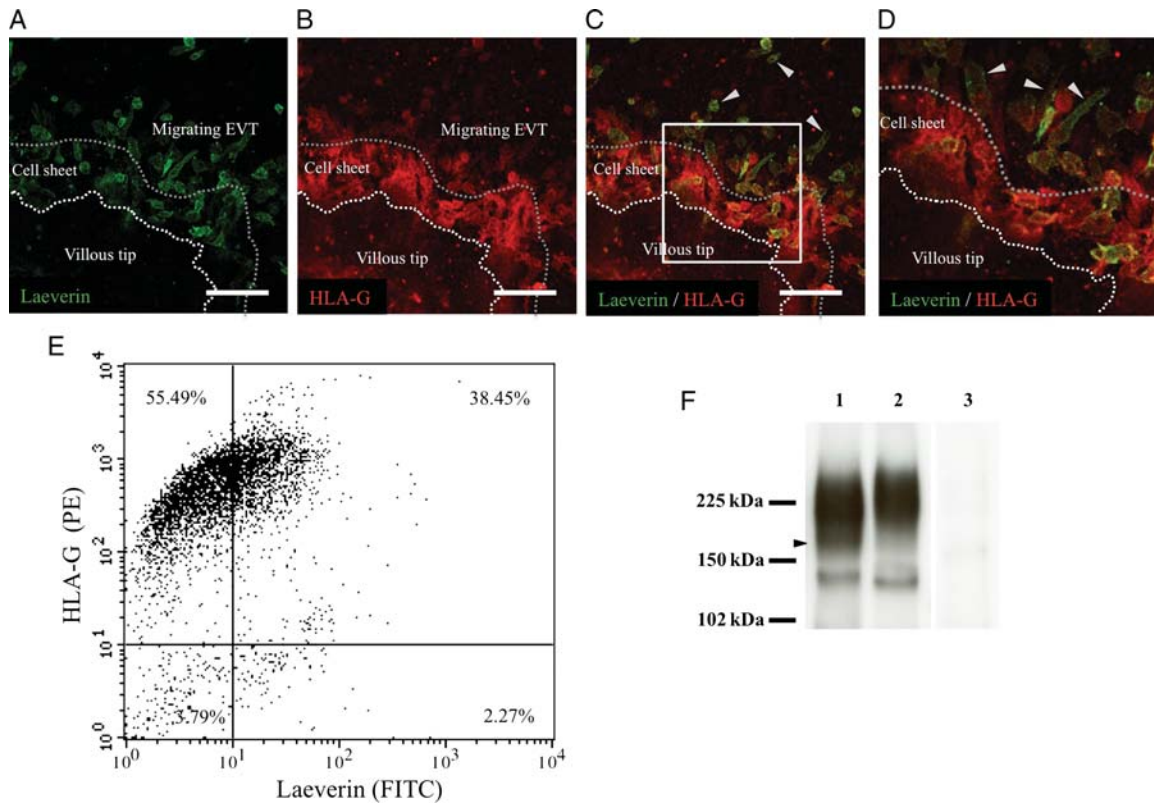
In an invasion assay, addition of recombinant laeverin significantly promoted EVT invasion in a dose-dependent manner (Fig. 6A), while the cell proliferation was not affected (Fig. 6B). Recombinant laeverin also stimulated invasion of laeverin-negative BeWo cells without affecting cell proliferation (Fig. 6C and D).

## Discussion

By immunohistochemical examination, the present study confirmed our previous report that laeverin is specifically expressed on EVT (Fujiwara et al., 2004; Maruyama et al., 2009) and demonstrated for the first time that laeverin expression on EVT is detectable from very early stage of human placentation (i.e. 4 weeks of gestation).

The specificity of laeverin expression on EVT was verified by its co-expression with HLA-G in double immunostaining. In addition, flow cytometric analysis using isolated EVT verified the cell surface expression of laeverin as well as HLA-G. Although other membrane-bound peptidases are rather ubiquitously expressed in various organs (Fujiwara et al., 1999), laeverin mRNA was only detectable in human placenta on our northern blot analysis (Fujiwara et al., 2004). Since no other cell population in the placenta expressed laeverin, we concluded that laeverin is a novel specific marker of human EVT that is expressed on the surface of EVT at early pregnancy and the term. To our knowledge, this is the first cell surface molecule that is EVT specific and co-localized with HLA-G.

