



Endovascular trophoblast expresses CD59 to evade complement-dependent cytotoxicity

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ABSTRACT

In the human placenta, extravillous trophoblasts (EVTs) invade maternal decidual tissues (interstitial trophoblasts) and maternal spiral arteries (endovascular trophoblasts). Although endovascular trophoblasts are directly exposed to maternal blood containing complement components, they are not eliminated by complement-dependent cytotoxicity (CDC). In this study, we investigated the expression and possible function of CD59, one of the membrane-bound complement regulators, in EVT.

Immunohistochemistry of early embryo implantation sites revealed that CD59 was hardly expressed on interstitial trophoblasts, whereas it was intensely expressed on endovascular trophoblasts. Using the human EVT-like cell line Swan71, we established CD59-silencing Swan71 cells (Sw_CD59sh) and non-silencing control Swan71 cells (Sw_CTRsh). *In vitro* cell apoptosis assay showed that Sw_CD59sh cells were significantly more susceptible to CDC as compared to Sw_CTRsh.

Our results suggest that CD59 confers some protection against maternal complement attack to the endovascular trophoblasts.

1. Introduction

In the human placenta, cytotrophoblasts show two distinct patterns of differentiation. In floating villi, cytotrophoblasts differentiate into multinuclear syncytiotrophoblasts to form the syncytial layer, where exchange of gas and nutrients takes place. At villus-anchoring sites, in contrast, cytotrophoblasts differentiate into EVT and form the stratified structure called the cell column. After losing their proliferative activity and acquiring invasive activity in the cell column, EVTs invade the maternal decidual tissue (interstitial trophoblasts) and the maternal spiral arteries (endovascular trophoblasts). Endovascular trophoblasts replace the endothelium and disrupt the muscular linings of the maternal spiral arteries, transforming them into flaccid large-caliber vessels (Pijnenborg et al., 2006). This process, known as “spiral artery remodeling”, induces arterial vasodilation to ensure adequate placental perfusion and consequently contributes to the establishment of a successful pregnancy (Ramsey, 1981). Indeed, insufficient spiral artery remodeling is believed to be one of the underlying causes for obstetric

complications such as preeclampsia and fetal growth restriction (Huppertz, 2008). In this regard, elucidation of the mechanism underlying the spiral artery remodeling may enable future development of preventative and therapeutic measures against these obstetric complications. Nevertheless, massive infiltration of the maternal spiral arteries by trophoblastic cells is a phenomenon unique to primate pregnancies (Cross et al., 2002). Due to the difficulty in obtaining adequate animal models, the precise mechanisms of spiral artery remodeling are still largely unknown.

We previously reported that maternal platelets are trapped on endovascular trophoblasts *in vivo* and co-culture of primary EVTs with human platelets induces their differentiation towards endovascular trophoblast-like phenotype *in vitro* (Sato et al., 2005). Accordingly, we conducted microarray analysis comparing primary EVTs with and without platelet co-culture aiming to extract possible markers of endovascular trophoblasts. First, we identified the top 184 genes whose expressions were significantly higher in the primary EVTs with platelet co-culture. Next, these 184 genes were assessed using the Functional

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Abbreviations

18S rRNA	18S ribosomal RNA	HHS	heat-inactivated human serum
CDC	complement-dependent cytotoxicity	HRP	horseradish peroxidase
Crry	complement receptor 1-related protein y	MAC	membrane attack complex
DAVID	database for annotation visualization and integrated discovery	NHS	normal human serum
EDTA	ethylenediaminetetraacetic acid	PBS	phosphate buffered saline
EVT	extravillous trophoblast	PI	propidium iodide
FCS	fetal calf serum	PRP	platelet-rich plasma
FSC	forward scatter	q-PCR	quantitative polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	RIPA	radio-immunoprecipitation assay
GFP	green fluorescent protein	RT-PCR	reverse transcriptase-polymerase chain reaction
HBSS	Hanks' Balanced Salt Solution	SDS	sodium dodecyl sulfate
		shRNA	short hairpin RNA
		SSC	side scatter
		TNP	trinitrophenyl

Annotation Tool (DAVID, GOTERM_CC_DIRECT), which allowed us to extract 20 groups with p-values of < 0.05 (Table 1). Finally, we extracted 36 genes belonging to the cell surface protein group “GO:0016020~membrane” and postulated that these 36 genes are possible candidates for the cell surface differentiation markers of endovascular trophoblasts (Table 2). Among these 36 genes, the present study focuses on CD59 and examines possible involvement of this molecule in the process of spiral artery remodeling.

CD59, an 18–20 kDa glycosylphosphatidylinositol-anchored protein, is one of the membrane-bound complement regulators. The complement is an innate immune system against foreign cells such as microorganisms as well as damaged host cells. It is activated through any of three pathways: the classical, alternative, and lectin pathways. All three of these pathways converge at the C3 convertase and share a common effector, MAC, which comprises a molecular complex of complement proteins including C5b, 6, 7, 8, and 9. Excess binding of MACs to the cell surface creates pores through the cytoplasmic membrane, leading to osmotic cell lysis and cell death (Bohana-Kashtan et al., 2004; Zipfel and Skerka, 2009; Ziporen et al., 2009). CD59 inhibits assembly of MAC by binding with C8 and/or C9 to block the incorporation of additional C9 copies into the complex (Cai et al., 2014).

Fetus-derived trophoblastic cells are immunologically foreign to the mother. Since syncytiotrophoblasts and endovascular trophoblasts make direct contact with maternal blood and thus are constantly exposed to maternal complements, it is imperative for these trophoblastic cells to possess some complement regulator(s) that can restrain maternal complement activation. In fact, it has been demonstrated that CD46 and CD55, both of which are membrane-bound complement regulators, are expressed on syncytiotrophoblasts as well as on endovascular trophoblasts (Hsi et al., 1991). Although CD59, another membrane-bound complement regulator, is reportedly expressed on syncytiotrophoblasts (Holmes et al., 1992), its expression on EVTs is still undetermined. Therefore, in this study, we examine the detailed expression profiles of CD59 in various subpopulations of EVTs *in vivo*. In addition, using human EVT-like cell line, we investigate the possible roles of CD59 in EVTs *in vitro*.

2. Materials and methods

2.1. Antibodies

Antibodies used in this study are listed in Table 3.

2.2. Tissue samples and cell lines

Tissue samples of human embryo implantation sites were collected from therapeutic hysterectomies for cervical intraepithelial neoplasia or uterine fibroid during normal pregnancies (9–16 weeks of gestation,

n = 9). Human chorionic villous tissues were aseptically obtained from legal abortions of normal pregnancies (6–11 weeks of gestation, n = 13). The gestational age was determined by gestational length assignment based on either date of last menstrual period or first-trimester crown-rump length. Informed consent for the use of these samples was obtained from each donor. The use of these samples was also approved by the Ethical Committee of Kyoto University Hospital.

The human EVT-like cell line Swan71, which was derived from a 7-week human chorionic villous tissue, was kindly gifted by Professor Gil Mor (Straszewski-Chavez et al., 2009). The human choriocarcinoma cell lines BeWo, JAR, and JEG3 were also used in this study. BeWo was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the others were purchased from the American Type Culture Collection (Manassas, VA, USA).

2.3. Generation of CD59-silencing Swan71 cells

CD59-silencing Swan71 cells (Sw_CD59sh) and non-silencing control Swan71 cells (Sw_CTRsh) were generated as described previously (Matsumoto et al., 2016). First, CD59-targeting shRNA and a non-silencing control were selected from the GIPZ Human Lentiviral shRNA Clone Gene Set (GE Healthcare Dharmacon Inc., Lafayette, CO, USA) (clone name/clone ID/antisense sequence: CD59 shRNA/V2LHS_224814/TCAGATGAACAATTGACGG; control shRNA/RHS4346). These vectors contain both GFP gene and puromycin-resistance gene, enabling us to sort out the transfected cells. Second, lentiviral particles were prepared using ViralPower Lentiviral Packaging Mix (Thermo Fisher Scientific, Waltham, MA, USA) and TurboFect Transfection Reagent (Thermo Fisher Scientific) as recommended by the manufacturer. Briefly, 293FT cells were seeded into six-well plates at 2×10^5 cells/well. After incubation for 24 h, 6 μ l of TurboFect Transfection Reagent, 1 μ g of plasmid DNA, 3 μ l of ViralPower Lentiviral Packaging Mix, and 400 μ l of Opti-MEM were mixed, cultured for 20 min, and dispensed into the six-well plates. After another 24 h, the culture medium was collected from each well, passed through a 0.45- μ m pore-sized filter (Merck, Billerica, MA, USA), and stored at -80°C until later use. Third, Swan71 cells were cultivated in 6-cm dishes at 2.5×10^5 cells/dish for 24 h, after which thawed lentiviral particles were added to each dish. After another 48 h of incubation, selection using 3 μ g/ml of puromycin (Gibco, Waltham, MA, USA) was started. The silencing efficacy of CD59 was checked by real-time q-PCR, western blotting, and flow cytometry as described below.

