Placenta 47 (2016) 105–112

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

CD9 suppresses human extravillous trophoblast invasion

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ARTICLE INFO

Article history:
Received 3 August 2016
Received in revised form
18 September 2016
Accepted 22 September 2016

Keywords:
CD9
Extravillous trophoblast
Invasion
Oxygen concentration
Vascular remodeling

ABSTRACT

During human placentation, the extravillous trophoblast (EVT) invades the maternal decidua and reconstructs maternal spiral arteries. However, the precise mechanisms that control EVT behavior have not yet been elucidated in detail. CD9 has been reported to be a cell-motility-related molecule. Since we previously observed that CD9 was expressed on human EVT, we examined the possible involvement of CD9 in the invasion process of EVT. Placental and umbilical samples were obtained from patients who underwent legal abortions, normal delivery, or hysterectomy. The expression of CD9 at the implantation site and on isolated EVT from a villous explant culture, an EVT-derived immortalized cell line, Swan71, and HUVEC was examined by immunocytochemical staining, flow cytometry, and RT-PCR. The effects of anti-CD9 functional antibody (ALB6) on EVT and Swan71 cell invasion were further examined by matrigel invasion assay along with shRNAmir gene knockdown treatment. CD9 was highly expressed on EVT at the boundary region of EVT invasion and intravascular EVT. EVT and Swan71 cell invasions were promoted by ALB6 or shRNAmir treatment. CD9 expression on Swan71 cells was reduced under hypo-oxygenic conditions, while its expression was increased by the co-culture with HUVEC. These findings suggest that CD9 could attenuate EVT invasion under the influence of an oxygen environment and maternal endothelial cells, proposing that CD9 is a potential regulator of human placental formation.

1. Introduction

During human placentation, trophoblasts proliferate and differentiate into an extravillous trophoblast (EVT) in the anchoring villi. Then, EVT invades the maternal decidua and reconstructs maternal spiral arteries, reducing arterial contractility to maintain adequate maternal blood flow into the intervillouss spaces [1]. Various biologically active molecules, such as several growth factors [2,3] and proteinases [4–6], and cell-cell and/or cell-extracellular matrix interactions mediated by adhesion molecules, such as cadherins and/or integrins [7], have been proposed to be important to regulate EVT invasion. The Eph-ephrin system was also reported to be involved in placentation by EVT [8,9]. Recently, the interaction between EVT and decidual immune cells, especially uterine natural killer cells, has been proposed to regulate EVT invasion [10]. In contrast to malignant cells, EVT invasion is confined spatially to the uterus and temporally to early pregnancy. However, the molecules described above cannot fully explain the spatio-temporal development and differentiation of EVT.

Previously, we reported that a cell surface molecule, CD9, was weakly expressed on EVT in the cell columns of first trimester placentae and highly expressed on EVT in the basal plate of placentae in the second and third trimesters and in the chorion laeve in the fetal membrane of term placentae [11]. CD9 was initially considered to be specific for acute lymphoblastic leukemia cells [12]. This antigen was also expressed on a variety of tumors

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http://dx.doi.org/10.1016/j.placenta.2016.09.014
0143-4004/

and normal human cells, including pre-B cells, activated T cells, and Schwann cells [13–15]. It has been shown that anti-CD9 mAbs induce the migration of Schwann cells [15] and endothelial cells [16], and regulate the adhesion of pre-B cells to bone marrow fibroblasts [17]. CD9 was also reported to play a critical in sperm-egg fusion in fertilization [18,19], suggesting the involvement of CD9 in cell adhesion, migration and cell fusion. Although the precise physiological role of CD9 is still unknown, CD9-targeted therapy for cancer was recently proposed [20].

By invasion assay, the binding of anti-CD9 mAb (ALB-6) to CD9 enhanced the number of invaded BeWo cells without affecting cell proliferation. Although these findings suggest the involvement of CD9 in human trophoblast invasion [21], this did not provide direct evidence because BeWo cells comprise a cell line derived from human choriocarcinoma. Therefore, in this study, we examined the effects of anti-CD9 mAb on EVT invasion using human EVT isolated from primary villous explant culture [22,23], which was originally established by Yagel et al. [24]. We also investigated the involvement of the CD9 molecule in EVT invasion by gene knockdown using a human first trimester trophoblast cell line, Swan71 [25].

2. Materials and methods

2.1. Antibodies, tissue samples, chorionic villous explant culture, and isolation of EVT

Antibodies used in this study are listed in Table 1.

Tissue samples at the implantation sites of a human conceptus during early placentation were obtained from two women who underwent therapeutic hysterectomy for cervical intraepithelial neoplasia or uterine myoma during normal pregnancy at 9–13 weeks of gestation (n = 3) and were subjected to immunohistochemical study.

Placental tissues were aseptically obtained from legal abortions of normal pregnancies (6–9 weeks of gestation, n = 35). EVT were isolated from human villous explant cultures as described previously [22,23,26].

HUVEC were separated from the umbilical cord as described previously [27,28]. The isolated HUVEC were cultured using HuMedia-EB2 (Kurabo, Osaka, Japan) containing 2% FCS, human epithelial growth factor (10 ng/mL), hydrocortisone (1 μg/mL), human basic fibroblast growth factor (3 ng/mL), heparin (10 μg/mL), gentamycin (50 mg/mL), and amphotericin B (50 μg/mL).

Informed consent for the use of these tissues in this study was obtained from all donors. Analysis of these samples was approved by the Ethical Committee of Kyoto University Hospital.

A human first trimester trophoblast cell line, Swan71, derived by telomerase-mediated transformation of a 7-week human cytotrophoblast was kindly provided by Prof. Gil Mor [25].

2.2. Immunohistochemistry

Frozen sections were prepared from tissue samples at the implantation sites as described previously [22,26,29]. The frozen sections were double-stained using anti-CD9 (ALB6), anti-CD31, or control mAb for 30 min at 4 °C along with anti-HLA-G mAb or anti-cytokeratin 7 mAb, as previously described [22]. Anti-TNP mAb (unrelated mAb, 50 μg/mL) was used to block non-specific binding [30]. EVT cultured in the collagen type I-coated culture chamber slide (Iwaki) were fixed in acetone at −20 °C for 5 min and double-stained using anti-CD9 mAb and PE-conjugated anti-human HLA-G1 mAb. The slides were examined with a confocal laser scanning microscope (Carl Zeiss Inc., Jena, Germany).