HLA-G is known as a specific marker for human EVT that is induced on the surface of EVT from the distal site of the cell column (McMaster et al., 1995). Immunohistochemical study of placental tissues at various developmental stages showed that expression profiles of laeverin were virtually identical to those of HLA-G. Notably, expressions of laeverin and HLA-G became detectable from the same portion in the distal site of the cell column. In the primary explant cultures of human chorionic villous tissues obtained from the first-trimester pregnancy, cell sheets followed by migrating cells grow out from the explanted villous tips. Since these structures resemble the cell column and the invading EVT *in vivo*, this primary villous explant culture is considered to reproduce the extravillous differentiation occurring at the villus-anchoring sites (Sato et al., 2003). In this culture system, immunoreactive HLA-G

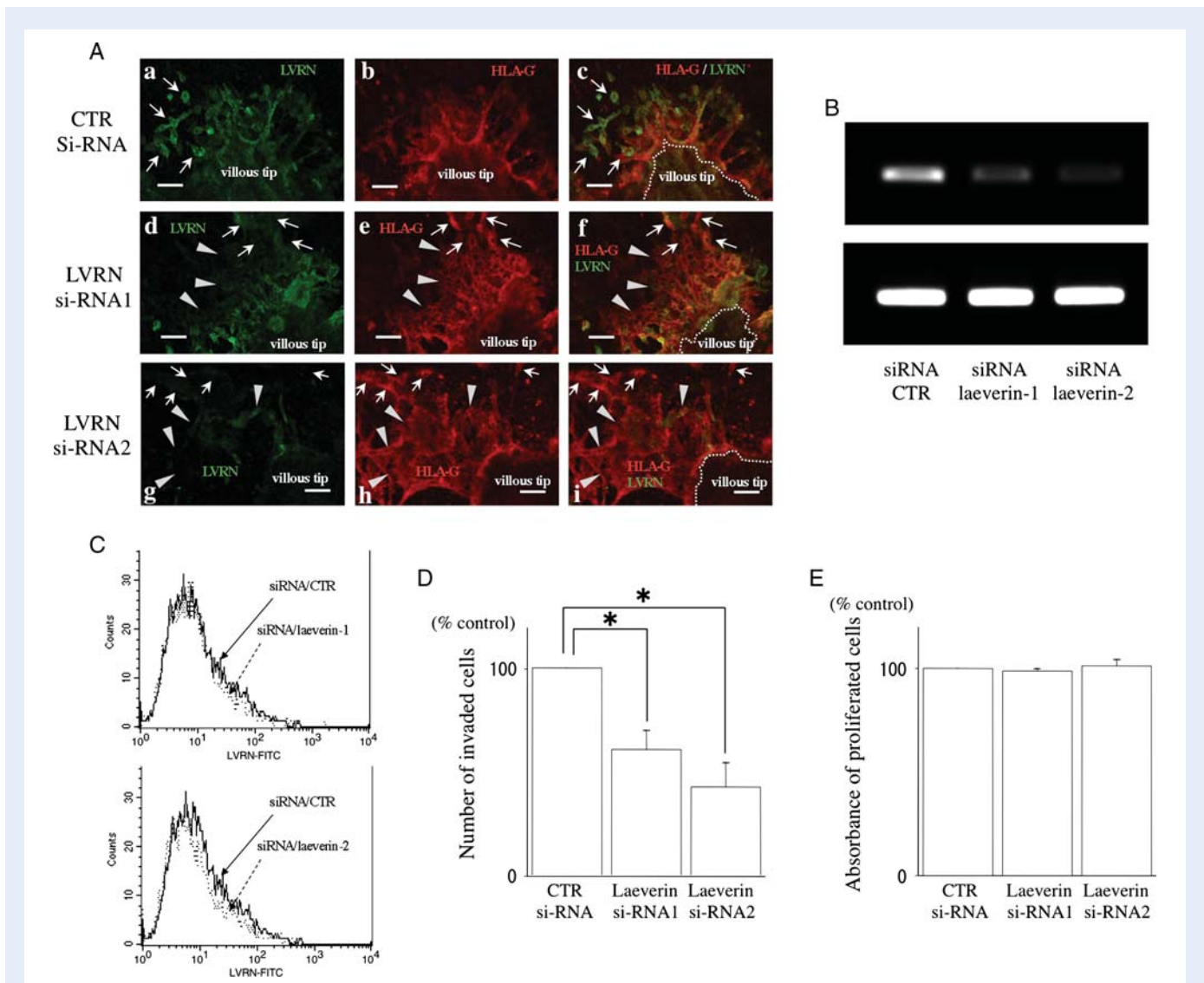


**Figure 4** Laeverin expression in primary villous explant culture and isolated EVT. (A–D) The explanted villous tissues were double stained with anti-laeverin (green) and anti-HLA-G (red) monoclonal antibodies. C is merged images of A and B. D is the magnified image of the boxed area indicated in C. EVT outgrew from the attached chorionic villous tips (white dotted lines). The outgrowing EVT were composed of two subpopulations, i.e. cell sheet (between white and gray dotted lines) and migrating EVT. HLA-G expression was constantly detected on all the outgrowing EVT including those in the cell sheet and the migrating EVT (B, C and D). By contrast, laeverin expression in the cell sheet region was patchy and heterogenous (A, C and D). On the other hand, laeverin was constantly expressed on most of the migrating EVT (C and D, arrowheads). Bars indicate 100  $\mu\text{m}$ . (E) Dot plot of double-staining flow cytometry using the isolated EVT cells stained with anti-laeverin polyclonal antibody (FITC) and anti-HLA-G monoclonal antibody (PE). Note that  $\sim 60\%$  of HLA-G-positive isolated EVT cells are laeverin negative. (F) Western blot analysis of placental tissue (38 weeks of gestation, lane 1), isolated EVT (8 weeks of gestation, lane 2) and BeWo cells (human choriocarcinoma cell line, lane 3) using anti-laeverin polyclonal antibody. Note that a 160 kDa protein band observed in the placental tissue (arrowhead) was