2.4. Human chorionic villous explant culture and isolation of EVTs

Primary EVTs were isolated from human chorionic villous explant cultures as described previously (Sato et al., 2003). Briefly, human chorionic villi (8–10 weeks of gestation, n = 13) were aseptically minced into small fragments (about 2 mm in diameter) and seeded onto

Table 1
Functional Annotation Chart (David ver. 6.8, GOTERM_CC_DIRECT).

GO Term	Count	%	P value	Genes
GO:0005829 ~ cytosol	57	34.3	1.38E-07	SH3RF1, SEC24A, CHMP5, TSNAX, FERMT2, ECHDC1, PPP5K2, PHF20, CTNNB1, APP, YAP1, RAPGEF2, FNDG3A, ERH1, SGK1, ROCK1, CYCS, HERC4, ACTN1, LYPLA1, TANK, HIF1A, KPN4A, SRP9, SERP1, SNRPG, MOB4, XIAP, SRSF10, CALD1, UGDH, UBA6, CMPK1, TFAM, RPS26, EIF3A, TGM2, HNRNPC, RASA2, GLRX, XPOT, ZMYM2, DNML, PDCD10, IREB2, RPS7, PTPN11, TJP1, CDKN1B, EIF4E, CHML, GLS, TCEB1, DNAJB4, TJP2, CALM2, UBE2E1
GO:0005737 ~ cytoplasm	75	45.2	6.53E-07	CPEB2, FERMT2, STRN, PHF20, PAWR, LNX1, CTNNB1, FUBP3, APP, QKI, YAP1, RAPGEF2, ERH1, NTSE, IBTK, SGK1, FMR1, HERC4, ACTN1, KRT10, LYPLA1, TXNDC9, GLCC1, YTHDF3, SRPK1, TANK, MTMR12, HIF1A, GNAQ, SERBP1, BTG3, NRK, ADAMTS1, NEK7, MOB4, PPP4R2, FAM98B, SRSF10, XIAP, UBA6, CMPK1, SEC14L1, HNRNP3, RPS26, EIF3A, DDX3X, NAA50, BTF3, SKIL, ACSL4, USP32, RASA2, KLF5, XPOT, DNMT3A, ZMYM2, DNML, PDCD10, IREB2, FAM208B, PTPN11, RPS7, CSORF30, RNF6, TJP1, EIF4E, CDKN1B, CHML, ETS2, PTP4A1, TGFBR3, TMPO, DNAJB4, TJP2, CALM2
GO:0005913 ~ cell-cell adherens junction	12	7.2	1.15E-04	RPS26, TJP1, DDX3X, CHMP5, SERBP1, CALD1, TMOD3, KTN1, TXNDC9, TMPO, TJP2, CTNNB1
GO:0005654 ~ nucleoplasm	43	25.9	1.41E-04	SNX18, HMG1, PPP4R2, PPARG, FAM98B, SRSF10, XIAP, FERMT2, UGDH, PHF20, CTNNB1, HNRNP3, RPS26, DDX21, YAP1, HNRNPC, SKIL, XPOT, IBTK, KLF5, DNMT3A, AEBP2, SGK1, FMR1, FAM208B, DDX5, HNRNPR, RPS7, SUZ12, RNF6, CDKN1B, HIF1A, CHML, ETS2, RBM39, KPN4A, TCEB1, DNAJB4, TJP2, CALM2, NEK7, UBE2E1, SNRPG
GO:0016020 ~ membrane	36	21.7	2.18E-04	KCNJ15, MOB4, GALT7, STRN, JAG1, CTNNB1, RPS26, EIF3A, FUBP3, ELOVL5, ITGAV, PGRMC2, DDX21, YAP1, HNRNPC, RAPGEF2, ACSL4, NTSE, FNDG3A, USP32, IBTK, DNML, OSBP18, FMR1, ATP11A, TSPAN13, KTN1, KRT10, DDX5, REEP3, RPS7, LAMP2, GNAQ, CD59, SERBP1, TMPO, WNT5A, SNX18, GALT7, CHMP5, FAM3C, ECHDC1, UGDH, CMPK1, CTNNB1, GPC4, RPS26, APP, NAA50, H2AFV, DDX3X, ITGAV, ST3GAL6, TGM2, HNRNPC, ACSL4, NTSE, GLRX, PDCD10, ACTN1, KRT10, LYPLA1, DDX5, RPS7, LAMP2, EIF4E, GNAQ, CD59, SERBP1, PTP4A1, TGFBR3, KPN4A, HSPA13, DNAJB4, CALM2, SRP9
GO:0070062 ~ extracellular exosome	40	24.1	0.001391138	PPARG, CPEB2, CHMP5, TSNAX, FERMT2, PHF20, PAWR, CTNNB1, GPC4, ZNF777, FUBP3, H2AFV, QKI, DDX21, YAP1, ERH1, NFKBIZ, SGK1, MICAL2, FMR1, CYCS, HERC4, KRT10, TXNDC9, SRPK1, SUZ12, AMIGO2, HIF1A, SERBP1, VGLL3, RBM39, HMG1, PPP4R2, ZNF468, FAM98B, SRSF10, XIAP, UGDH, CMPK1, HNRNP3, TFAM, EIF3A, ZNF704, DDX3X, BTF3, HNRNPC, SKIL, GLRX, DNMT3A, AEBP2, ZNF567, MCAM, DDX5, FAM208B, RPS7, PTPN11, RNF6, TJP1, CDKN1B, PTP4A1, ETS2, TMPO, CALM2, UBE2E1
GO:0005634 ~ nucleus	64	38.6	0.003304327	HNRNP3, FMR1, HNRNPC, DDX5, HNRNPR, RPS7
GO:0030529 ~ intracellular ribonucleoprotein complex	6	3.6	0.006490817	SRSF10, FMR1, TANC1, HNRNPR
GO:0043679 ~ axon terminus	4	2.4	0.008170331	HNRNP3, HNRNPC, DDX5, HNRNPR, SNRPG
GO:0071013 ~ catalytic step 2 spliceosome	5	3.0	0.008239392	TJP1, STRN, RAPGEF2, TJP2, CTNNB1
GO:0005923 ~ bicellular tight junction	5	3.0	0.016540201	SH3RF1, APP, MOB4, EIF4E, SEC24A, DNML, SERBP1, FMR1, TSNAX, RAPGEF2, RASA2, CTNNB1
GO:0048471 ~ perinuclear region of cytoplasm	12	7.2	0.018773628	MOB4, SLC38A2, ELOVL5, SRSF10, TANC1, STRN, ACSL4, RAPGEF2
GO:0043025 ~ neuronal cell body	8	4.8	0.019522728	APP, FMR1
GO:0019013 ~ viral nucleocapsid	3	1.8	0.024075999	SLC38A2, ELOVL5, SRSF10, GNAQ, FMR1, TANC1, NPTN, HNRNPR
GO:1990812 ~ growth cone filopodium	2	1.2	0.025624418	CDKN1B, DNML, BTRD1, SKIL, HNRNPC, RAPGEF2, TANK, CTNNB1, PTPN11
GO:0030425 ~ dendrite	8	4.8	0.026217121	PDCD10, SEC24A, GALT7, ROCK1, ST8SIA4, CD59, ST3GAL6, FNDG3A, PAQR3, HS3ST3B1, RNF13
GO:0043234 ~ protein complex	9	5.4	0.026526046	SGK1, SEC24A, DNML, ELOVL5, OSBP18, CD59, KTN1, YIPF5, TMPO, ACSL4, REEP3, SERP1, SSR1, RNF13
GO:0000139 ~ Golgi membrane	11	6.6	0.032157174	EDNRB, RNF6, OSBP18, GNAQ, PHF20, TMPO
GO:0005789 ~ endoplasmic reticulum membrane	14	8.4	0.035560839	
GO:0031965 ~ nuclear membrane	6	3.6	0.048379035	

GO; Gene Ontology, CC; Cellular Component.

