Up-Regulation of α_5-Integrin by E-Cadherin Loss in Hypoxia and Its Key Role in the Migration of Extravillous Trophoblast Cells during Early Implantation

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During early pregnancy, cytotrophoblast cells differentiate into extravillous trophoblast (EVT) cells and invade the uterine spiral arteries. This physiological process is essential for the development of maternal-fetal circulation. Because EVT cells are exposed to a low-oxygen environment during this process, we investigated the role of hypoxia in the mechanism that regulates the invasive behavior of EVT cells. Real-time PCR and immunofluorescent analysis were performed to investigate how hypoxia influences the expression of E-cadherin in villous explants cultures and in trophoblast-derived BeWo cells. We determined that hypoxia induced E-cadherin down-regulation through Snail up-regulation in villous explant cultures. The influence of E-cadherin loss was examined by analyzing the expression of α_5-integrin and phosphorylated focal adhesion kinase (FAK) by Western blot and evaluating trophoblast invasion using a matrigel invasion assay. E-cadherin loss induced the up-regulation of α_5-integrin, which leads to the tyrosine phosphorylation of FAK, resulting in an increase in the invasive activity of EVT cells. An α_5-integrin neutralizing antibody inhibited the invasion of EVT cells by attenuating FAK tyrosine phosphorylation. Immunohistochemical analysis using clinical placental bed biopsies revealed that α_5-integrin was up-regulated and FAK tyrosine phosphorylated (Try^{861}) as EVT cells invade the uterine myometrium, whereas E-cadherin expression was down-regulated. These results suggest that α_5-integrin up-regulation induced by E-cadherin loss under hypoxia has a crucial role in regulating the migration of EVT cells. This finding should help us reach a better understanding of the pathogenesis of critical gestational diseases, such as preeclampsia. **(*Endocrinology* 150: 4306–4315, 2009)**

During early pregnancy, cytotrophoblast cells differentiate to extravillous trophoblast (EVT) cells, which migrate along the lumina of uterine spiral arterioles, replacing the maternal endothelial lining as far as the inner third of the myometrium and changing the vascular smooth muscle, which is responsible for maintaining vessel integrity (1–3). In this stage, EVT cells are exposed to a low-oxygen environment (4). Recent reports indicated that low-oxygen tension influences cell adhesion and migration in a number of biological contexts (5, 6), including trophoblast invasion (7).

The transcriptional response to hypoxia is mediated by the transcription factor hypoxia-inducible factor (HIF),

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Abbreviations: ECM, Extracellular matrix; EVT, extravillous trophoblast; FAK, focal adhesion kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; siRNA, small interfering RNA.

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which activates transcription by binding to hypoxic response elements scattered throughout the genome (8). Caniggia *et al.* (7) reported that HIF-1α is elevated during early pregnancy and appears to be crucial for mediating the biological effects of oxygen on early trophoblast differentiation. However, little is known about the mechanisms that govern how HIF-1α regulates the migration and invasion of EVT cells.

Snail, a zinc-finger transcription factor, is considered one of the key genes regulated by HIF-1α (9). Snail represses the transcription of E-cadherin, which helps cell-cell interactions, by binding to E-box elements found in the E-cadherin promoter region (10). E-cadherin loss has been found to promote the invasion of various cancer cells through increased levels of Snail under hypoxia (11). In normal placentas, E-cadherin was shown to be highly expressed in cytotrophoblast stem cells of anchoring villi and was believed to be down-regulated or absent in invading EVT cells (12). It was also shown that E-cadherin expression was up-regulated in interstitial and vascular EVT cells in placental bed biopsies from patients with preeclampsia (13). Therefore, it has been suggested that E-cadherin loss plays a key role in the invasive behavior of EVT cells that occurs in normal placental development. However, the molecular consequences of E-cadherin loss remain obscure.

During the transition of EVT cells to an invasive state, there is considerable trophoblast cell-extracellular matrix (ECM) remodeling marked by specific integrin switching (from the expression of $\alpha_6\beta_4$ to $\alpha_5\beta_1$ and $\alpha_1\beta_1$) (12–15). In preeclampsia, in which both interstitial and endovascular invasion are abnormally shallow, EVT cells show significant defects in differentiation (12, 13, 15, 16). Therefore, it is assumed that the expression and conversion of integrin subunits is involved in the regulation of normal EVT differentiation. Recently we identified α_5-integrin up-regulation as a molecular mechanism by which E-cadherin loss promotes tumor progression in ovarian cancer (17). Evidence from these studies suggests that loss of E-cadherin induces cell-ECM adhesion through α_5-integrin up-regulation and deposition of a fibronectin-rich ECM because α_5-integrin is a cell surface receptor for fibronectin (18). Therefore, we hypothesized that E-cadherin loss, induced by low-oxygen tension, regulates trophoblast-ECM remodeling via α_5-integrin up-regulation.

Remodeling of cell-ECM contacts via integrins generates a cascade of phosphorylation of signal transduction molecules, including focal adhesion kinase (FAK) (19). FAK is located at focal adhesions, tyrosine phosphorylated in response to cell adhesion to ECM, and is known to be critical in integrin-mediated signal transduction pathways (20). FAK plays a key role in regulating gene expression and cell behavior, such as cell spreading and migration (21, 22). MacPhee *et al.* (23) recently reported that FAK was highly expressed and activated during early placental development and was also regulated by low-oxygen tension. Therefore, we decided to analyze the involvement of FAK activation in the invasion of EVT cells under hypoxia.

We show here that hypoxia induces E-cadherin down-regulation through Snail up-regulation in villous explant cultures as well as trophoblast-derived BeWo cells. E-cadherin loss induced the up-regulation of α_5-integrin, which led to the tyrosine phosphorylation of FAK, resulting in increased invasive activity. Immunohistochemical analysis using placental bed biopsies revealed that α_5-integrin was up-regulated and FAK tyrosine phosphorylated as EVT cells invaded the uterine myometrium, whereas E-cadherin expression was down-regulated. These results support the hypothesis that E-cadherin loss induced by low-oxygen tension promotes the invasive behavior of EVT cells and may also play an important role in early placental development.