hardly detectable in the isolated EVT. There was no specific band detected for BeWo cells, in which laeverin mRNA was undetectable by RT-PCR.

was constantly detected in the cell sheets as well as on migrating EVT cells. In contrast, laeverin expression in the cell sheets was rather patchy and heterogeneous. In agreement with this, double-staining flow cytometry using laeverin and HLA-G antibodies revealed that only  $\sim 40\%$  of the HLA-G-positive isolated EVT cells were also positive for laeverin. In this regard, although immunohistochemical study failed to detect any difference in the expression profiles between HLA-G and laeverin, it is likely that HLA-G expression is induced on EVT at earlier stage than laeverin during extravillous differentiation occurring in the cell column. Additionally, there are considerable differences in laeverin expression profiles between the explant cultures (*in vitro*) and the villous-anchoring sites (*in vivo*). This suggests that certain additional factors such as decidua-derived factor(s) at the implantation site play an important role in the induction of laeverin expression on EVT.

Initially, laeverin protein was purified from placental tissues using CHL-2 antibody that had been raised against human chorion laeve

and its partial amino acid sequence was identified from the main 160-kDa protein band. In the present study, western blot analysis of placental tissue using a polyclonal antibody raised against recombinant laeverin detected additional minor band at 130 kDa and broad protein band ranging from 200 to 270 kDa. The same immunohistochemical staining profiles observed with the monoclonal and polyclonal antibodies indicate that this polyclonal antibody specifically reacts with native laeverin. Thus, it is likely that there are several isoforms of laeverin protein *in vivo*. Based on its entire amino acid sequence, molecular mass of laeverin core protein is estimated to be 113 kDa, suggesting that native laeverin has certain molecular modifications such as glycosylation. After de-glycosylation of N-linked sugar chains, all of these three laeverin bands were converged on a single protein band at 115 kDa. This indicates that native laeverin is an N-linked glycoprotein and suggests that several glycoforms of laeverin protein are attributable to differences in the extent of N-glycosylation. Interestingly, the 160-kDa band was