Table 2
Extracted genes from 'GO:0016020 ~ membrane'.

Symbol	Gene Title
NT5E	5'-nucleotidase ecto (NT5E)
ATP11A	ATPase phospholipid transporting 11A (ATP11A)
CD59	CD59 molecule (CD59)
DDX5	DEAD-box helicase 5 (DDX5)
DDX21	DEAD-box helicase 21 (DDX21)
ELOVL5	ELOVL fatty acid elongase 5 (ELOVL5)
GNAQ	G protein subunit alpha q (GNAQ)
MOB4	MOB family member 4, phocein (MOB4)
RAPGEF2	Rap guanine nucleotide exchange factor 2 (RAPGEF2)
SERPBP1	SERPINE1 mRNA binding protein 1 (SERBP1)
YAP1	Yes associated protein 1 (YAP1)
ACSL4	Acyl-CoA synthetase long-chain family member 4 (ACSL4)
CTNNB1	Catenin beta 1 (CTNNB1)
DNM1L	Dynamitin 1 like (DNM1L)
EIF3A	Eukaryotic translation initiation factor 3 subunit A (EIF3A)
FUBP3	Far upstream element binding protein 3 (FUBP3)
FNDC3A	Fibronectin type III domain containing 3A (FNDC3A)
FMR1	Fragile X mental retardation 1 (FMR1)
HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRNPC)
IBTK	Inhibitor of Bruton tyrosine kinase (IBTK)
ITGAV	Integrin subunit alpha V (ITGAV)
JAG1	Jagged 1 (JAG1)
KRT10	Keratin 10 (KRT10)
KTN1	Kinectin 1 (KTN1)
LAMP2	Lysosomal associated membrane protein 2 (LAMP2)
OSBPL8	Oxysterol binding protein like 8 (OSBPL8)
GALNT7	Polypeptide N-acetylgalactosaminyltransferase 7 (GALNT7)
KCNJ15	Potassium voltage-gated channel subfamily J member 15 (KCNJ15)
PGRMC2	Progesterone receptor membrane component 2 (PGRMC2)
REEP3	Receptor accessory protein 3 (REEP3)
RPS26	Ribosomal protein S26 (RPS26)
RPS7	Ribosomal protein S7 (RPS7)
STRN	Striatin (STRN)
TSPAN13	Tetraspanin 13 (TSPAN13)
TMPO	Thymopoietin (TMPO)
USP32	Ubiquitin specific peptidase 32 (USP32)

GO; Gene Ontology.

collagen type I-coated 10-cm dishes (Iwaki, Chiba, Japan). After 4 h of incubation to permit the attachment of the villous fragments to the dishes, RPMI 1640 (Sigma-Aldrich Co., Ayrshire, UK) containing 10% FCS (Life Technologies, Grand Island, NY, USA) was gently added and the attached villous fragments were incubated for an additional 48 h. The cells growing out from the adherent villous tips were dispersed in 0.05% trypsin (Difco, Detroit, MI, USA)/0.05% EDTA (Nakalai, Kyoto, Japan) solution. The cell suspension was collected, passed through a 40- μ m pore-sized Nylon Cell Strainer (Becton Dickinson, Bedford, MA, USA) to remove the villous fragments, and re-plated into collagen type

I-coated six-well plates (Iwaki). After 4 h of incubation, non-adherent cells and debris were cleared away by gentle washing with PBS. The remaining cells were defined as primary EVT.

In five samples, the minced chorionic villi were divided into two groups. In one group, the whole process of EVT isolation was carried out under hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂); in the other group, the whole process was carried out under standard normoxic conditions (20% O₂, 5% CO₂, and 75% N₂). The expressions of CD59, CD46, and CD56 in the primary EVTs derived from the hypoxic cultures and in those derived from the normoxic cultures were compared using real-time q-PCR as described below.

2.5. Treatment of primary EVTs with decidua-conditioned medium

To obtain a mixture of soluble factors derived from the decidua, decidua-conditioned media were prepared as described previously (Sato et al., 2003). Briefly, 10 pieces of ~2 mm-diameter decidual fragments (8–10 weeks of gestation, n = 5) were soaked in RPMI containing 10% FCS and put on a 3.5-cm dish coated with collagen type I. After incubation for 4 h to allow adherence of the explants, they were washed gently with PBS and then cultured in 1 ml of RPMI for an additional 24 h. After this incubation, the supernatant was collected as decidua-conditioned medium, passed through a 0.45 μ m-pore filter (Millipore, Bedford, MA, USA), and stored at –80 °C until use.

The primary EVTs (8–10 weeks of gestation, n = 5) were dispersed in RPMI containing 1% FCS and plated in each well of a collagen type I-coated 6-well plate (Iwaki). Five hundred microliter of decidua-conditioned medium with or without heat inactivation (boiling at 95 °C for 5 min) was added to each well and incubated for 24 h. In each experiment, we used the decidua-conditioned medium derived from the women from whom the EVTs were isolated. The expressions of CD59 in the primary EVTs treated with decidua-conditioned medium with and without heat inactivation were compared using real-time q-PCR as described below.

2.6. Double immunohistochemistry

Frozen sections of the human embryo implantation sites (9–16 weeks of gestation, n = 9) were prepared as described previously (Sato et al., 2003). Briefly, the fresh tissue samples were fixed in 4% paraformaldehyde (Nakalai) at 4 °C for 1 h, embedded in OCT compound (Miles, Elkhart, IN, USA), snap-frozen in liquid nitrogen, and stored at –80 °C until use. The embedded tissue samples were cut into 6- μ m-thick sections and additionally fixed with acetone (Nakalai) at –20 °C for 5 min.

The prepared serial sections were incubated with anti-CD59

Table 3
List of antibodies used in this study.

Antibody	Manufacturer
Mouse anti-human CD59 mAb (clone MEM-43, IgG2a)	AbD Serotec (Oxford, UK)
Rabbit anti-human CD59 pAb	Proteintech (Rosemont, IL, USA)
Rabbit anti- β -actin pAb	Abcam (Cambridge, UK)
eFluor 615-conjugated mouse anti-human cytokeratin 7 mAb (clone LP5K, IgG2b)	eBioscience (San Diego, CA, USA)
PE-conjugated anti-CD31 mAb (clone 158-2B3, 5 μ g/ml)	Ancell Corporation (Bayport, MN, USA)
Rabbit anti-human C5b-9 complex pAb	Abcam (Cambridge, UK)
Mouse IgG2a isotype control mAb (clone #20102, IgG2a)	R&D Systems (Minneapolis, MN, USA)
Negative control rabbit immunoglobulin	Dako (Glostrup, Denmark)
Mouse anti-TNP mAb (unrelated IgG1)	raised in our laboratory (Tsujimura et al., 1990)
HRP-conjugated goat anti-rabbit immunoglobulin pAb	Santa Cruz Biotechnology (Dallas, TX, USA)
FITC-conjugated rabbit anti-mouse immunoglobulin pAb	Dako (Glostrup, Denmark)
Rhodamine-conjugated goat anti-mouse immunoglobulin pAb	Santa Cruz Biotechnology (Dallas, TX, USA)
TRITC-conjugated swine anti-rabbit immunoglobulin pAb	Dako (Glostrup, Denmark)

mAb, monoclonal antibody; pAb, polyclonal antibody.

monoclonal antibody (clone MEM-43, 5 µg/ml) or isotype-matched control monoclonal antibody (IgG2a, 5 µg/ml) for 30 min and then stained with FITC-conjugated rabbit anti-mouse polyclonal antibody (dilution 1:40) for 30 min. After incubation with unrelated anti-TNP monoclonal antibody (50 µg/ml) to block non-specific binding (Tsujiura et al., 1990), the slides were treated with eFluor 615-conjugated anti-cytokeratin 7 monoclonal antibody (clone LP5K, 5 µg/ml) or PE-conjugated anti-CD31 monoclonal antibody (clone 158-2B3, 5 µg/ml) for 30 min. Cytokeratin 7 is known as a specific marker for all trophoblast-lineage cells (Haigh et al., 1999). After washing in PBS, the glass slides were mounted with an anti-fade reagent (Life Technologies Corp., Carlsbad, CA, USA) and examined under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Some serial sections were also stained with hematoxylin and eosin (HE) and examined under a light microscope.