Materials and Methods

Materials

Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). Anti-Snail (RB1400) monoclonal antibody was purchased by ABGENT (San Diego, CA). Anti-E-cadherin (36) monoclonal antibody and Matrigel were purchased from BD Biosciences (Bedford, MA). Antibodies against FAK (polyclonal) and phospho-FAK (pTyr^{861}) (polyclonal) were purchased from Sigma Aldrich Corp. (St. Louis, MO). Anti-α_5-integrin antibody (polyclonal) was from Santa Cruz Biotechnology (Santa Cruz, CA) and anticytokeratin-7 antibody (OV-TL 12/30) was from Dako (Glostrup, Denmark).

Isolation and purification of primary trophoblast cells

Human placental tissues were collected from normal pregnancies that were voluntarily terminated by dilation and curettage between 8 and 9 wk of gestation. Written informed consent was obtained from each patient before obtaining the placental explants. Placental tissues were collected in ice-cold PBS, transported to the laboratory on ice, and processed immediately. Primary trophoblast cells were prepared by the method described previously (24). Briefly, placental tissues were rinsed thoroughly in cold PBS, dissected from the membranes and cut into small pieces (1–2 mm). The tissue fragments were washed three times with Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. These fragments were then cultured in Medium 199 containing 20% FBS. The culture media were replaced every 24 h for 7–14 d until nonadherent cells could be removed. To ensure that the cultured cells possessed the phenotype of trophoblast cells, adherent cells were characterized by immunofluorescence analysis by confirming that more than 90% of cells were cytokeratin-7 positive as described previously (25).

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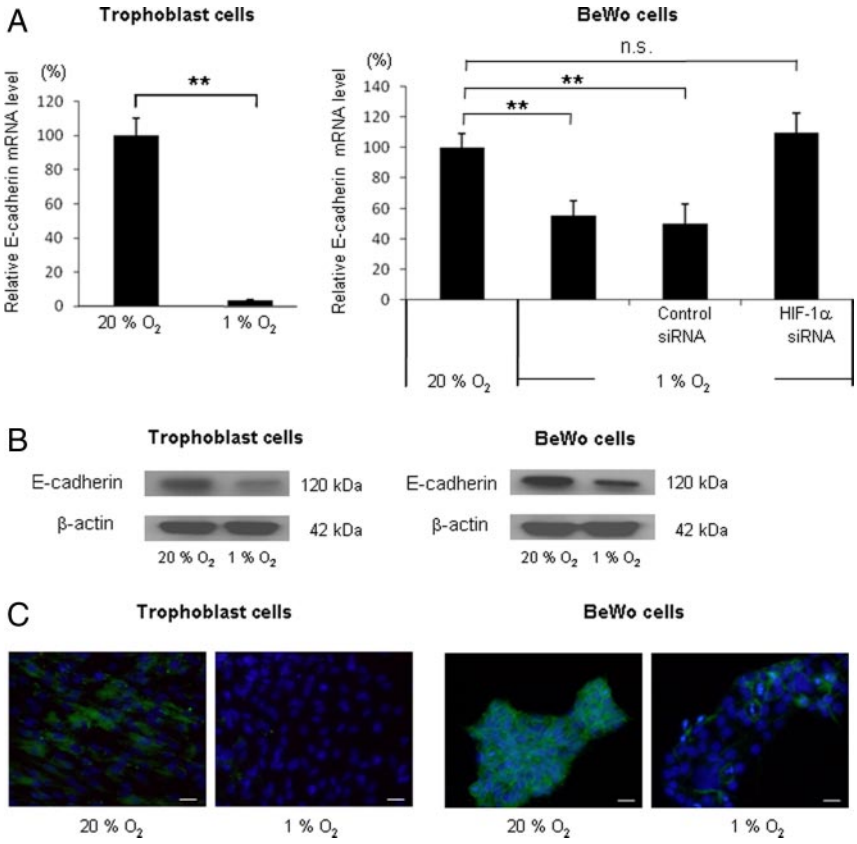

FIG. 1. Hypoxia induced the down-regulation of E-cadherin in primary cultured trophoblast cells and trophoblast-derived BeWo cells. A, *Left panel,* Trophoblast cells isolated from human first-trimester placental tissue were cultured under 20% O_2 or 1% O_2. After RNA extraction, the relative expression of E-cadherin mRNA normalized to GAPDH was measured using TaqMan quantitative real-time RT-PCR. The expressional level in normoxia (20% O_2) was set at 100. A, *Right panel*, HIF-1α siRNA or control siRNA was transfected into BeWo cells cultured under 1% O_2. Thirty hours after the transfection, the relative expression of E-cadherin mRNA was measured. *Columns* show average value for three independent experiments. *Bars*, SD. **, Significant differences are indicated by $P < 0.01$. B and C, Effect of hypoxia on E-cadherin protein expression in primary cultured trophoblast cells and BeWo cells. B, Western blot analysis. Cells were cultured under 20% O_2 or 1% O_2 and the lysates were immunoblotted with an E-cadherin antibody and reprobed with β-actin as internal loading control. Blots were representative of three experiments. C, Immunohistochemistry. Cells were stained with an E-cadherin antibody followed by Alexa-488 (*green*) secondary antibody and nuclei stained with Hoechst 33342 (*blue*). One percent of O_2 exposure drastically reduced E-cadherin expression. *Bar*, 100 μm. n.s., Not significant.

Cell culture

The human choriocarcinoma cell line BeWo was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in Ham's F12 medium (Sigma) containing 15% FBS. Oxygen tension in the medium was routinely measured by a blood gas analyzer, and the low oxygen condition in our system (1% O_2 incubator: 20–23 mm Hg) is thought to be approximately close to the physiological oxygen tension during early pregnancy (26).