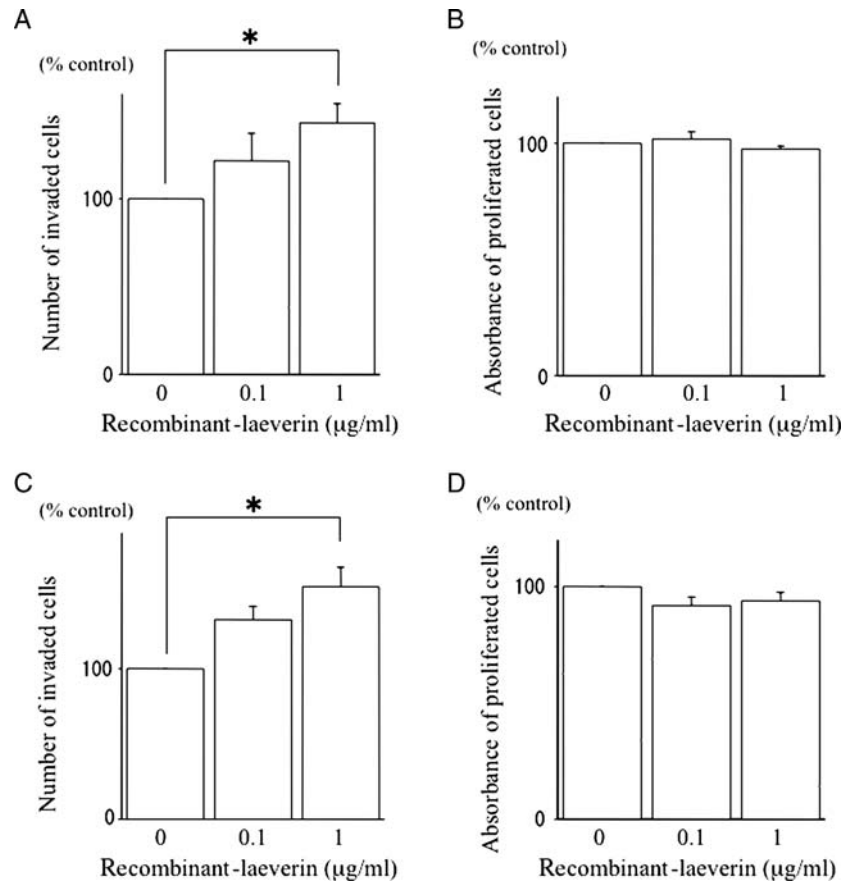


hardly detectable in the isolated EVT cells. Considering that these isolated cells only contain EVT of early differentiation stage occurring at villus-anchoring sites, EVT might acquire a 160-KDa isoform of laeверин protein in the later stage of extravillous differentiation that occurs in the maternal decidua or spiral artery.

Next, we examined the possible role of laeверин in the EVT function. In the invasion assay, silencing the endogenous laeверин expression with specific siRNAs resulted in the inhibition of invasion of the isolated EVT. Although the siRNAs sufficiently reduced laeверин

mRNA levels as shown by RT–PCR, the reduction in the cell-surface laeверин protein level evaluated by flow cytometry was less than expected, suggesting that laeверин is a stable protein whose turnover is relatively slow. Interestingly, when the siRNAs were applied to the primary villous explant culture, we observed that reduction in laeверин protein expression was greater on migrating EVT cells than in the cell sheet region. Thus, it is possible that the isolated EVT cells with migratory activity are more susceptible to siRNA than those without migratory activity. This could explain why relatively small reduction