2.3. Flow cytometry

Flow cytometry was carried out as previously described [29]. The isolated EVT were incubated with FITC-conjugated anti-CD9 or control mAb for 30 min at 4 °C. Swan71 cells cultured under 1 or 20% oxygen conditions for 48 h were stained by CD9 or control mAb. Cell surface labeling was analyzed by FITC fluorescence detection using a FACScalibur (Becton Dickinson).

3. Invasion assay

An invasion assay for isolated EVT was carried out as previously described [22,23]. EVT were cultured on cell culture inserts (6.4 mm in diameter; Beckton Dickinson Labware, Bedford, MA, USA) containing polyethylene terephthalate membranes with 8-μm-diameter pores, which were placed in each well of a 24-well tissue culture plate (Beckton Dickinson Labware). In this assay, the lower surface of the filter of the culture insert was coated with Matrigel (150 μg/mL, Collaborative Research Co., Bedford, MA, USA) and air-dried aseptically. The lower well was filled with 800 μL of RPMI 1640 medium with 1% FCS. The isolated EVT were trypsinized and 4×10^4 cells/200 μL of RPMI 1640 medium with 1% FCS in the presence or absence of anti-CD9 (ALB6, 5 μg/mL) or control mAb (anti-TNP, 5 μg/mL), and 800 μL of the same medium was added to the culture well. After 12 h, the cells that reached the lower surface were fixed with 100% methanol at −20 °C for 5 min and immunostained with mouse anti-human cytokeratin 7 mAb to visualize EVT. The filters were examined under a confocal laser scanning microscope. The numbers of cytokeratin 7-positive cells were counted using NIH Image 1.63 [22,23]. This assay was performed in duplicate chambers and repeated 5 times using EVT isolated from different chorionic samples.

The average was defined as the migrated cell number under each experimental condition. The result is expressed as the migrated cell number as a percentage of that of the control

Table 1
List of antibodies used in this study.

Antibody	Company
Mouse anti-human CD9 mAb (ALB-6, IgG1 class)	Cosmo Bio Co., Ltd. (Tokyo, Japan)
eFluor 615-conjugated mouse anti-human cytokeratin 7 mAb (clone LP5K, IgG2b)	eBioscience (San Diego, CA, USA)
FITC-conjugated mouse anti-human CD9 mAb	Beckman Coulter (Brea, CA, USA)
PE-conjugated mouse anti-human HLA-G mAb (clone MEM-G/9, IgG1)	Abcam (Cambridge, UK)
Rabbit anti-human β-actin pAb (ab8227)	Abcam (Cambridge, UK)
Rabbit ant-human CD9 mAb (clone EPR2949)	Abcam (Cambridge, UK)
FITC-conjugated mouse anti-human CD31 mAb (clone 158–2B3, IgG1)	Ancell Corporation (Bayport, MN, USA)
FITC-conjugated and non-conjugated mouse IgG1 (clone DAK-GO1)	Dako (Glostrup, Denmark)
FITC-conjugated rabbit anti-mouse pAb	Dako (Glostrup, Denmark)
Mouse anti-trinitrophenyl (TNP) mouse mAb (unrelated mAb, IgG1)	Raised in our lab. (Tsujimura et al., 1990, reference No. 25)
Rabbit anti-human HIF-1α mAb (clone EP1215Y)	Epitomics, Inc. (Burlingame, CA, USA)
Horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig pAb	Santa Cruz Biotechnology (Santa Cruz, CA, USA)

(without mAb). The differences were analyzed by one-way analysis of variance followed by Scheffe's F-test for multiple comparisons.

4. RNA isolation and RT-PCR analysis

Total RNA extracted from isolated human EVT was reverse-transcribed using a First Strand cDNA Synthesis Kit (Pharmacia, Inc., Piscataway, NJ, USA). Thirty cycles of PCR were performed with oligonucleotides from the human CD9 cDNA as primers [31] (sense primer 5′-ACTGTTCTTCGGCTTCCTCT-3′: position 321–340; antisense primer 5′-AAAATCCCAAAAATCTTCAT-3: position 774–793) or with human S26 primers [32] (sense primer 5′-GGTCCGTGCCTCCAAGATGA-3′: position 8–27; antisense primer 5′-TAAATCGGGGTGGGGGTGTT-3′: position 308–327). S26 was used as a positive control, and negative controls (without cDNA) were included for all amplifications. PCR products were sequenced to confirm their identity.

5. Silencing of endogenous CD9 with shRNAmir

shRNA for CD9 and validated shRNAmir for control were obtained from GE Healthcare Dharmacon Inc. (Tokyo, Japan) and two effective shRNAmir, CD9 shRNA-1 and CD9 shRNA-2, were selected from the Human GIPZ lentiviral shRNAmir target gene set (RHS4531-NM_001769). Lentivirus particles were produced using 293FT cells and used to transfect Swan71 cells with *Trans*-Lenti shRNA Packaging Kit and Ca Phos (TLP5913, Thermo Fisher Scientific K.K., Tokyo, Japan) according to the instruction manual. The expression levels of CD9 mRNA and protein in each sample were confirmed with RT-PCR and flow cytometry, respectively, as described above.

6. Co-culture of Swan71 cells with HUVEC

HUVEC were cultured in 6-well dishes (Iwaki) using HuMedia-EB2 (Kurabo, Osaka, Japan) containing 2% FCS, human epithelial growth factor (10 ng/mL), hydrocortisone (1 μg/mL), human basic fibroblast growth factor (3 ng/mL), heparin (10 μg/mL), gentamycin (50 mg/mL), and amphotericin B (50 μg/mL). Swan71 cells were used to inoculate the 1-μm-pored insert well (Cell Culture Insert, Kurabo) and co-cultured with HUVEC for 2 days.