In vitro immunofluorescence study

Primary trophoblast cells and BeWo cells were plated on eight-well chamber slides. For analysis of E-cadherin expression, the cells were cultured under the indicated conditions (20% O_2 or 1% O_2) for 72 h, respectively. After incubation, they were fixed with 3.7% paraformaldehyde in PBS for 15 min and permeabilized with ice-cold 100% methanol for 10 min at −20 C followed by staining with an anti-E-cadherin antibody (36) for 18 h at 4 C. After washing, samples were incubated with Alexa Fluor 488-labeled goat antimouse IgG (Molecular Probes, Eugene, OR) and finally stained with Hoechst 33342. Images were analyzed on a TE2000-U microscope (Nikon Corp., Tokyo, Japan) with an IEEE 1394 digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and Lumina Vision software (Mitani Corp., Fukui, Japan).

Western blot analysis

Cells (5×10^5) were plated onto six-well plates and lysed with radioimmunoprecipitation assay buffer [150 mmol/liter NaCl, 50 mmol/liter Tris-HCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride, 1 mmol/liter NaOVO4, protease inhibitor cocktail (Sigma)]. Fifteen micrograms of cell lysate were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane followed by incubating with the primary antibodies indicated in Figs. 1B, 2B, 3B, and 4 and then with a corresponding secondary horseradish peroxidase-conjugated IgG. The proteins were visualized with an enhanced chemiluminescence system (PerkinElmer Life Science, Boston, MA).

Real-time RT-PCR

Real-time quantitative RT-PCR was performed using the Prism7500 TaqMan PCR detector with the following probes from Applied Biosystems (Foster City, CA): Snail (Hs00195591), CDH1 (Hs00170423), α_5-integrin (Hs01547690), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs00266705). Total RNA was extracted using TRIzol and was transcribed into cDNA using a high-capacity cDNA kit (Applied Biosystems). Relative levels of mRNA gene expression were calculated using the $2^{-\Delta\Delta CT}$ method as described (27).

Transient transfection

The expression vector encoding HIF-1α (pcDNA3.1-HIF1α) was a generous gift from Dr. L. Eric Huang (Harvard Medical School, Boston, MA) (25). BeWo cells grown in 60-mm^2 dishes were transfected with 2 μg of a control vector (pcDNA3.1) or pcDNA3.1-HIF1αusing FuGENE 6 (Roche, Nutley, NJ) according to the manufacture's instructions.

Knockdown using small interfering RNA (siRNA)

BeWo cells were grown in six-well plates at 40–50% confluency and transfected with HIF-1α, Snail, or E-cadherin siRNA (ID 199319, ID115742, ID146381, respectively; Ambion, Austin, TX) using X-tream gene siRNA transfection reagent (Roche) according to the manufacturer's instruction. A siRNA concentration of 100 nM was determined to be optimal. Controls included a GAPDH

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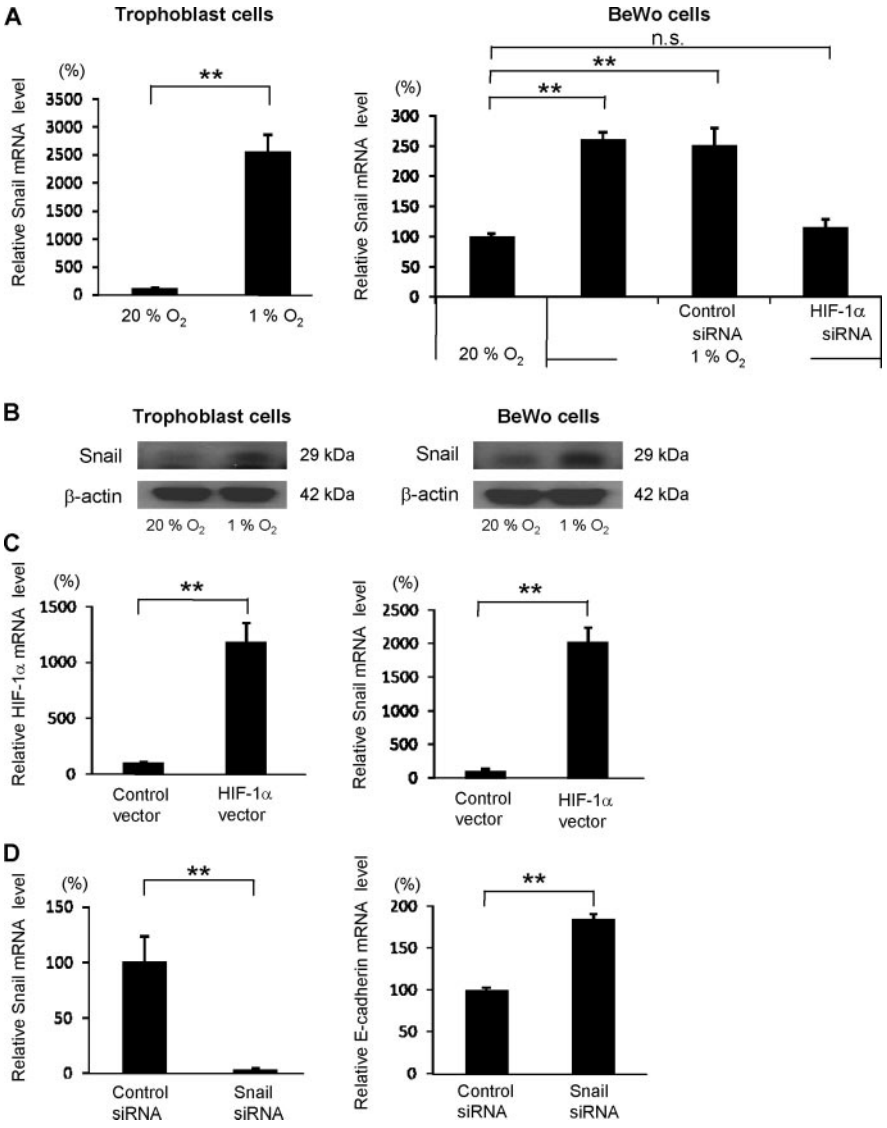