2.7. Immunocytochemistry

NHS and HHS were obtained from healthy volunteer women (22–40 years old, $n = 7$) as described previously (Goswami et al., 2016). Briefly, the whole blood was allowed to coagulate at room temperature for 30 min, and then centrifuged at $1300 \times g$ for 10 min. The supernatant was collected as human serum. Half of the serum was kept intact to serve as NHS, which contains active complement components. The remaining half was incubated in a water bath at 56 °C for 30 min to inactivate the complement components and thereby generate HHS. Both NHS and HHS were aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until later use.

Sw_CD59sh and Sw_CTRsh were cultivated on a slide glass with eight separate circular culture spaces (Matsunami Glass Ind., Osaka, Japan) as described previously (Horie et al., 2012). After 24 h of starvation in RPMI alone under standard normoxic conditions (20% O_2), the medium was changed to RPMI containing 20% NHS or HHS. After 2 h of incubation under hypoxic conditions (1% O_2) to reproduce the

reported environment at the early fetal-maternal interface (Jauniaux et al., 2001), the cells were fixed with 4% paraformaldehyde and stained using anti-human C5b-9 complex (MAC) polyclonal antibody (10 µg/ml) followed by TRITC-conjugated swine anti-rabbit polyclonal antibody (dilution 1:20) for 30 min. After the slide was mounted, images were captured of five different areas in each circular culture space (center, upper, lower, left, and right) at a magnification of $\times 400$ using the confocal laser scanning microscope. Quantification of MAC deposition was assessed using the particle analyzing function of Image J software (ver. 1.48, <https://imagej.nih.gov/>) as described previously (Lee et al., 2014). The experiment was repeated five times and results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests.

2.8. Flow cytometry

Flow cytometry was performed as previously described (Matsumoto et al., 2016). Briefly, primary EVT, Sw_CD59sh, and Sw_CTRsh cells were suspended in HBSS (Gibco) containing 0.1% bovine serum albumin and 0.1% NaN_3 . These cells were incubated with anti-CD59 monoclonal antibody (100 µg/ml, 10 µl) or the control monoclonal antibody (100 µg/ml, 10 µl) at 4 °C for 30 min followed by FITC-conjugated rabbit anti-mouse polyclonal antibody (dilution 1:40, 20 µl) or rhodamine-conjugated goat anti-mouse polyclonal antibody (dilution 1:40, 20 µl) at 4 °C for 30 min. After washing twice, the cells were re-suspended in HBSS. Cell surface labeling was assessed using MACS Quant (Miltenyi Biotec, Bergisch Gladbach, Germany). The debris was removed using FSC and SSC and the doublets were excluded using FSC-height and FSC-area. Additionally, since Sw_CD59sh and Sw_CTRsh cells constitutively express GFP, those negative for GFP were gated out.

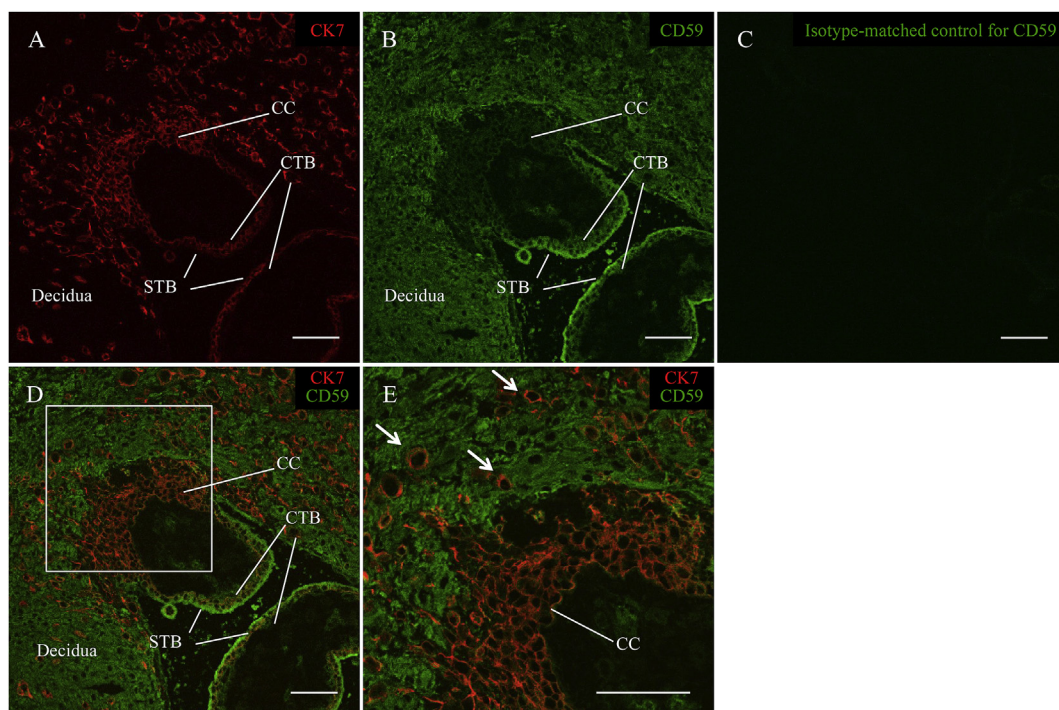


Fig. 1. CD59 expression at the villus-anchoring site at 16 weeks of gestation. (A) Immunoreactive signals against cytokeratin 7 (CK7), a marker for all trophoblast-lineage cells, are labeled with red. (B) Immunoreactive signals against CD59 are labeled with green. (C) Immunostaining against isotype-matched control for CD59, yielding no signal. (D) Merged image of (A) and (B). (E) Magnified image of the boxed area indicated in (D). CD59 is expressed strongly on syncytiotrophoblast (STB) and relatively weakly on cytotrophoblasts (CTB). Although intense expression of CD59 is diffusely distributed on decidua cells, its expression is hardly detectable in the cell column (CC) or on interstitial trophoblasts (E, arrows). Scale bars indicate 100 µm.

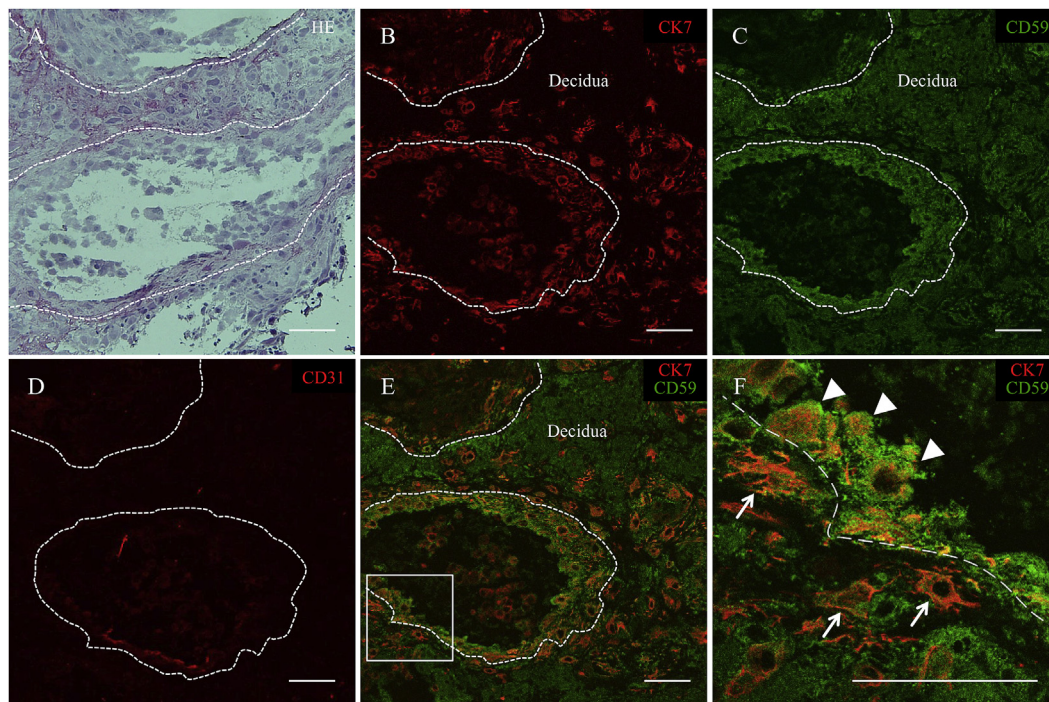


Fig. 2. CD59 expression surrounding the maternal spiral artery at 16 weeks of gestation. (A) Hematoxylin and eosin (HE) staining. (B) Immunoreactive signals against cytokeratin 7 (CK7), a marker for all trophoblast-lineage cells, are labeled with red. (C) Immunoreactive signals against CD59 are labeled with green. (D) Immunoreactive signals against CD31, a marker for endothelial cells, are labeled with red. (E) Merged image of (B) and (C). (F) Magnified image of the boxed area indicated in (E). Two spiral arteries are outlined by dotted lines. Endovascular trophoblasts invading the vessel wall (F, arrowheads) strongly express CD59, whereas CD59 expression is hardly detectable on interstitial trophoblasts located in the surrounding decidua (F, arrows). Scale bars indicate 100 μ m.