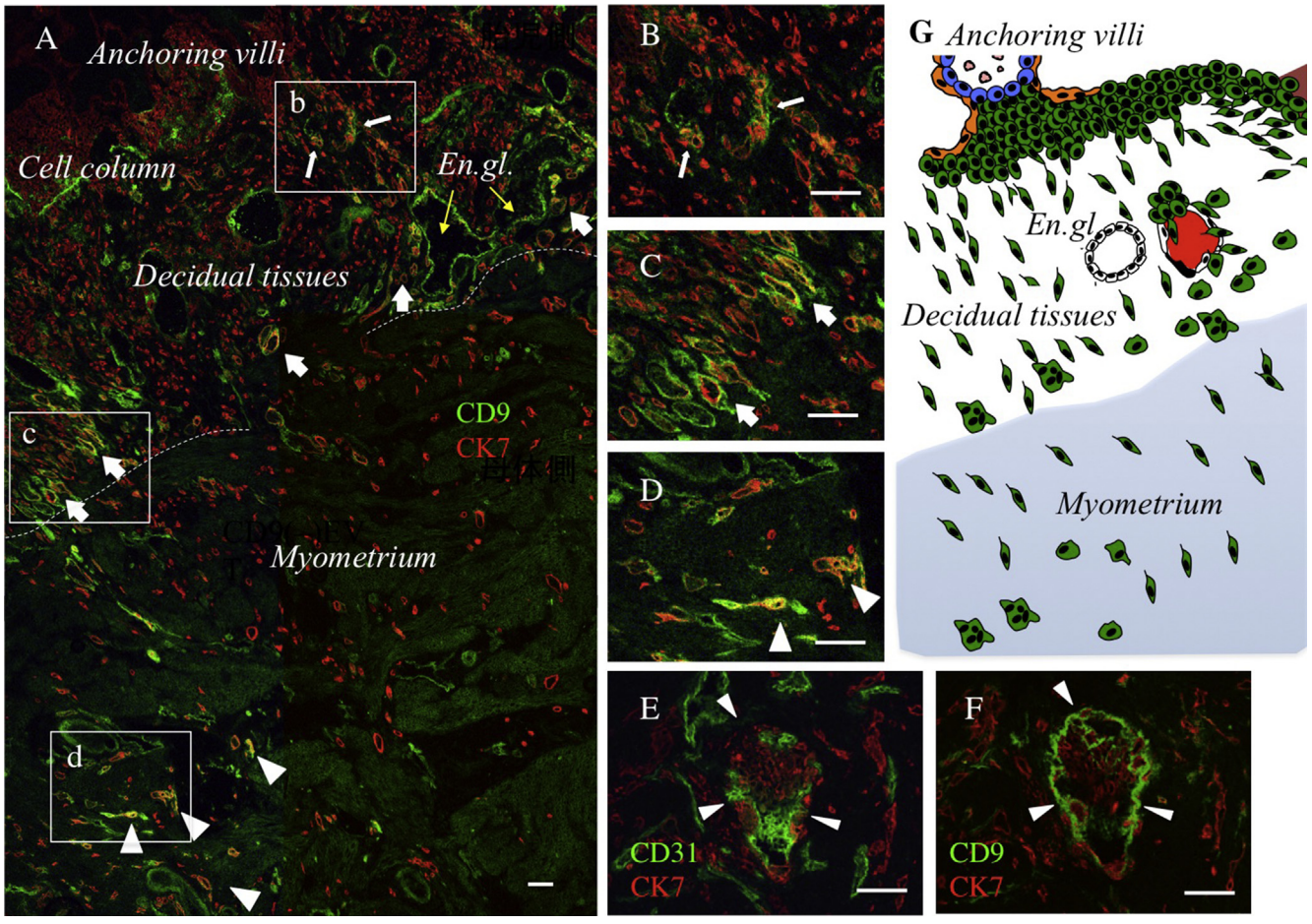

Fig. 1. *CD9 expression at the embryo implantation site.* A-F, immunohistochemical staining of the embryo implantation site at 9 weeks of gestation. B, C, and D are magnified images of areas within the white squares of (b), (c), and (d) in A, respectively. The expression of cytokeratin-7 (CK-7) was clearly detected on EVT. CD9 was expressed on the endometrial glandular epithelial cells (En-gl, small yellow arrows) and endothelial cells. CD9 expression on EVT was decreased from the cell column to invading EVT, while high-level expression of CD9 was observed on EVT crowded around the basal layer of the endometrium (C, big arrows) and on EVT located at the boundary region of EVT invasion (D, big arrowheads). Marked expression of CD9 was also observed on certain population of EVT which invaded the maternal vessels (B, small arrows). E and F, sequential sections of a spiral artery. An endothelial cell marker, CD31-positive maternal endothelial cells being replaced by CK7-positive EVT, of reconstructing maternal vessels (E). High-level expression of CD9 was observed on these reconstructing cells lining the epithelial layer (Fig. 1F, G). H, a color illustration of Figure A. Bars show 100 μm.

7. Western blotting

Swan71 cells were lysed in RIPA buffer containing 0.2 mg/mL phenylmethylsulfonyl fluoride (Wako Pure Chemicals, Osaka, Japan) and 10 μg/mL leupeptin (Peptide Institute, Osaka, Japan). Each supernatant was electrophoresed in 10% SDS-polyacrylamide gel with 10% glycerol and transferred onto an Immobilon PVDF membrane (Millipore). The PVDF membrane was incubated with rabbit anti-human HIF-1α mAb, CD9 mAb, or β-actin pAb followed by HRP-conjugated goat anti-rabbit Ig pAb. HRP was visualized using an ECL Western Blotting System (GE Healthcare, Piscataway, NJ, USA).

8. Results

8.1. CD9 expression at the implantation site

The expression of cytokeratin-7 was clearly detected on EVT that invaded maternal tissues from the cell column to muscle layer through decidual tissues. On the other hand, CD9 was expressed on the endometrial glandular epithelial cells [33] and endothelial cells [16], as described previously. CD9 expression on EVT was decreased from EVT in the cell column to invading EVT, while high-level expression of CD9 was observed on EVT that were crowded around the basal layer of the endometrium. These EVT containing multinuclear giant cells seemed to cease invasion (Fig. 1A, C, big arrows). Although numbers were decreased as compared with EVT in the decidual tissues, a large population of EVT further invaded the myometrium. Notably, high-level expression of CD9 was observed again on EVT located at the boundary region of EVT invasion around the third layer of the myometrium (Fig. 1A, D, big arrowheads). Marked expression of CD9 was also observed on certain populations of EVT that invaded the maternal vessels (Fig. 1A, B, small arrows). When the expression of CD31, an endothelial cell marker, was examined in the maternal vessels with EVT invasion, CD31-positive maternal endothelial cells were replaced by CD9 and CK7 double-positive EVT that seemed to reconstruct maternal vessels, lining the epithelial layer (Fig. 1E, F).