**Figure 6** Effects of recombinant laeверin on migration of isolated EVT and BeWo cells. **(A and B)** Invasion assay (A,  $n = 4$ ) and proliferation assay (B,  $n = 3$ ) using isolated EVT. Recombinant laeверin significantly promoted EVT invasion in a dose-dependent manner (A) without affecting the cell proliferation (B). **(C and D)** Invasion assay (C,  $n = 7$ ) and proliferation assay (D,  $n = 3$ ) using BeWo cells. Recombinant laeверin also stimulated the invasion of BeWo cells (C) without affecting the cell proliferation (D). Error bars indicate SEM. \* $P < 0.05$ .

in laeверin protein expression on the isolated EVT yielded marked inhibition of their invasion. In any case, the result of this invasion assay indicates that endogenous laeверin on the surface of the isolated EVT cells acts to promote their invasion.

In the other invasion assay, addition of recombinant laeверin promotes invasion of laeверin-positive isolated EVT cells as well as laeверin-negative BeWo cells, suggesting that enzymatic activity of laeверin is important for its migration-promoting effect. Laeверin can degrade several placenta-derived peptide hormones, such as angiotensin III, endokinin C and kisspeptin-10 (Maruyama *et al.*, 2007). Among these, kisspeptin-10 has been demonstrated to suppress trophoblast migration (Bilban *et al.*, 2004). Since kisspeptin-10 is detectable in serum (Armstrong *et al.*, 2009), significant amount of this peptide should be present in our invasion assay system that contains 10% FCS. It is also possible that Matrigel contains certain motility-suppressive peptides such as kisspeptin-10. Thus, it is likely that recombinant as well as endogenous laeверin promotes invasion of the isolated EVT and BeWo cells by degrading cell motility-suppressive peptides such as kisspeptin-10 *in vitro*. At villous-anchoring site, laeверin expression is induced from the distal site of the cell column

where EVT acquires invasive capacity. These findings suggest that laeверin inactivates motility-suppressive peptides derived from the maternal decidua to initiate EVT migration from the distal end of the cell column.

Western blotting and immunohistochemistry of the placenta of various gestational stages indicate that EVT cells in the term placenta express higher level of laeверin than those in the early placenta. Considering that invasive capacity of EVT cells is lower in the term as compared with the early pregnancy, higher laeверin expression at term is not consistent with the migration-stimulatory effect of laeверin, suggesting the presence of additional functions of laeверin.

In conclusion, the present study showed that laeверin, which has several isotypes sharing a common 115-kDa core protein with various extent of N-glycosylation, is a specific cell-surface marker of human EVT throughout pregnancy. *In vitro* functional analysis using primary EVT cells suggests that cell-surface laeверin inactivates motility-suppressive peptides to induce EVT migration. Further investigation of laeверin will contribute to clarifying the mechanism(s) that regulates differentiation and function of human EVT.

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## Authors' roles

A.H., Y.S., K.S., M.M., H.M. and A.H. performed experiments. H.F. designed this study and wrote paper. I.K. discussed the results.

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## Conflict of interest

None declared.