2.9. Western blotting

Western blotting was performed as described previously (Matsumoto et al., 2016). Briefly, Sw_CD59sh or Sw_CTRsh cells were lysed in RIPA buffer (50 mM Tris-HCl, 1% NP-40, 5 mM EDTA, 1% deoxycholic acid, and 100 mM NaCl; pH 7.4) containing 0.2 mg/ml of phenylmethylsulfonyl fluoride hydrochloride (Wako Pure Chemical, Osaka, Japan) and 10 mg/ml of leupeptin (Peptide Institute, Osaka, Japan). Each lysate was centrifuged at $20,600 \times g$ for 20 min to collect the supernatant. The supernatant including 10 μ g of protein was separated by electrophoresis on 10% SDS-polyacrylamide gel containing 10% glycerol and transferred onto Immobilon-P PVDF membrane with 0.45 μ m-pore (Millipore). After blocking in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membrane was incubated with rabbit anti-human CD59 polyclonal antibody (diluted 1:500) or rabbit anti-human β -actin polyclonal antibody (diluted 1:5000) followed by HRP-conjugated goat polyclonal antibody against rabbit immunoglobulin (diluted 1:5000). HRP was then detected using an ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA).

2.10. Cell proliferation assay

Cell proliferation assay was performed as described previously (Tani et al., 2016). Swan71, Sw_CD59sh, or Sw_CTRsh cells (5×10^3 cells/100 μ l of RPMI with 1% FCS) were cultured in the wells of a 96-well plate (Corning). After 24 h, the absorbance value reflecting the number of viable cells in each well was assessed using the Premix WST-1 Cell Proliferation Assay System (Takara, Kusatsu, Japan) according to the manufacturer's instructions. Each proliferation assay was performed in triplicate and the result was expressed as a percentage of the absorbance value in the control (Swan71 cells). The same experiment was repeated five times and the results were assessed by unpaired Student's *t*-test.

2.11. Matrigel invasion assay

Matrigel invasion assay was performed as described previously (Tani et al., 2016). Swan71, Sw_CD59sh, or Sw_CTRsh cells (1×10^5 cells/200 μ l of RPMI with 1% FCS) were seeded on a cell culture insert with an 8- μ m pore-sized membrane (Corning) that was placed in each well of a 24-well plate (Corning) filled with 800 μ l of RPMI plus 10% FCS. Membranes of the culture insert were aseptically pre-coated with 20 μ g of Matrigel (Collaborative Research, Bedford, MA, USA). After 24 h, the cells that had reached the lower surface of each membrane were fixed with 100% methanol for 5 min and stained with hematoxylin. Each membrane was observed under a light microscope at a magnification of $\times 200$ and the numbers of stained cells were individually counted in five different areas (center, upper, lower, left, and right). The sum of the cell numbers from these five areas was recorded as the "invaded cell number". Each invasion assay was performed in triplicate and the result was expressed as a percentage of the invaded cell number in the control (Swan71 cells). The same experiment was repeated five times and the results were assessed by unpaired Student's *t*-test.

2.12. Cell apoptosis assay

The Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to quantify dead cells resulting from CDC. In this kit, dead cells were detected as double positive for annexin V and PI. Sw_CD59sh and Sw_CTRsh cells were plated into 12-well plates at 1×10^5 cells/well in RPMI containing 10% FCS. After confirming the full confluence, the culture medium was replaced with RPMI alone. After 48 h of the serum-starving culture, culture medium was replaced with RPMI containing 20% NHS or HHS prepared as above. After an additional 24 h of incubation under hypoxic conditions (1% O_2) to reproduce the reported environment at the early fetal-maternal interface (Jauniaux et al., 2001), these cells were washed in PBS twice and

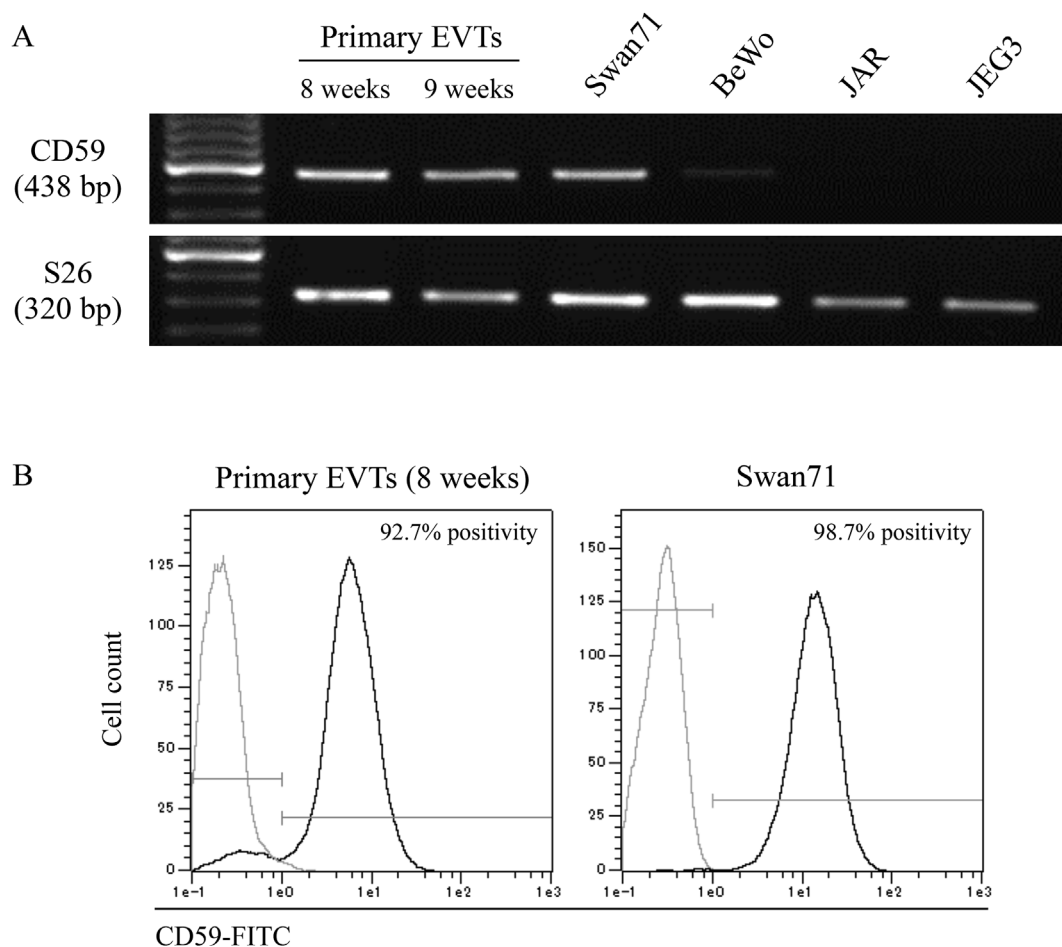


Fig. 3. CD59 expressions in primary EVTs and trophoblastic cell lines. (A) Primary EVTs are isolated from explant cultures of the chorionic villi at 8 and 9 weeks of gestation. mRNA expressions of CD59 in these primary EVTs and in the trophoblastic cell lines (Swan71, BeWo, JAR, and JEG3) are evaluated by 28-cycle RT-PCR. Human ribosomal S26 serves as an internal control. Note that the specific bands at 438 bp corresponding to CD59 are clearly detected in the primary EVTs and Swan71 cells, whereas they are hardly detectable in BeWo, JAR, and JEG3 cells. (B) Protein expressions of CD59 are evaluated by flow cytometry. Cell-surface expressions of CD59 protein are confirmed on the primary EVTs as well as on Swan71 cells.

suspended in $1 \times$ Binding Buffer at a concentration of 1×10^6 cells/ml. A 100- μ l sample of the cell suspension containing 1×10^5 cells was incubated with APC-conjugated annexin V (5 μ l) and PI (5 μ l) for 15 min at room temperature in the dark. After the addition of 400 μ l of $1 \times$ Binding Buffer, the number of cells that were double positive for annexin V and PI was computed using MACS Quant. The experiments were repeated five times and the results were assessed by one-way ANOVA followed by Tukey's multiple comparison tests.