FIG. 2. Hypoxia induced Snail up-regulation via HIF-1α. A, Real-time quantitative RT-PCR analysis. The effect of hypoxia on the expression of Snail mRNA is seen in primary cultured trophoblast cells (*left panel*) and trophoblast-derived BeWo cells (*right panel*). After cells were cultured under 20% O_2 or 1% O_2 and RNA proceeded, the relative expression of Snail mRNA was measured. HIF-1α siRNA or control siRNA was transfected into BeWo cells cultured under 1% O_2. Thirty hours after the transfection, RNA was isolated and the relative expression of Snail mRNA was measured. B, Western blot analysis. Cells were cultured under 20% O_2 or 1% O_2 and the lysates were immunoblotted with a Snail antibody and reprobed with β-actin as internal loading control. Blots were representative of three experiments. C, The effects of HIF-1α overexpression on Snail expression in BeWo cells. BeWo cells were treated with the expression vector encoding HIF-1α or control vector. Thirty hours after the transfection, HIF-1α overexpression was confirmed (*left panel*) and the relative expression of Snail (*right panel*) mRNA was measured. D, The effects of Snail mRNA knockdown on E-cadherin expression in BeWo cells. BeWo cells were treated with Snail siRNA or control siRNA. Thirty hours after the transfection, Snail knockdown was confirmed (*left panel*) and the relative expression of E-cadherin (*right panel*) mRNA was measured. *Columns* show average value for three independent experiments. *Bars*, SD. **, Significant difference are indicated by $P < 0.01$. n.s., Not significant.

siRNA to confirm transfection efficiency and a scrambled siRNA sequence as negative control. Forty-eight hours after the transfection, the cells were proceeded.

Matrigel invasion assay

In vitro cellular invasion was assayed by determining the ability of cells to invade through a synthetic basement membrane. Briefly, polycarbonate filters (8 μm pore size) coated with 20 μg Matrigel (BD Biosciences) were placed in a modified Boyden chamber. BeWo cells (1×10^5/well) were plated to the top chamber in serum-free medium and incubated with 30% FBS containing medium as a chemoattractant in the bottom chamber. After the cells were allowed to invade through the Matrigel barrier for 18 h, the filter was fixed and stained with Gentian violet. Noninvading cells were removed using a cotton swab and invading cells on the underside of the filter were enumerated using an inverted microscope.

Immunohistochemistry

Placental bed biopsy tissues were obtained from the site of implantation immediately after cesarean delivery at Osaka Medical Center and Research Institute for Maternal and Child Health (Osaka, Japan). Six placental bed samples were obtained from pregnant women at 27–36 wk of gestation at the time of cesarean section. Informed consent was obtained from each patient before obtaining the placental bed biopsy. The tissues were formalin fixed, paraffin embedded, and cut into 5-μm slices. The slides were deparaffinized in xylene and dehydrated with 100% ethanol before antigen unmasking was performed by boiling the slides in target retrieval solution (pH 9.0) (Dako). After being placed in 3% H_2O_2 and blocked with blocking solution (Dako), the slides were incubated with the primary cytokeratin-7 antibody (OV-TL 12/30) at 1:200, E-cadherin antibody (36) at 1:200, α_5-integrin antibody (polyclonal) at 1:200, and phospho-FAK ($pTyr^{861}$) (polyclonal) at 1:200 for 18 h at 4 C. After washing with PBS, the slides were stained using the Envision system (Dako) and then counterstained with Mayer's hematoxylin. Slides were examined by the qualified pathologist (M.N.) to confirm the presence of EVT cells indicative of true placental bed. Decidua and myometrium were analyzed separately for interstitial trophoblast distribution, the presence of spiral arteries, and glandular structures.

Statistical analysis

Data are expressed as the mean ± SD. Statistical analysis was performed using the Student's *t* test or one-way ANOVA for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

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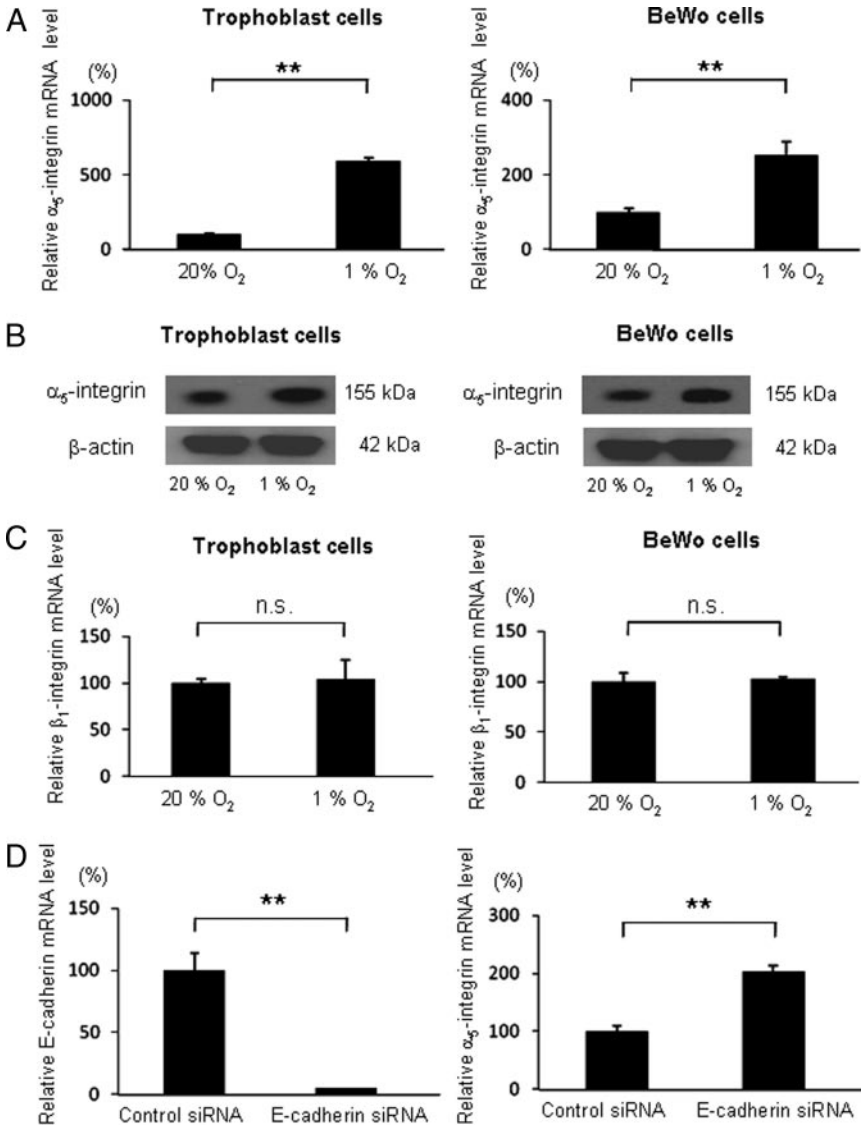