8.2. CD9 expression on isolated human EVT

To confirm CD9 expression on isolated EVT, we performed double immunocytochemical staining. The majority of isolated cells as EVT were positive for HLA-G, a cell marker for human EVT, as described previously (Fig. 2B) [26]. CD9 expression was detected on these EVT (Fig. 2A, C, D). In addition, RT-PCR analysis of cDNA derived from isolated EVT (n = 4, from 6 to 9 weeks of gestation) showed specific single bands at 473 bp, which were sequenced to confirm their identity with CD9 (Fig. 2E). Furthermore, cell surface expression of CD9 on the isolated EVT was confirmed by flow cytometrical analysis (Fig. 2F).

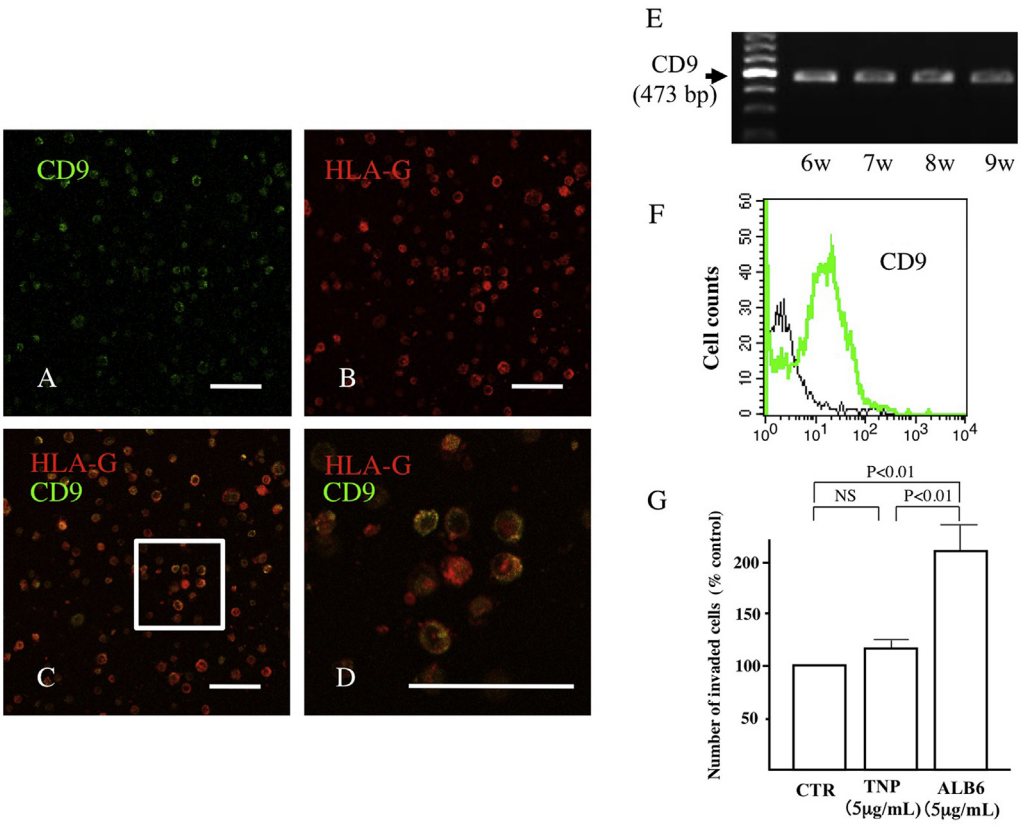

Fig. 2. *CD9 expression on isolated human EVT and the effects of anti-CD9 mAb (ALB6) on EXT invasion.* A-D, double staining of the isolated EVT by anti-CD9 mAb (green) and anti-HLA-G1 mAb (red). The majority of isolated EVT were positively stained by anti-HLA-G mAb (B). CD9 expression was detected on these EVT (A, 2C, and 2D). D is a magnified image of the area within the white square in C. E, RT-PCR analysis of cDNA derived from isolated EVT (n = 4, from 6 to 9 weeks of gestation). Specific single bands at 473 bp, which were sequenced to confirm their identity with CD9, were clearly observed. F, Cell surface expression of CD9 on the isolated EVT was also confirmed by flow cytometry. G, By invasion assay, the number of invaded EVT in the group treated with ALB6 was significantly higher than that in the CTR mAb-treated group. Bars show 100 μm.

8.3. The effects of anti-CD9 mAb (ALB6) on invasion and proliferation of EVT

To estimate the physiological roles of CD9 in EVT function, we examined the effects of anti-CD9 mAb (ALB6) on the invasion and proliferation of cultured EVT. By invasion assay, the number of invaded EVT in the group treated with ALB6 was significantly higher than that in the CTR mAb-treated group (Fig. 2G). On the other hand, no significant effect of anti-CD9 mAb on the cell proliferation of EVT was observed during a 24 h-culture (data not shown). Since this mAb did not affect the proliferation of EVT within 24 h, the stimulatory effects of ALB6 observed in the invasion assays indicated an enhanced invasive property in EVT, rather than enhanced cell proliferation.

The same experiments were performed using Swan71 cells, showing similar results whereby anti-CD9 mAb (ALB6) promoted the invasion of Swan71 cells (Fig. 3A) without affecting cell proliferation (data not shown).

8.4. The effects of CD9 gene knockdown on Swan71 cell invasion and proliferation

When Swan71 cells were transfected with CTR shRNAmir and two kinds of anti-CD9 shRNAmir, reductions of mRNA expression (Fig. 3B) and cell surface protein expression of CD9 were observed in Swan71 cells treated with both anti-CD9 shRNAmir, CD9 shRNA-1 (Fig. 3C) and CD9 shRNA-2 (Fig. 3D). Under these conditions, Swan71 cell invasion was significantly promoted by both anti-CD9 shRNAmir (Fig. 3E), while cell proliferation was not affected (Fig. 3F).

8.5. The effects of oxygen concentration on CD9 expression on Swan71 cells

When Swan71 cells were cultured under the conditions at a 1% oxygen concentration, the increase of protein expression of HIF-1α in Swan71 cells was observed (Fig. 4A). Under this condition, reductions of mRNA expression (Fig. 4B) and cell surface protein expression (Fig. 4C, D) of CD9 on Swan71 cells were observed.