## References

- Aplin JD. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*. *J Cell Sci* 1991; **99**(Pt 4):681–692.
- Armstrong RA, Reynolds RM, Leask R, Shearing CH, Calder AA, Riley SC. Decreased serum levels of kisspeptin in early pregnancy are associated with intra-uterine growth restriction and pre-eclampsia. *Prenat Diagn* 2009; **29**:982–985.
- Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W et al. Kisspeptin-10, a KiSS-1/metastatin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *J Cell Sci* 2004; **117**:1319–1328.
- Bischof P, Martelli M, Campana A, Itoh Y, Ogata Y, Nagase H. Importance of matrix metalloproteinases in human trophoblast invasion. *Early Pregnancy* 1995; **1**:263–269.
- Cho SG, Li D, Stafford LJ, Luo J, Rodriguez-Villanueva M, Wang Y, Liu M. KiSS1 suppresses TNF $\alpha$ -induced breast cancer cell invasion via an inhibition of RhoA-mediated NF- $\kappa$ B activation. *J Cell Biochem* 2009; **107**:1139–1149.
- Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ. Integrin switching regulates normal trophoblast invasion. *Development* 1994; **120**:3657–3666.
- Fujiwara H. Membrane-bound peptidases regulate human extravillous trophoblast invasion. *Placenta* 2007; **28**(Suppl A):S70–S75.
- Fujiwara H, Imai K, Inoue T, Maeda M, Fujii S. Membrane-bound cell surface peptidases in reproductive organs. *Endocr J* 1999; **46**:11–25.
- Fujiwara H, Higuchi T, Yamada S, Hirano T, Sato Y, Nishioka Y, Yoshioka S, Tatsumi K, Ueda M, Maeda M et al. Human extravillous trophoblasts express laeverin, a novel protein that belongs to membrane-bound gluzincin metallopeptidases. *Biochem Biophys Res Commun* 2004; **313**:962–968.
- Lala PK, Chakraborty C. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. *Placenta* 2003; **24**:575–587.
- Maruyama M, Hattori A, Goto Y, Ueda M, Maeda M, Fujiwara H, Tsujimoto M. Laeverin/aminopeptidase Q, a novel bestatin-sensitive leucine aminopeptidase belonging to the M1 family of aminopeptidases. *J Biol Chem* 2007; **282**:20088–20096.
- Maruyama M, Arisaka N, Goto Y, Ohsawa Y, Inoue H, Fujiwara H, Hattori A, Tsujimoto M. Histidine 379 of human laeverin/aminopeptidase Q, a nonconserved residue within the exopeptidase motif, defines its distinctive enzymatic properties. *J Biol Chem* 2009; **284**:34692–34702.
- McMaster MT, Librach CL, Zhou Y, Lim KH, Janatpour MJ, DeMars R, Kovats S, Damsky C, Fisher SJ. Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. *J Immunol* 1995; **154**:3771–3778.
- Menier C, Saez B, Horejsi V, Martinuzzi S, Krawiec-Radanne I, Bruel S, Le Danff C, Reboul M, Hilgert I, Rabreau M et al. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum Immunol* 2003; **64**:315–326.
- Navenot JM, Wang Z, Chopin M, Fujii N, Peiper SC. Kisspeptin-10-induced signaling of GPR54 negatively regulates chemotactic responses mediated by CXCR4: a potential mechanism for the metastasis suppressor activity of kisspeptins. *Cancer Res* 2005; **65**:10450–10456.
- Nishioka Y, Higuchi T, Sato Y, Yoshioka S, Tatsumi K, Fujiwara H, Fujii S. Human migrating extravillous trophoblasts express a cell surface peptidase, carboxypeptidase-M. *Mol Hum Reprod* 2003; **9**:799–806.
- Pattillo RA, Gey GO, Delfs E, Mattingly RF. Human hormone production *in vitro*. *Science* 1968; **159**:1467–1469.
- Pijnenborg R, Bland JM, Robertson WB, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta* 1983; **4**:397–413.
- Sato Y, Fujiwara H, Higuchi T, Yoshioka S, Tatsumi K, Maeda M, Fujii S. Involvement of dipeptidyl peptidase IV in extravillous trophoblast invasion and differentiation. *J Clin Endocrinol Metab* 2002; **87**:4287–4296.
- Sato Y, Higuchi T, Yoshioka S, Tatsumi K, Fujiwara H, Fujii S. Trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype. *Development* 2003; **130**:5519–5532.
- Sato Y, Fujiwara H, Zeng BX, Higuchi T, Yoshioka S, Fujii S. Platelet-derived soluble factors induce human extravillous trophoblast migration and differentiation: platelets are a possible regulator of trophoblast infiltration into maternal spiral arteries. *Blood* 2005; **106**:428–435.
- Tsujimura K, Park YH, Miyama-Inaba M, Meguro T, Ohno T, Ueda M, Masuda T. Comparative studies on FcR (FcRII, FcRIII, and FcR  $\alpha$ ) functions of murine B cells. *J Immunol* 1990; **144**:4571–4578.
- Vincent S, Marty L, Fort P. S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eucaryotic cells and tissues. *Nucleic Acids Res* 1993; **21**:1498.