FIG. 3. Hypoxia induced α_5-integrin up-regulation in primary cultured trophoblast cells and trophoblast-derived BeWo cells. A, Trophoblast cells isolated from human first-trimester placental tissue were cultured under 20% O_2 or 1% O_2 (*left panel*). RNA was extracted and the relative expression of α_5-integrin mRNA was measured. The expressional level in normoxia (20% O_2) was set at 100. Parallel experiments and quantitative analyses were performed using BeWo cells (*right panel*). B, Western blot analysis. Cells were cultured under 20% O_2 or 1% O_2 and the lysates were immunoblotted with an α_5-integrin antibody and reprobed with β-actin as internal loading control. Blots were representative of three experiments. C, Hypoxia did not induce β_1-integrin up-regulation in primary cultured trophoblast cells (*left panel*) and trophoblast-derived BeWo cells (*right panel*). D, E-cadherin mRNA knockdown using siRNA transfection (*left panel*) led to the up-regulation of α_5-integrin mRNA expression (*right panel*). BeWo cells were transfected with E-cadherin siRNA or with control siRNA. Forty-eight hours after the transfection, E-cadherin knockdown was confirmed (*left panel*), and the relative expression of α_5-integrin (*right panel*) mRNA was measured. The expressional level when control siRNA transfected was set at 100. *Columns* show average value for three independent experiments. *Bars*, SD. **, Significant difference are indicated by $P < 0.01$.

Results

Hypoxia induces E-cadherin down-regulation in trophoblast cells

Oxygen tension has been shown to be a crucial regulator when cytotrophoblast cells differentiate into EVT cells. Therefore, to determine the effect of low-oxygen tension (1% O_2) on E-cadherin expression in trophoblast cells, we first examined E-cadherin expression using villous explant cultures (Fig. 1A, *left panel*) and trophoblast-derived BeWo cells (Fig. 1A, *right panel*). Exposure of cells to 1% O_2 for 8 h resulted in a significant reduction of E-cadherin mRNA (by 96% in villous explant cultures and 44% in BeWo cells, respectively). Western blot analysis (Fig. 1B) and immunofluorescent analysis (Fig. 1C) with anti-E-cadherin antibody revealed that E-cadherin was inhibited in cells cultured in a 1% O_2 environment and strongly expressed in a 20% O_2 environment.

Hypoxia induced Snail up-regulation via HIF-1α, which led to E-cadherin down-regulation

Because Snail is known to be the most prominent repressor of E-cadherin (28), we determined the effect of low-oxygen tension on the Snail mRNA level to clarify the mechanism by which E-cadherin expression was down-regulated under hypoxia. Snail mRNA was significantly up-regulated (25-fold) under 1% O_2 compared with under 20% O_2 in villous explant cultures (Fig. 2A, *left panel*), and a 2.5-fold up-regulation was shown in BeWo cells (Fig. 2A, *right panel*). Snail up-regulation was also confirmed by Western blotting (Fig. 2B).

Snail is also known to be regulated by HIF-1α (27). Therefore, we examined the involvement of HIF-1α in Snail up-regulation under low oxygen tension. The knockdown of HIF-1α with siRNA prevented not only the down-regulation of E-cadherin but also the up-regulation of Snail under 1% O_2 (Figs. 1A and 2A, *right panel*). On the other hand, the overexpression of HIF-1α led to 20-fold up-regulation of Snail mRNA, suggesting that hypoxia induced Snail up-regulation through HIF-1α, one of the most prominent HIFs (Fig. 2C). In contrast, Snail knockdown using siRNA (97% reduction, Fig. 2D, *left panel*) up-regulated E-cadherin expression (Fig. 2D, *right panel*). These findings suggest that in a low-oxygen environ-

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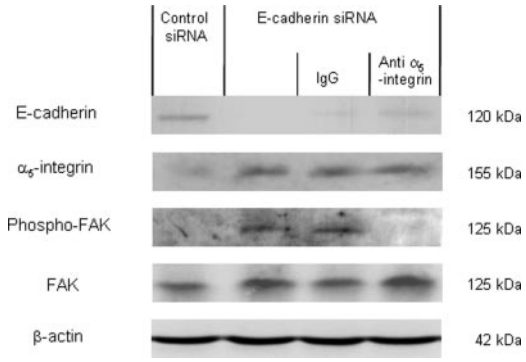

FIG. 4. Western blot analysis. BeWo cells were transfected with the E-cadherin siRNA or control siRNA. Cell lysates were resolved by SDS-PAGE and immunoblotted with the antibodies indicated in the figure. E-cadherin knockdown led to the up-regulation of α_5-integrin and the tyrosine phosphorylation of FAK (Tyr^{861}), whereas the total levels of FAK were unchanged. An α_5-integrin neutralizing antibody (10 μg/ml) attenuated FAK phosphorylation. The membrane was stripped and reprobed with β-actin as internal loading control. Blots were representative of three experiments.

ment, trophoblast cells lose E-cadherin expression via Snail up-regulation mediated by HIF-1α.