8.6. The effects of co-culture with HUVEC on CD9 expression in Swan71 cells

When Swan71 cells were co-cultured with HUVEC, the cell surface expressions of CD9 on Swan71 cells was increased (Fig. 4E). The increase in the total proteins of CD9 on Swan71 cells was also observed by Western blot analysis (Fig. 4F).

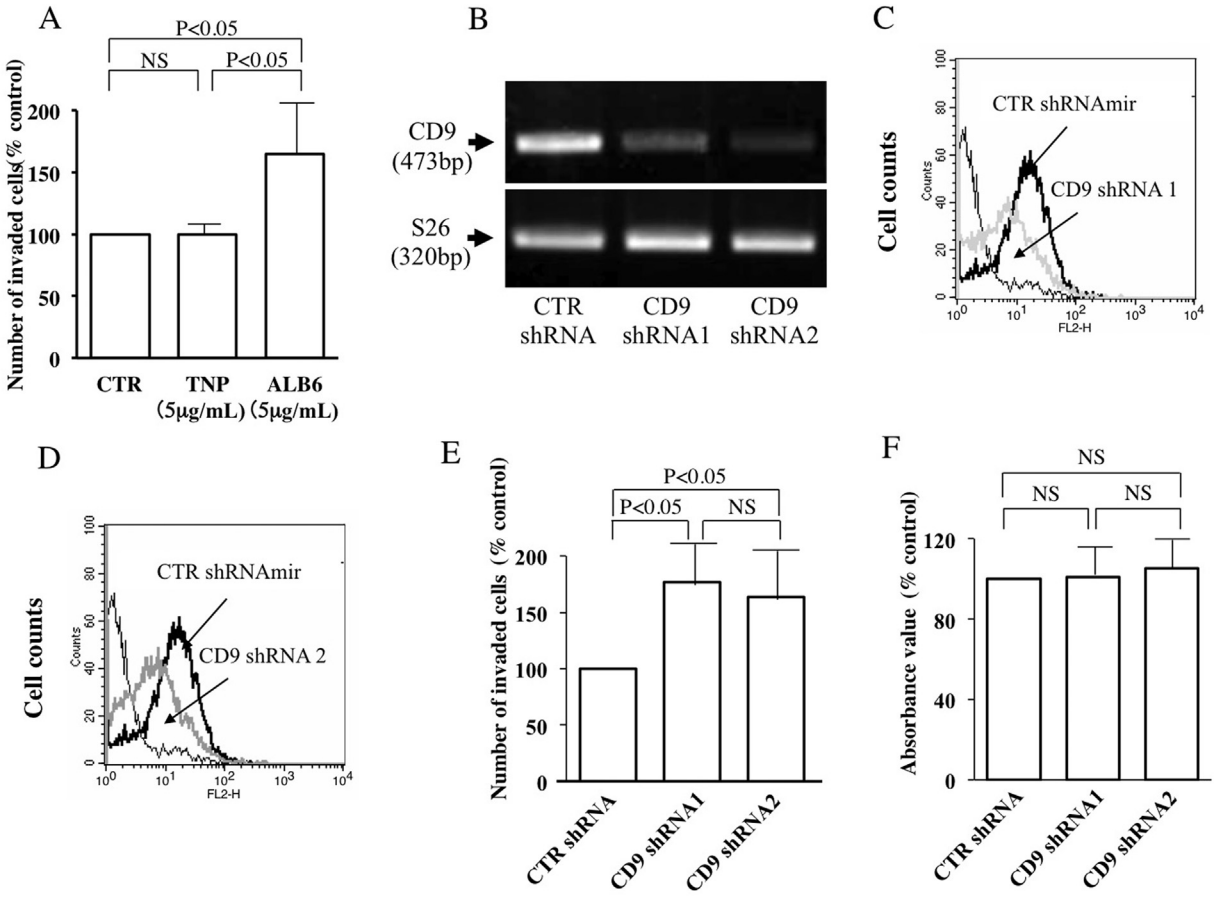

Fig. 3. *The effects of CD9 gene knockdown on Swan71 cell invasion and proliferation*. A, By invasion assay, the number of invaded Swan71 cells was significantly higher than that in the CTR mAb-treated group. B–F, Swan71 cells were transfected with CTR shRNAmir and two kinds of anti-CD9 shRNAmir, CD9 shRNA-1 and CD9 shRNA-2. B, Reduction of mRNA expression was observed by RT-PCR. C and D, Reduction of cell surface expression of CD9 on Swan71 cells was observed by treatment with both CD9 shRNA-1 (C) and CD9 shRNA-2 (D). Invasion of Swan71 cells was significantly promoted by both anti-CD9 shRNAmir (E), while cell proliferation was not affected (F).