2.13. RT-PCR and real-time q-PCR

Total RNA was extracted from primary EVTs, Swan71, BeWo, JAR, JEG3, Sw_CD59sh, and Sw_CTRsh cells using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. First Strand cDNA Synthesis Kit was used for cDNA synthesis as recommended by the manufacturer (GE Healthcare). Conventional PCR was performed using a thermal cycler. Real-time q-PCR was carried out by amplifying the target gene and reference genes with a Light Cycler 480-II (Roche Diagnostics, Mannheim, Germany). Relative mRNA expression levels were estimated using the $\Delta\Delta$ Ct method, and were normalized to GAPDH or 18S rRNA as appropriate according to the fact that expression of GAPDH itself is up-regulated under hypoxic conditions (Higashimura et al., 2011).

Primers utilized in conventional PCR are as follows (gene name/forward primer sequence/reverse primer sequence): CD59/GGACAAT CACAATGGGAATCCAAG/CAGCAAGAGAAAGCGGACTACG and S26/

GGTCCGTGCCTCCAAGATGA/TAAATCGGGGTGGGGGTGTT. Primers utilized in real-time q-PCR are as follows (gene name/forward primer sequence/reverse primer sequence): (CD46/TTCCTGGCGCTTTCCTG GGT/GTTGGTGGCTCCTCACAGGC), (CD55/TCCTGGCGAGAAGGACT CAGTGA/AGCCTTGTGGCACCTCGCA), (CD59/TGTCCTTCCTTCCA GGTCTGT/TGACGGCTGTTTGCAGTCAGC), (GAPDH/GAGTCAACG GATTTGGTCGTATTGG/GCCATGGGTGGAATCATATTGGAAC), and (18S rRNA/CTCAACACGGGAAACCTCAC/AGCATGCCAGAGTCTCG TTC).

2.14. Gene expression microarray

The primary EVTs (2×10^5 cells/2 ml of RPMI with 1% FCS) derived from chorionic villous explant cultures (7–10 weeks of gestation, $n = 8$) were plated in collagen type I-coated 6-well plates. The cell culture inserts (Corning, Corning, NY, USA) with 1- μ m-pore-sized membrane filter were pre-coated with collagen type I (1 μ g/ml, Koken, Tokyo, Japan) and placed on each well of the 6-well plates. Five hundred microliter of RPMI with or without 2×10^8 of human platelets, which had been isolated from healthy volunteer women (22–40 years old, $n = 8$) as described previously (Sato et al., 2005), were poured into the cell culture insert. After 48 h of co-incubation, total RNA was extracted from the primary EVTs. Gene expression in the primary EVTs with and without platelet co-culture was analyzed using the Affymetrix HG-U133 plus 2.0 gene chip (Affymetrix, Santa Clara, CA, USA), as previously described (Murakami et al., 2016). Robust multiarray

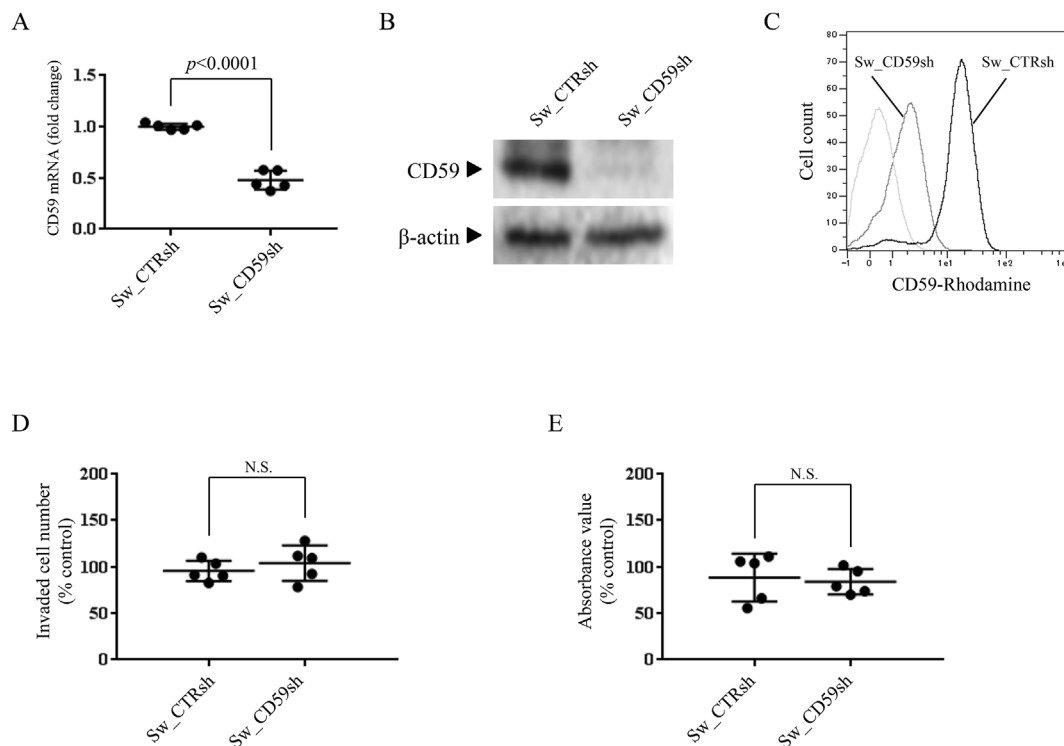


Fig. 4. Establishment of Sw_CD59sh and Sw_CTRLsh. (A) Swan71 cells were transfected with anti-CD59 shRNA or control shRNA to obtain CD59-silencing Swan71 (Sw_CD59sh) and non-silencing control Swan71 (Sw_CTRLsh). Real-time q-PCR verifies significant reduction of CD59 mRNA expression in Sw_CD59sh. (B) Western blotting shows that whole protein expression of CD59 is markedly reduced in Sw_CD59sh. (C) Flow cytometry shows that cell-surface CD59 protein expression is reduced on Sw_CD59sh. (D) Effect of CD59-silencing on invasion of Swan71 cells. Invasive activity of Sw_CD59sh is similar to that of Sw_CTRLsh in matrigel invasion assay. Data are presented as mean $\pm$ SD. “N.S.” indicates “not significant”. (E) Effect of CD59-silencing on proliferation of Swan71 cells. Proliferative activity of Sw_CD59sh is similar to that of Sw_CTRLsh in cell proliferation assay. Data are presented as mean $\pm$ SD. “N.S.” indicates “not significant”.

average normalization was conducted using R (ver. 3.1.0, <https://www.r-project.org>). Of the 54,675 gene probes, we selected the 20,823 with annotated gene symbols, and of these, we retained the top half with the highest average expression across all samples, and then determined 184 significantly up-regulated genes in the primary EVT co-cultured with platelets, by a false discovery rate of $q\text{-value} = 0$ and a standard deviation of > 0.75 . DAVID (ver. 6.8, <https://david.ncicrf.gov/>) was performed using these 184 genes as a signature of the primary EVTs co-cultured with platelets. In order to discover a key molecule supporting interaction between EVTs and platelets, we focused on GO-TERM_CC_DIRECT and then 20 groups were extracted with p-values of < 0.05 .

2.15. Statistical analysis

Data were presented as mean $\pm$ standard deviation. Group comparisons were performed using unpaired Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison tests as appropriate. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). *P*-values of < 0.05 were considered significant.

3. Results

3.1. Gene expression microarray

Using the top 184 genes whose expressions were significantly higher in the primary EVTs with platelet co-culture than those without, GOTERM_CC_DIRECT, one of DAVID Functional Annotation Tools, extracts 20 groups with p-values of < 0.05 (Table 1). Among the 20 groups, we focused on “GO:0016020 ~ membrane”, which encompasses the cell surface proteins, because these proteins could be possible

candidates for the cell surface differentiation markers of endovascular trophoblasts. Thirty-six genes belonging to “GO:0016020 ~ membrane” were detailed in Table 2.