Hypoxia increases α_5-integrin expression through E-cadherin down-regulation

Current studies emphasized the importance of the modulation of integrin signaling by E-cadherin. We recently identified α_5-integrin up-regulation as a molecular mechanism by which E-cadherin loss promotes tumor progression, thereby providing an explanation for the association of E-cadherin loss with an increase in metastasis in ovarian cancer (17). α_5-Integrin (VLA-5) exists as a heterodimer with β_1-integrin and is the major fibronectin binding integrin. Caniggia *et al.* reported that α_5-integrin expression is a marker associated with the trophoblast intermediate phenotype and is elevated in a low-oxygen environment (7). Therefore, we investigated whether E-cadherin loss under hypoxia affects α_5-integrin expression in trophoblast cells. α_5-Integrin mRNA was significantly up-regulated by 5.9-fold under 1% O_2 compared with under 20% O_2 in villous explant cultures (Fig. 3A, *left panel*), and 2.5-fold up-regulation was shown in BeWo cells (Fig. 3A, *right panel*). α_5-Integrin up-regulation was also confirmed by Western blotting (Fig. 3B).

On the other hand, the expression of β_1-integrin was not altered under hypoxia (Fig. 3C), and α_1-integrin expression was not detected at the mRNA level (data not shown). To determine the involvement of E-cadherin in α_5-integrin expression, E-cadherin expression was transiently knocked down by 95% using siRNA (Fig. 4C, *left panel*). E-cadherin siRNA induced the 2.3-fold induction of α_5-integrin compared with scrambled siRNA (Fig. 4C, *right panel*, $P < 0.01$). These data suggested that a low-oxygen tension reduced E-cadherin expression, which led to the up-regulation of α_5-integrin in trophoblast cells.

E-cadherin loss regulates FAK signaling

Cell-matrix adhesion requires not only integrins but also downstream molecules, such as FAK, which is a key signal transducer of outside-inside signaling (23, 29). Because α_5-integrin is known to activate several signal cascades, including MAPK and phosphatidylinositol 3-kinase through FAK phosphorylation, which also may promote the invasion of EVT cells (30), we analyzed the effect of E-cadherin down-regulation on the tyrosine phosphorylation of FAK. Transfection with E-cadherin siRNA (transfection efficiency 80%) not only up-regulated α_5-integrin protein as well as mRNA, shown in Fig. 2, but also induced the phosphorylation of FAK (Tyr^{861}), whereas the total level of FAK was unchanged. On the other hand, an α_5-integrin-neutralizing antibody attenuated FAK phosphorylation (Fig. 4). These data suggested that the loss of E-cadherin regulates FAK signaling through α_5-integrin up-regulation in trophoblast cells.

Loss of E-cadherin promotes the invasion of EVT cells via α_5-integrin up-regulation

Because HIF-deficient placentas exhibit poor maternal invasion (31), we determined whether hypoxia regulates trophoblast invasion through integrin-mediated adhesion to the ECM using a modified Boyden chamber assay. In a low-oxygen environment (1% O_2), BeWo cells displayed significantly increased invasion (3.3-fold) into Matrigel compared with that found in normoxia ($P < 0.01$) (Fig. 5A). To clarify how hypoxia induces invasion, Snail mRNA was down-regulated using siRNA, resulting in the decreased invasion of BeWo cells by 73% ($P < 0.01$ *vs.* scrambled siRNA) (Fig. 5B). However, the E-cadherin-neutralizing antibody restored the invasion of BeWo cells transfected with Snail siRNA (Fig. 5B), approximately to the level of the scrambled siRNA. These data suggest that Snail down-regulation suppressed the invasion of trophoblast cells via E-cadherin up-regulation. In contrast, E-cadherin down-regulation led to the 2.5-fold increased invasion of BeWo cells ($P < 0.01$ *vs.* scrambled RNA) and α_5-integrin-neutralizing antibody attenuated the invasion induced by E-cadherin siRNA (Fig. 5C), indicating that E-cadherin loss promoted the invasion of trophoblast cells via α_5-integrin up-regulation. Taken together, these data suggest that hypoxia induces Snail up-regulation, which leads to E-cadherin inhibition in trophoblast cells. In a low-oxygen environment, Snail up-regulation by HIF-1α reduced E-cadherin expression, resulting in increased adhesion and invasion of trophoblast cells via increased α_5-integrin cell surface expression.

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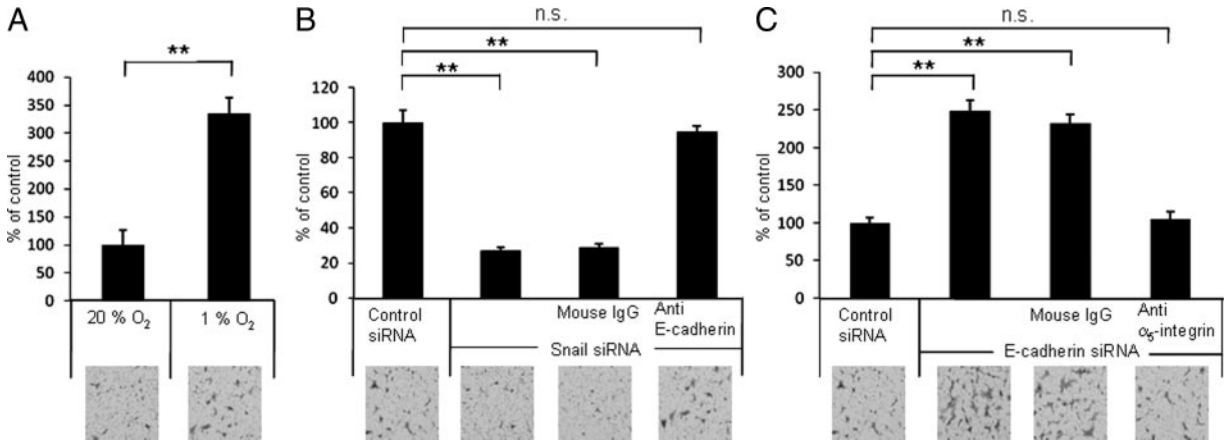