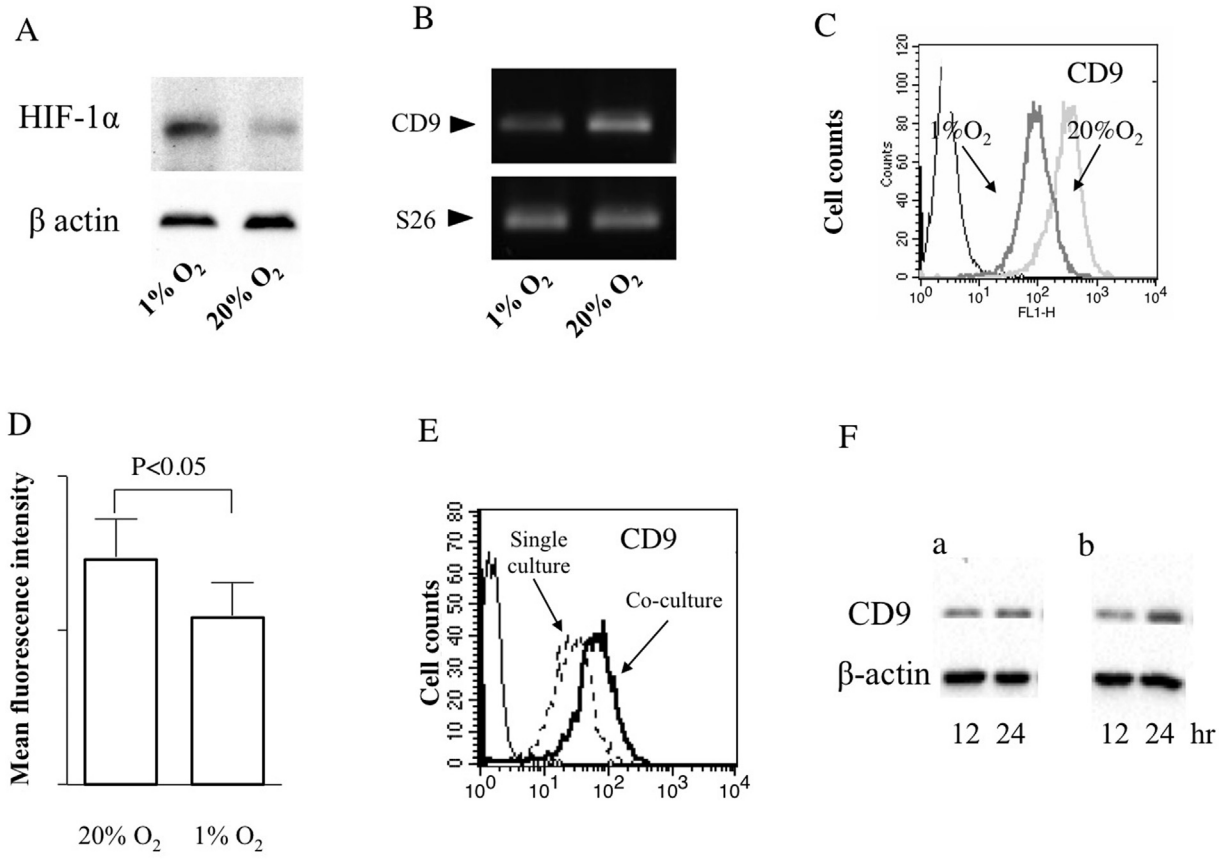

Fig. 4. *The effects of oxygen concentration and co-culture with HUVEC on CD9 expression on Swan71 cells*. When Swan71 cells were cultured under the conditions at a concentration of 1% oxygen, the protein expression of HIF-1α in Swan71 cells was increased (A). Under this condition, reductions of mRNA expression (B) and cell surface protein expression (C and D) of CD9 were observed in Swan71 cells. However, when Swan71 cells were co-cultured with HUVEC, the cell surface expression of CD9 on Swan71 cells (E) was increased. The increase of total proteins of CD9 on Swan71 cells was also observed by Western blot analysis (F). F-a, control culture of Swan71 cells without HUVEC. F-b, Swan71 cells co-cultured with HUVEC.

9. Discussion

We previously reported that in chorionic villi of the first trimester, CD9 was weakly expressed in the EVT of the cell columns, whereas CD9 was only minimally present on the invading interstitial trophoblasts in the decidua. Since, the EVT in the basal plate in the second and third trimester placentae showed intense expression of CD9, we speculated that the expression of CD9 on EVT was inversely correlated with the invasive phenotype of EVT [11]. To support our previous speculation, in this study, high-level expression of CD9 was detected on EVT around the basal layer of the endometrium, which contained multinuclear giant cells in the placenta at 9 weeks of gestation. Marked expression of CD9 was also observed on EVT located at the boundary region of EVT invasion. These findings suggest the inhibitory role of CD9 in the early invasion of EVT.

To examine the above speculation, we performed *in vitro* experiments. Firstly, we observed that a functional mAb against CD9, ALB6, promoted the invasion of human EVT isolated from a primary villous explant culture obtained from women at an early stage of pregnancy. This mAb also enhanced Swan71 cell invasion. These results are in agreement with our previous observation that ALB6 promoted BeWo cell invasion [21], and indicate that the CD9 molecule is involved in human EVT invasion. However, since there is little information on whether ALB6 inhibits or activates CD9 function, we cannot determine the precise role of CD9 in EVT invasion from these data alone. Consequently, using anti-CD9 shRNA, we further examined the effects of the down-regulation of the CD9 gene on Swan71 cell invasion, and observed that the decrease of CD9 expression led to the promotion of Swan71 cell invasion. Taken together, we conclude that CD9 expressed on EVT plays an inhibitory role in EVT invasion. In accordance with this conclusion, it was reported that anti-CD9 mAb and antisense oligonucleotide against the CD9 gene enhanced murine trophectoderm-outgrowth on the monolayer of uterus epithelial cells without affecting the attachment of blastocysts to epithelial cells [34].

The mechanism of the inhibitory effects of CD9 on EVT invasion remains unclear. CD9 is known to be associated with β1-related integrins in other cells [35,36]. By analyzing the affinity-purified proteins, we previously confirmed that both integrin α3 and α5 expressed on human EVT in the chorion laeve were associated with CD9, suggesting the functional relationship between CD9 and these integrin molecules. On the other hand, it is generally accepted that integrin α5β1 expressed on EVT plays a crucial role in EVT invasion [7,37–39]. By raising human anti-integrin α5 functional mAb (CHL3), we also demonstrated that human EVT invasion is regulated by interaction with fibronectin through integrin α5 [40]. Although we did not directly confirm the association of integrin α5 with CD9 on isolated EVT, integrin α5 may be one of the candidates mediating regulation of EVT invasion by CD9.

It was reported that the clustering of CD9 regulates cell-cell

fusion by virus-derived proteins [41]. Using BeWo cells, Muroi et al. demonstrated that the overexpression of CD9 induced the expression of ERVWE1 (SYNCYTIN-1), which is a membrane protein originating from the envelope gene of human endogenous retrovirus-W and mediates the fusion of mononucleated cytotrophoblasts into multinucleated syncytiotrophoblasts [42]. EVT that arrested invasion in the deep region frequently transformed into multinuclear giant cells by cell fusion. Although the mechanism remains unknown, considering the high-level expression of CD9 on EVT in the deep sites observed in this study and in the basal plate in the second and third trimester placentae [11], it is reasonable to deduce that CD9 is relevant to the transformation of EVT into multinuclear giant cells.