3.2. CD59 expression at the human embryo implantation site

In floating villi, CD59 was expressed strongly on syncytiotrophoblasts and relatively weakly on cytotrophoblasts (Fig. 1B and D). At villus-anchoring sites, CD59 expression was hardly detectable on EVTs in the cell column or on the interstitial trophoblasts (Fig. 1E), whereas strong expression was diffusely detected on the decidual cells (Fig. 1B, D and 1E). In the vicinity of spiral arteries (Fig. 2A), CD59 was strongly expressed on the endovascular trophoblasts that were located inside the spiral arteries (Fig. 2C, E, and 2F). As is the case with the interstitial trophoblasts at the villus-anchoring sites, CD59 expression was hardly detectable on the interstitial trophoblasts surrounding the spiral arteries (Fig. 2F).

3.3. CD59 expression on primary EVTs and various trophoblastic cell lines

In RT-PCR analysis, CD59 was equally expressed in the primary EVTs and Swan71 cells, whereas its expression was hardly detectable in the choriocarcinoma cell lines BeWo, JAR, and JEG3 (Fig. 3A). Flow cytometric analysis confirmed the cell surface expression of CD59 protein on the primary EVTs as well as on Swan71 cells (Fig. 3B).

3.4. Establishment of CD59-silencing Swan71 cells

Swan71 cells were transfected with anti-CD59 shRNA or control shRNA to obtain CD59-silencing Swan71 cells (Sw_CD59sh) and non-silencing control Swan71 cells (Sw_CTRLsh), respectively. Substantial reduction of CD59 mRNA expression in Sw_CD59sh as compared to

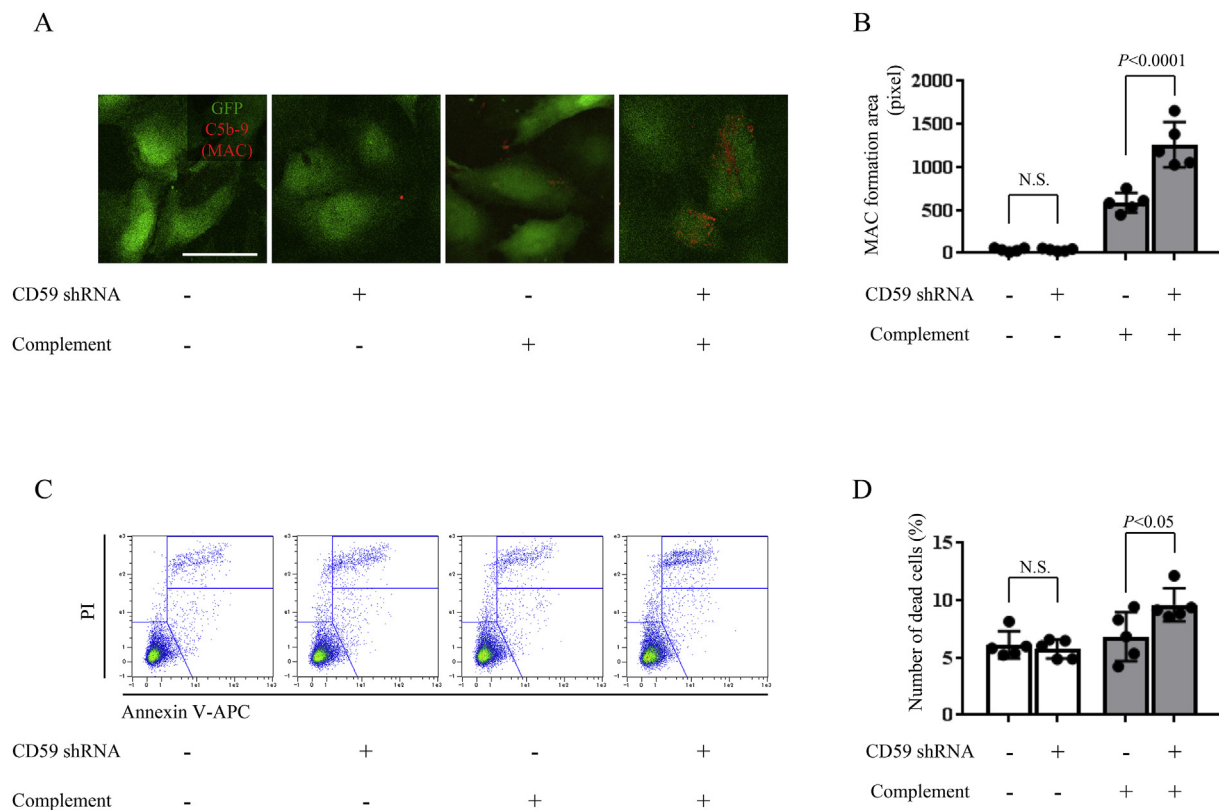


Fig. 5. Effects of CD59-silencing on MAC formation and on CDC in Swan71 cells. CD59-silencing Swan71 (Sw_CD59sh) and non-silencing control Swan71 (Sw_CTRLsh), both of which constitutively express GFP, are treated with NHS that contains active complement components or with HHS that is devoid of active complement components under 1% O₂ conditions. After 2 h, cell-surface MAC formations are visualized by immunocytochemistry (A and B). After 24 h, numbers of dead cells are calculated by cell apoptosis assay (C and D). (A) Representative photos showing that cell-surface immunoreactive MAC signals (red spots) on Sw_CD59sh (shown as “CD59 shRNA +”) are apparently larger than those on Sw_CTRLsh (shown as “CD59 shRNA -”) after NHS treatment (shown as complement “+”), whereas immunoreactive MAC signals are hardly detectable on either Sw_CD59sh or on Sw_CTRLsh after HHS treatment (shown as complement “-”). Scale bars indicate 50 μ m. (B) Total area of the immunoreactive MAC signals on Sw_CD59sh is significantly larger than that on Sw_CTRLsh after NHS treatment. On the other hand, the MAC area is not significantly different between Sw_CD59sh and Sw_CTRLsh after HHS treatment. Data are presented as mean $\pm$ SD. “N.S.” indicates “not significant”. (C) Dead cells are characterized as double positive for annexin V and PI, which are plotted in the upper-right boxed areas. (D) Number of dead cells is significantly larger on Sw_CD59sh than on Sw_CTRLsh after NHS treatment. On the other hand, dead cell number is not significantly different between Sw_CD59sh and Sw_CTRLsh after HHS treatment. Data are presented as mean $\pm$ SD. “N.S.” indicates “not significant”.

Sw_CTRLsh was confirmed by real-time q-PCR analysis (Fig. 4A). Western blotting also confirmed that whole protein expressions of CD59 was markedly reduced in Sw_CD59sh as compared to Sw_CTRLsh (Fig. 4B). In addition, silencing of cell-surface CD59 protein in Sw_CD59sh was demonstrated by flow cytometric analysis (Fig. 4C). Invasive and proliferative activities were not significantly different between Sw_CTRLsh and Sw_CD59sh (Fig. 4D and E).

3.5. Effects of CD59-silencing on MAC formation and on CDC in Swan71 cells

To examine whether trophoblastic CD59 provides some protection against MAC formation and subsequent CDC, Sw_CD59sh and Sw_CTRLsh, both of which constitutively express GFP, were treated with NHS containing active complement components or HHS that was devoid of active complement components for 2 h under 1% O₂ conditions. Immunoreactive MAC was detected only after treatment with NHS but not after treatment with HHS (Fig. 5A). After treatment with NHS, the summed area of immunoreactive signals for MAC was significantly larger on Sw_CD59sh than on Sw_CTRLsh (Fig. 5B).

In the cell apoptosis assay, dead cells were detected as double positive for annexin V and PI (right-upper boxed area in Fig. 5C). After 24 h of treatment with NHS under 1% O₂ conditions, the number of dead Sw_CD59sh was significantly larger than that of dead Sw_CTRLsh (Fig. 5D). The numbers of dead cells were not significantly different

between Sw_CD59sh and Sw_CTRLsh after HHS treatment (Fig. 5D), in keeping with the results observed in MAC formation. By contrast, the same experiment performed under standard normoxic (20% O₂) conditions revealed that numbers of dead cells are similar between Sw_CD59sh and Sw_CTRLsh, regardless of the presence or absence of complement (Supplementary Fig. 1).