FIG. 5. Matrigel invasion assay. A, BeWo cells were plated on a modified Boyden chamber coated with Matrigel and incubated under 20% O_2 or 1% O_2 for 18 h to permit the penetration of the filters. B, Invasion analysis of BeWo cells after Snail knockdown. Equal numbers (1×10^5) of Snail down-regulated BeWo cells were plated on the top chamber with serum-free media in the presence of 10 μg/ml of blocking antibodies against E-cadherin or control IgG and allowed to invade through the Matrigel barrier. C, Invasion analysis of BeWo cells after E-cadherin knockdown. E-cadherin down-regulated BeWo cells were plated on the top chamber in the presence of 10 μg/ml of blocking antibodies against α_5-integrin or control IgG and allowed to invade. Invading cells on the underside of the filter were enumerated using an inverted microscope after cells were removed from the top aspect of the filter. Experiments were performed in triplicate with a minimum of 10 grids per filter counted. Representative photographs are shown at low magnification (×40). **, Significant difference are indicated by $P < 0.01$. n.s., Not significant.

Expression of E-cadherin, α_5-integrin, and activated FAK in EVT cells from placental bed biopsies

The data thus far indicate that α_5-integrin is up-regulated on E-cadherin loss and that it induces adhesion and invasion *in vitro*. In view of these encouraging *in vitro* experiments, we sought to determine the expressional level of E-cadherin, α_5-integrin, and phospho-FAK (Try^{861}) using immunohistochemistry of placental bed biopsies. Samples were obtained from six women undergoing a cesarean section who had no clinical or biochemical evidence of preeclampsia at 27–36 wk of gestation. All samples were stained with an anticytokeratin-7 antibody to detect cytotrophoblast cells (Fig. 6, B and G). In the uterine decidua, cytotrophoblast cells showed a strong expression of E-cadherin, whereas α_5-integrin and FAK were not expressed (Fig. 6 and supplemental Fig. S1). On the other hand, in the uterine myometrium, in which EVT cells invade deeply, trophoblast cells show a reduced expression of E-cadherin, whereas α_5-integrin is strongly expressed and FAK activated (Fig. 6 and supplemental Fig. S1). In contrast, in placental beds from the patients with preeclampsia, cytotrophoblast cells (cytokeratin-7 positive) were rarely seen in uterine myometrium but were highly present in uterine deciduas and showed a strong expression of E-cadherin (supplemental Fig. S2). Given that trophoblast cells are exposed to a low-oxygen environment during differentiation into EVT cells and uterine invasion, these immunohistochemical data support our hypothesis that E-cadherin loss induced by low-oxygen tension regulates trophoblast-ECM remodeling via α_5-integrin up-regulation, which is an essential process if EVT cells are to invade the uterus.

Discussion

Preeclampsia, which complicates approximately 7% of all pregnancies, remains a major cause of maternal and perinatal morbidity and mortality. An absent or incomplete invasion of trophoblast cells into maternal spiral arteries is often seen in the placental beds of women with preeclampsia, suggesting that a failure of EVT invasion could cause this condition. Therefore, elucidation of the regulatory mechanisms controlling the invasiveness of EVT cells in human placentas would be helpful to arrive at a

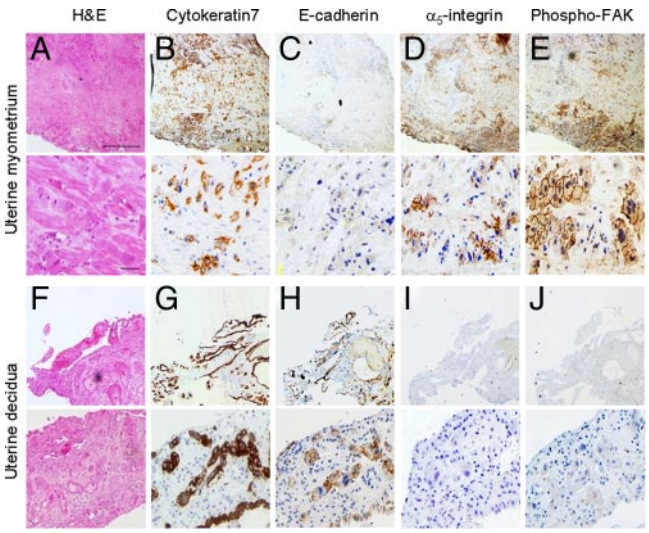

FIG. 6. Serial cross-sections of placental bed tissues obtained from a patient at 27 wk of gestation. Decidua (*lower panels*) and myometrium (*upper panels*) are shown. A and F, Hematoxylin and eosin (H&E) staining. B and G, The section was immunostained with cytokeratin-7 to visualize cytotrophoblasts. Immunolocalization of E-cadherin (C and H), α_5-integrin (D and I), and phospho-FAK (Tyr^{861} (E and J) is shown. Representative pictures are shown at low (×40, *upper panels*) and high (×200, *lower panels*) magnification. *Bar*, 50 μm.

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better understanding of the pathogenesis of this critical gestational disease.