In this study, we found that the oxygen concentration and soluble factors from endothelial cells are potential regulators of CD9 expression on EVT. It has been widely accepted that the oxygen concentration is an important regulator of EVT invasion [43]. It was also reported that the placental oxygen concentration was 2.5 times lower than that of decidual tissues, and this oxygen gradient from the placenta to decidua continued until 16 weeks of gestation [44]. Therefore, the increase of CD9 expression on the EVT invading toward the basal layer of the endometrium is a change compatible with the local gradient of the oxygen concentration. On the other hand, a comparative staining of endovascular EVT with anti-CD31and anti-CD9 mAbs using sequential tissue sections showed that CD31-positive maternal endothelial cells lining the maternal artery were being replaced by EVT, and that EVT newly lining the maternal vessel expressed CD9, resulting in the clear expression of CD9 along the basement membrane line (Fig. 1E, F). Since CD9 expression on Swan71 cells was promoted by a high oxygen concentration and soluble factors from HUVEC *in vitro*, it is speculated that CD9 expression was induced on endovascular EVT in response to the high oxygen environment of the maternal artery and the interaction with maternal endothelial cells.

In conclusion, this study showed that CD9 was highly expressed on EVT including multinuclear giant cells at the boundary region of EVT invasion and intravascular EVT. From the findings of *in vitro* experiments, we propose that CD9 attenuates the invading process of EVT under the influence of an oxygen environment and maternal endothelial cells. Further clarification of the precise mechanisms of the CD9-mediated regulation of EVT invasion will provide important information on the regulatory mechanisms of human placentation. In addition, since the insufficient invasion of EVT toward the maternal spinal artery or the excess invasion into muscle layer may cause pregnancy-induced hypertension or placenta accreta, respectively, the possible involvement of CD9 in placental diseases should be elucidated.

Authorship contributions

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

Conflict of interest statements

Each author has no conflicts of interest or no competing financial interests for this work.

Acknowledgements

The authors are grateful to Ms. M. Takemura and Ms. I. Sugiyama for their technical assistance and preparation of the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research (no. 26293358).

References

[1] P.F. O'Tierney-Ginn, G.E. Lash, Beyond pregnancy: modulation of trophoblast invasion and its consequences for fetal growth and long-term children's health, J. Reprod. Immunol. 104–105 (2014) 37–42.
[2] J.D. Aplin, Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*, J. Cell. Sci. 99 (1991) 681–692.
[3] P.K. Lala, C. Chakraborty, Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury, Placenta 24 (2003) 575–587.
[4] P. Bischof, M. Martelli, A. Campana, Y. Itoh, Y. Ogata, H. Nagase, Importance of matrix metalloproteinases in human trophoblast invasion, Early Pregnancy 1 (1995) 263–269.
[5] J. Liu, C. Chakraborty, G.H. Graham, Y.P. Barbin, S.J. Dixon, P.K. Lala, Non-catalytic domain of uPA stimulates human extravillous trophoblast migration by using phospholipase C, phosphatidylinositol 3-kinase and mitogen-activated protein kinase, Exp. Cell. Res. 286 (2003) 138–151.
[6] J. Pollheimer, V. Fock, M. Knöfler, Review: the ADAM metalloproteinases - novel regulators of trophoblast invasion? Placenta 35 (Suppl.:S57–S63) (2014).
[7] C.H. Damsky, C. Librach, K.H. Lim, M.L. Fitzgerald, M.T. McMaster, M. Janatpour, Y. Zhou, S.K. Logan, S.J. Fisher, Integrin switching regulates normal trophoblast invasion, Development 120 (1994) 3657–3666.
[8] K. Red-Horse, M. Kapidzic, Y. Zhou, K.T. Feng, H. Singh, S.J. Fisher, EPHB4 regulates chemokine-evoked trophoblast responses: a mechanism for incorporating the human placenta into the maternal circulation, Development 132 (2005) 4097–4106.
[9] H. Fujiwara, Y. Nishioka, H. Matsumoto, K. Suginami, A. Horie, H. Tani, N. Matsumura, T. Baba, Y. Sato, Y. Araki, I. Konishi, Eph-ephrin A system regulates human choriocarcinoma-derived JEG-3 cell invasion, Int. J. Gynecol. Cancer 23 (2013) 576–582.
[10] A.E. Wallace, R. Fraser, J.E. Cartwright, Extravillous trophoblast and decidual natural killer cells: a remodelling partnership, Hum. Reprod. Update 18 (2012) 458–471.
[11] T. Hirano, T. Higuchi, M. Ueda, T. Inoue, N. Kataoka, M. Maeda, H. Fujiwara, S. Fujii, CD9 is expressed in extravillous trophoblasts in association with integrin α3 and integrin α5, Mol. Hum. Reprod. 5 (1999) 162–167.
[12] J.H. Kersey, W.L. Tucker, C.S. Abramson, R. Newman, R. Sutherland, M. Greaves, A human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody, J. Exp. Med. 153 (1981) 726–731.
[13] L.K. Jennnings, C.F. Fox, W.C. Kouns, C.P. McKay, L.R. Ballou, H.E. Schlutz, The activation of human platelets mediated by anti-human platelet p24/CD9 monoclonal antibodies, J. Biol. Chem. 265 (1990) 3815–3822.
[14] A. Masselis-Smith, G.S. Jensen, J.G. Seehafer, J.R. Slupsky, A.R.E. Shaw, Anti-CD9 monoclonal antibodies induce homotypic adhesion of pre-B cell lines by a novel mechanism, J. Immunol. 144 (1990) 1607–1613.
[15] E.S. Anton, M. Hadjiargyrou, P.H. Patterson, W.D. Matthew, CD9 plays a role in Schwann cell migration in vitro, J. Neurosci. 15 (1995) 584–595.
[16] C. Klein-Soyer, D.O. Azorsa, J.P. Cazenave, F. Lanza, CD9 participates in endothelial cell migration during in vitro wound repair, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 360–369.
[17] A. Masselis-Smith, A.R.E. Shaw, CD9 regulated adhesion. Anti-CD9 monoclonal antibody induce pre-B cell adhesion to bone marrow fibroblasts through de novo recognition of fibronectin, J. Immunol. 152 (1994) 2768–2777.
[18] K. Miyado, G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, M. Okabe, E. Mekada, Requirement of CD9 on the egg plasma membrane for fertilization, Science 287 (2000) 321–324.
[19] K. Klinovska, N. Sebkova, K. Dvorakova-Hortova, Sperm-egg fusion: a molecular enigma of mammalian reproduction, Int. J. Mol. Sci. 15 (2014) 10652–10668.
[20] Y. Murayama, K. Oritani, S. Tsutsui, Novel CD9-targeted therapies in gastric cancer, World J. Gastroenterol. 21 (2015) 3206–3213.
[21] T. Hirano, T. Higuchi, H. Katsuragawa, T. Inoue, N. Kataoka, K.R. Park, M. Ueda, M. Maeda, H. Fujiwara, S. Fujii, CD9 is involved in invasion of human trophoblast-like choriocarcinoma cell line, BeWo cells, Mol. Hum. Reprod. 5 (1999) 168–174.
[22] Y. Sato, T. Higuchi, S. Yoshioka, K. Tatsumi, H. Fujiwara, S. Fujii, Trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype, Development 130 (2003) 5519–5532.
[23] Y. Sato, H. Fujiwara, B. Zeng, T. Higuchi, S. Yoshioka, S. Fujii, Platelet-derived soluble factors induce human extravillous trophoblast migration and differentiation: platelets are a possible regulator of trophoblast infiltration into maternal spiral arteries, Blood 106 (2005) 428–435.
[24] S. Yagel, R.F. Casper, W. Powell, R.S. Parhar, P.K. Lala, Characterization of pure human first-trimester cytotrophoblast cells in long-term culture: growth pattern, markers, and hormone production, Am. J. Obstet. Gynecol. 160 (1989) 938–945.
[25] S.L. Straszewski-Chavez, V.M. Abrahams, A.B. Alvero, P.B. Aldo, Y. Ma, S. Guller, R. Romero, G. Mor, The isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, Swan 71, Placenta 30 (2009) 939–948.
[26] A. Horie, H. Fujiwara, Y. Sato, K. Suginami, H. Matsumoto, M. Maruyama,