3.6. Effects of decidual factors and hypoxia on CD59 expression in primary EVT

To investigate possible involvement of the surrounding micro-environments in the regulation of CD59 expression in EVTs, effects of decidual-conditioned medium and hypoxic cultures were examined using primary EVTs. Treatment with decidual-conditioned medium did not significantly change CD59 expression in primary EVTs (Fig. 6A). On the other hands, the primary EVTs isolated from hypoxic (1% O₂) chorionic villous explant cultures expressed significantly lower levels of CD59 as compared to those isolated from the normoxic (20% O₂) counterparts (Fig. 6B). Similar down-regulation by hypoxic cultures was also observed for CD55, another member of the membrane-bound complement regulators (Supplementary Fig. 2).

4. Discussion

In this study, we confirmed the previously reported finding that

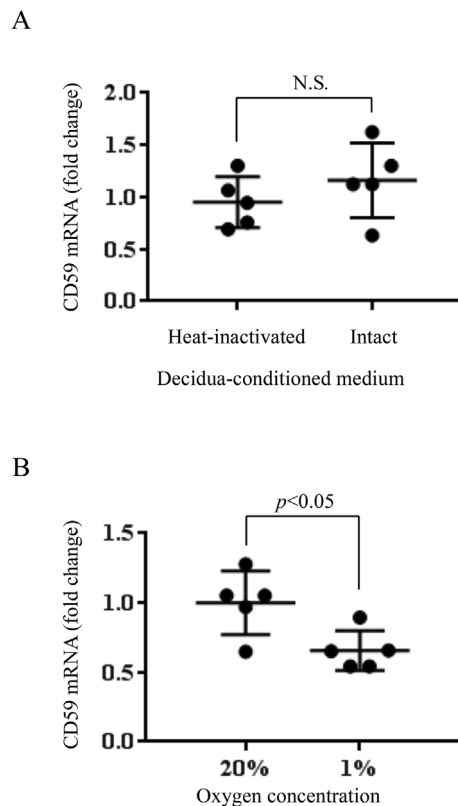


Fig. 6. Effects of decidual factors and hypoxia on CD59 expression in primary EVTs. (A) CD59 mRNA expression in the primary EVTs treated with intact decidua-conditioned medium is not significantly different from CD59 mRNA expression in those treated with heat-inactivated decidua-conditioned medium. (B) The primary EVTs isolated from chorionic villous explant cultures under 1% O₂ conditions express significantly lower levels of CD59 mRNA as compared to those isolated from chorionic villous explant cultures under 20% O₂ conditions. Data are presented as mean $\pm$ SD. "N.S." indicates "not significant".

CD59 is intensely expressed on decidual cells as well as on syncytiotrophoblasts and relatively weakly expressed on cytotrophoblasts (Holmes et al., 1992). In addition, we newly demonstrated that, among trophoblastic cells of extravillous lineage, CD59 is highly expressed on endovascular trophoblasts. Fetus-derived trophoblasts are immunologically foreign to the mother due to the presence of paternal alloantigens. Thus, syncytiotrophoblasts and endovascular trophoblasts, both of which make direct contact with the maternal blood, could be attacked by maternal complements (Girardi et al., 2006). In this respect, it is reasonable to hypothesize that these trophoblastic cells have acquired cell surface CD59 as a protective measure against CDC.

RT-PCR as well as flow cytometry showed that the primary EVTs were positive for CD59 expression. Nevertheless, since the primary EVTs lack the proliferative activity that is required for passage culture, application of the gene-silencing strategy to the primary EVTs is not feasible. Among the cell lines that have frequently been used as trophoblastic models *in vitro*, only Swan71 cells, an immortalized EVT-like cell line (Straszewski-Chavez et al., 2009), express substantial levels of CD59. Therefore, in this study, we selected Swan71 cells for the CD59-silencing experiments. CD59-silencing did not affect either the invasive or the proliferative activity of Swan71 cells, which are major characteristics of EVTs and cytotrophoblasts, respectively. In the presence of human complements, CD59-silencing promoted the cell surface MAC formation as well as the death of the Swan71 cells. Such effects were not observed in the absence of complements. This is agreement with the results of the previous study demonstrating that CD59-silencing enhances CDC in bone marrow mesenchymal stem cells (Xiao et al., 2017). These findings support the idea that endovascular trophoblasts,

which are constantly exposed to the maternal complements, up-regulate their CD59 expression to protect themselves against maternal complement attack.

In human villous explant culture, cell sheets and migrating spindle-shaped cells that correspond to cell column and interstitial trophoblasts, respectively grow out from the adherent explanted villous tips. Although primary EVTs that had been isolated from this explant culture were largely positive for CD59, our immunohistochemistry showed that CD59 expression was hardly detectable in the cell column or on the interstitial trophoblasts. Such discrepancy suggests that some micro-environment factors such as decidual tissue (Sharma et al., 2016) and low oxygen tension (Genbacev et al., 1997) that are absent in the *in vitro* villous explant culture system could down-regulate CD59 expression on the EVTs *in vivo*. In agreement with this, we here demonstrated that primary EVTs isolated from hypoxic chorionic villous explant cultures express lower levels of CD59 as compared to those isolated from the normoxic counterparts, although decidual factors as a whole did not change CD59 expression of primary EVTs. Positive correlation between CD59 expression and the surrounding oxygen tension can at least in part explain our immunohistochemical finding that CD59 is highly expressed on the endovascular trophoblasts located inside the spiral arteries where oxygen tension is high.

Intriguingly, enhancement of CDC by CD59 silencing was only demonstrated in the experiments carried out under the hypoxic conditions (1% O₂), and not in those carried out under the standard normoxic conditions (20% O₂). In analogy with CD59, we have observed that CD55, another member of the membrane-bound complement regulators, is also down-regulated under the 1% O₂ conditions. In this respect, under 20% O₂ conditions, defense mechanism exerted by CD55 could offset the expected enhancement of CDC by CD59 silencing, which may be one of the reasons why enhancement of CDC was only seen under 1% O₂ conditions. This is consistent with the previous report demonstrating that hypoxic conditions increase complement susceptibility on lung cancer cells by giving up some of their available defense mechanisms (Okroj et al., 2009).

In mice, a lack of Crry, one of the membrane-bound complement regulators, results in embryonic lethality that is attributable to aberrant activation of maternal complements against the Crry^{-/-} embryo (Xu et al., 2000). In a mouse model of antiphospholipid syndrome, maternal complement activation by antiphospholipid antibodies bound to the trophoblastic cell membrane has been demonstrated to be one of the main causes of fetal loss and growth restriction (Holers et al., 2002). In humans, preeclamptic women are characterized by elevated levels of circulating complement activation products as well as by increased placental deposition of complement components (Lynch et al., 2008; Buurma et al., 2012; Lokki et al., 2014). Furthermore, mutations in complement regulators such as CD46 and complement factor I have been associated with preeclampsia (Salmon et al., 2011). Collectively, these findings strongly suggest that aberrant activation of maternal complement is one of the underlying causes for pathological pregnancies including recurrent fetal loss, preeclampsia, and fetal growth restriction. Considering that placental histology from such pathological pregnancies often reveals insufficient spiral artery remodeling (Huppertz, 2008), it could be possible that excessive complement activation is associated with inadequate shallow invasion of endovascular trophoblasts. In these respects, CD59 may restrict complement activation to protect endovascular trophoblasts against CDC, and consequently support their deep invasion to accomplish adequate spiral artery remodeling. This concept is also supported by that fact that MAC deposition is not observed on the endovascular trophoblasts in a normal pregnancy (Girardi et al., 2006).

5. Conclusion

We have demonstrated that among trophoblastic cells of extra-villous lineage, CD59 was most intensely expressed on the endovascular

trophoblasts. *In vitro*, CD59 silencing accelerated CDC in a human EVT-like cell line. These findings suggest that CD59 confers protection against maternal complement attack to ensure the survival of the EVTs in the maternal blood stream. Such protective role of CD59 may facilitate the process of spiral artery remodeling. In this study, however, we could not provide any evidence for a direct linkage between CD59 and spiral artery remodeling. Further studies are required to elucidate the molecular mechanisms of spiral artery remodeling, which may lead to new preventative strategies against pathological pregnancy such as preeclampsia and fetal growth restriction.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mce.2019.04.006>.

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