Whereas villous trophoblast cells remain in the fetal compartment and undergo terminal differentiation and fusion to form the multinucleate syncytiotrophoblast, EVT cells infiltrate the endomyometrium, anchoring the placenta and modifying maternal spiral arteries, playing a crucial role in the developing vascular connection between mother and fetus (1, 32). Here we show that during early placentation, EVT cells lose E-cadherin expression via Snail up-regulation under low-oxygen tension, which leads to the acquisition of the invasive phonotype through FAK activation followed by α_5-integrin up-regulation.

Over the past decades, considerable effort has been made toward understanding the mechanism controlling embryo implantation in humans. Early placental development occurs in a low-oxygen environment. O_2 sensing via HIF activity in the placenta is critical for trophoblast differentiation and invasion (7). As cytotrophoblast cells differentiate into intermediate trophoblast cells in trophoblastic columns, E-cadherin expression is down-regulated from the base to the tip of the trophoblastic column, suggesting the important role of E-cadherin loss in intermediate trophoblast cells during implantation (12). Moreover, the interaction of fibronectin with integrin $\alpha_5\beta_1$ significantly contributes to the anchorage of placental cells to uterine ECM (32), and FAK signaling appears to regulate the integrin-dependent processes of cell motility and proliferation during early placental development (23). However, these findings were reported independently and, as a result, the molecular basis and connections of placental development remain poorly understood.

We identified α_5-integrin as a key integrin up-regulated by E-cadherin loss under hypoxia in EVT cells and have shown that blocking α_5-integrin inhibited the invasive activity of EVT cells. These findings are consistent with previous studies documenting the important role of $\alpha_5\beta_1$-integrin in trophoblast migration. Irving and Lala (33) reported that the binding of $\alpha_5\beta_1$-integrin to the fibronectin substrate enhanced trophoblast migration. Gleeson *et al.* (34) reported that IGF-binding protein 1 stimulates human trophoblast migration through $\alpha_5\beta_1$-integrin via MAPK. In another set of experiments, anti-α_5-integrin antibody interfered with the outgrowth of EVT cell column and caused the rounding of spindle-shaped EVT cells, which could indicate a loss of cell motility in these cells (35). However, the mechanism by which $\alpha_5\beta_1$-integrin is up-regulated during early placental development has not been documented in detail. Indeed, Damsky *et al.* (15) reported that $\alpha_5\beta_1$-integrin is up-regulated in differentiating and invading trophoblast cells but that the adhesion of trophoblast cells to fibronectin via $\alpha_5\beta_1$-integrin generates an invasion-restraining signal. These conflicting results may be due, at least in part, to the type or different concentrations of ECM proteins used in each assay system. Further elucidation is needed to clarify the role of $\alpha_5\beta_1$-integrin in EVT cells.

We have shown here that FAK activation was observed in EVT cells, which invade uterine myometrium deeply, as well as in E-cadherin down-regulated BeWo cells. These findings are consistent with the previous report by MacPhee *et al.* (23) which described the crucial role of FAK during early placental development. In their paper, explants of 5–8 wk of gestation cultured in 3% O_2 showed increased EVT outgrowth and higher expression of α_5-integrin activated FAK than explants cultured in 20% O_2. On the other hand, E-cadherin is required for the maintenance of stable adherent junctions and plays a critical role in the suppression of tumor invasion (36). Shih *et al.* (37) suggested that down-regulation of E-cadherin contributes to the motility and invasiveness of implantation site intermediate trophoblast cells. In the initiation of ovarian cancer progression, hypoxia contributes to the transformation into the metastatic phenotype by inactivating E-cadherin via Snail up-regulation (38). These findings strengthen our conviction that trophoblast cells exposed in a low-oxygen tension acquire an invasive phenotype such as α_5-integrin and activated FAK as a result of E-cadherin loss through Snail up-regulation.

Here we used the phospho-FAK antibody, which recognizes tyrosine 861 (FAK Tyr^{861}) phosphorylation to show FAK activation in EVT cells, although several key tyrosine residues become phosphorylated on FAK activation. Among those, the phosphorylation of FAK at tyrosine 861 is especially interesting because it is known to regulate the migration of prostate carcinoma cells with increasing metastatic potential (39) as well as vascular endothelial cells (40). Our conclusion is that FAK Tyr^{861} phosphorylation could play a crucial role in the invasion of EVT cells, although other tyrosine residues might also be activated.

Immunohistochemical findings using placental beds from uncomplicated pregnancies revealed that EVT cells reduced E-cadherin expression as they invaded the myometrium deeply, accompanied by the up-regulation of α_5-integrin and the activation of FAK (Fig. 6 and supplemental Fig. S1). On the other hand, in preeclamptic placental tissues, cytotrophoblast cells in the decidua showed strong reactivity with anti-E-cadherin, although invading EVT cells were hardly seen in the myometrium (supplemental Fig. S2). Considering that the abnormally shallow invasion of EVT cells into maternal spiral arteries is often seen in the placental bed of women with preeclampsia (16),

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E-cadherin loss is likely to play a key role in EVT cell invasion of maternal spiral arteries, which contributes to the transformation of spiral arteries into vessels to promote uteroplacental circulation. Further studies using various placental bed biopsies are required to investigate the contribution of α_5-integrin or FAK activation to early placentation.

We have shown here that hypoxia induces E-cadherin down-regulation through Snail up-regulation in villous explant cultures. The E-cadherin loss induces the up-regulation of α_5-integrin, which leads to the tyrosine phosphorylation of FAK, resulting in increased invasive activity of EVT cells. Immunohistochemical analysis using placental bed biopsies revealed that α_5-integrin was up-regulated and FAK tyrosine phosphorylated as EVT cells invaded the uterine myometrium, whereas E-cadherin expression was down-regulated. Our findings suggest one possible mechanism by which EVT cells acquire the invasive phenotype, which plays a crucial role in early placental development. The elucidation of such mechanisms is likely to lead us to valuable insights into the pathogenesis of critical gestational diseases, such as preeclampsia.

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