I. Konishi, A. Hattori, Laeverin/aminopeptidase Q induces trophoblast invasion during human early placentation, Hum. Reprod. 27 (2012) 1267–1276.
[27] E.A. Jaffe, R.L. Nachman, C.G. Becker, C.R. Minick, Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria, J. Clin. Investig. 52 (1973) 2745–2756.
[28] K. Furukawa, H. Fujiwara, Y. Sato, B.X. Zeng, H. Fujii, S. Yoshioka, E. Nishi, T. Nishio, Platelets are novel regulators of neovascularization and luteinization during human corpus luteum formation, Endocrinology 148 (2007) 3056–3064.
[29] Y. Sato, H. Fujiwara, T. Higuchi, S. Yoshioka, K. Tatsumi, M. Maeda, S. Fujii, Involvement of dipeptidyl peptidase IV in extravillous trophoblast invasion and differentiation, J. Clin. Endocrinol. Metab. 87 (2002) 4287–4296.
[30] K. Tsujimura, Y.H. Park, I.M. Miyama, T. Meguro, T. Ohno, M. Ueda, T. Masuda, Comparative studies on FcR (FcRII, FcRIII, and FcR α) functions of murine B cells, J. Immunol. 144 (1990) 4571–4578.
[31] C. Boucheix, P. Benoit, P. Frachet, M. Billard, R.E. Worthington, J. Gagnon, G. Uzan, Molecular cloning of the CD9 antigen. A new family of cell surface proteins, J. Biol. Chem. 266 (1991) 117–122.
[32] S. Vincent, L. Marty, P. Fort, S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eukaryotic cells and tissues, Nucleic. acids. Res. 21 (1993) 1498.
[33] K.R. Park, T. Inoue, M. Ueda, T. Hirano, T. Higuchi, M. Maeda, I. Konishi, H. Fujiwara, S. Fujii, CD9 is expressed on human endometrial epithelial cells in association with integrins α6, α3 and β1, Mol. Hum. Reprod. 6 (2000) 252–257.
[34] W.M. Liu, Y.J. Cao, Y.J. Yang, J. Li, Z. Hu, E.K. Duan, Tetraspanin CD9 regulates invasion during mouse embryo implantation, J. Mol. Endocrinol. 36 (2006) 121–130.
[35] E. Rubinstein, F. Le Naour, M. Billard, M. Prenant, C. Boucheix, CD9 is an accessory subunit of the VLA integrin complexes, Eur. J. Immunol. 24 (1994) 3005–3013.
[36] K. Nakamura, R. Iwamoto, E. Mekada, Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin α3β1 at cell-cell contact sites, J. Cell. Biol. 129 (1995) 1691–1705.
[37] J.A. Irving, P.K. Lala, Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II, and IGFBP-1, Exp. Cell. Res. 217 (1995) 419–427.
[38] J.D. Aplin, T. Haigh, C.J. Jones, H.J. Church, L. Vicovac, Development of cytotrophoblast columns from explanted first-trimester human placental villi: role of fibronectin and integrin α5β1, Biol. Reprod. 60 (1999) 828–838.
[39] L.M. Gleeson, C. Chakraborty, T. McKinnon, P.K. Lala, Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through α5β1 integrin via mitogen-activated protein kinase pathway, J. Clin. Endocrinol. Metab. 86 (2001) 2484–2493.
[40] B.X. Zeng, H. Fujiwara, Y. Sato, Y. Nishioka, S. Yamada, S. Yoshioka, M. Ueda, T. Higuchi, S. Fujii, Integrin α5 is involved in fibronectin-induced human extravillous trophoblast invasion, J. Reprod. Immunol. 73 (2007) 1–10.
[41] K. Singethan, N. Müller, S. Schubert, D. Lüttge, D.N. Krementsov, S.R. Khurana, G. Krohne, S. Schneider-Schaulies, M. Thali, J. Schneider-Schaulies, CD9 clustering and formation of microvilli zippers between contacting cells regulates virus-induced cell fusion, Traffic 9 (2008) 924–935.
[42] Y. Muroi, T. Sakurai, A. Hanashi, K. Kubota, K. Nagaoka, K. Imakawa, CD9 regulates transcription factor GCM1 and ERVWE1 expression through the cAMP/protein kinase A signaling pathway, Reproduction 138 (2009) 945–951.
[43] O. Genbacev, Y. Zhou, J.W. Ludlow, S.J. Fisher, Regulation of human placental development by oxygen tension, Science 277 (1997) 1669–1672.
[44] E. Jauniaux, A. Watson, G. Burton, Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation, Am. J. Obstet. Gynecol. 184 (2001) 998–1003.