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Platelet-derived factors dysregulate placental sphingosine-1-phosphate receptor 2 in human trophoblasts

BIOGRAPHY

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KEY MESSAGE

The placental S1P receptor subtypes are differentially expressed across gestation and located on diverse cell types of the human placenta. Because platelets contain considerable amounts of S1P, the down-regulation of S1PR2 in response to platelet-derived factors may represent a negative feedback regulation to prevent exaggerated S1P signalling in villous trophoblasts.

ABSTRACT

Research question: Sphingosine-1-phosphate (S1P) is an essential and bioactive sphingolipid with various functions, which acts through five different G-protein-coupled receptors (S1PR1–5). What is the localization of S1PR1–S1PR3 in the human placenta and what is the effect of different flow rates, various oxygen concentrations and platelet-derived factors on the expression profile of S1PR in trophoblasts?

Design: Expression dynamics of placental S1PR1–S1PR3 were determined in human first trimester ($n = 10$), pre-term ($n = 9$) and term ($n = 10$) cases. Furthermore, the study investigated the expression of these receptors in different primary cell types isolated from human placenta, verified the findings with publicly available single-cell RNA-Seq data from first trimester and immunostaining of human first trimester and term placentas. The study also tested whether the placental S1PR subtypes are dysregulated in differentiated BeWo cells under different flow rates, different oxygen concentrations or in the presence of platelet-derived factors.

Results: Quantitative polymerase chain reaction revealed that *S1PR2* is the predominant placental S1PR in the first trimester and reduces towards term ($P < 0.0001$). *S1PR1* and *S1PR3* increased from first trimester towards term ($P < 0.0001$). S1PR1 was localized in endothelial cells, whereas S1PR2 and S1PR3 were predominantly found in villous trophoblasts. Furthermore, *S1PR2* was found to be significantly down-regulated in BeWo cells when co-incubated with platelet-derived factors ($P = 0.0055$).

Conclusion: This study suggests that the placental S1PR repertoire is differentially expressed across gestation. *S1PR2* expression in villous trophoblasts is negatively influenced by platelet-derived factors, which could contribute to down-regulation of placental S1PR2 over time of gestation as platelet presence and activation in the intervillous space increases from the middle of the first trimester onwards.

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INTRODUCTION

Sphingosine-1-phosphate (S1P) is an essential and bioactive sphingolipid associated with the regulation of various cellular functions such as differentiation, proliferation, apoptosis, cell motility and calcium signalling (*Bryan and Del Poeta, 2018*; *Maceyka* et al., *2012*). Moreover, S1P regulates vascular development during embryogenesis and has differential effects on motility, contraction, angiogenesis and vascular permeability, all of which are important to both maintenance and termination of pregnancy (*Spiegel and Milstien, 2003*; *Yamamoto* et al., *2010*). The structure of S1P consists of a polar head group, a phosphate group and a long-chain sphingoid base backbone, the sphingosine group (*Książek* et al., *2015*). It originates from phosphorylation of sphingosine by either sphingosine kinase 1 (SPHK1) or sphingosine kinase 2 (SPHK2). The concentration of S1P is controlled by two specific S1P phosphatases or an S1P lyase, which either remove the phosphate group or irreversibly degrade S1P (*Maceyka* et al., *2012*; *Tukijan* et al., *2018*). The major source of plasma S1P are red blood cells, vascular endothelial cells and activated platelets, making S1P a blood-borne lipid mediator, found in association with albumin and lipoproteins such as high-density lipoprotein (HDL) (*Patanapirunhakit* et al., *2021*). Although studies have shown that erythrocytes are the major source of S1P in blood plasma, platelets are also found to generate and store large quantities of S1P. Recent studies suggest that S1P is essential in thrombus formation due to its increased release during blood clot formation. The augmentation of S1P plasma concentrations by activated platelets is also assumed to play an important role in the repair of injured endothelial vessels (*Tukijan* et al., *2018*). S1P signalling is mediated by five different G-protein-coupled receptors (S1PR1–5), which were originally referred to as Edg for endothelial differentiation gene (S1PR1/Edg-1, S1PR2/Edg-5, S1PR3/Edg-3, S1PR4/Edg-6 and S1PR5/Edg-8) (*Kerage* et al., *2014*). The expression of these receptors is tissue- and cell type-specific. While S1PR1 is ubiquitously expressed, S1PR2 and S1PR3 are more restricted in expression and are mainly found in the gastrointestinal, vascular and central nervous systems. S1PR4 is prevailingly expressed in lymphoid tissues, whereas S1PR5 is primarily found in the nervous system (*Takabe* et al., *2008*).

Various cell types express different combinations of these receptors and the distribution of the receptors on different cell types and the coupling of receptors to different G-subunits allows S1P to influence numerous pathways in different ways. Because the commercial availability of antibodies for S1PR4 and S1PR5 is limited and their expression has been reported to be restricted to lymphoid tissue and the central nervous system, the main focus in this study was on S1PR1–3 (*Obinata and Hla, 2019*).

In fact, S1P signalling is increasingly becoming the focus of pregnancy research (see *Fakhr* et al., *2021*, for a comprehensive review). Its functions have an impact on female reproductive organs before and during pregnancy, including progesterone production in the corpus luteum, differentiation of the endometrium (i.e. decidualization) and placenta development. In human pregnancy, trophoblast invasion into the maternal decidua, as well as fusion of mononucleated cytotrophoblasts into the multinucleated syncytiotrophoblast, are mandatory steps in placentation, both of which are described to be regulated by S1P. Moreover, S1P is suggested to regulate vascular function and immune cell trafficking at the maternal–fetal interface and to play a powerful role in establishing an adequate uteroplacental microvasculature and development of immune tolerance of the mother towards the fetus (*Nagamatsu* et al., *2014*). However, an increasing body of evidence suggests that S1P signalling pathways are dysregulated in pregnancy pathologies such as pre-eclampsia and intrauterine growth restriction (IUGR). Accordingly, circulating S1P plasma concentrations were significantly higher in women diagnosed with pre-eclampsia when compared with controls (*Charkiewicz* et al., *2017*). Besides, placental S1P receptor subtypes are dysregulated in placental tissues from pregnancies complicated by severe pre-eclampsia (*Dobierzewska* et al., *2016*; *Li* et al., *2014*). Both aberrant trophoblast differentiation and turbulent blood flow (high-velocity jets and vortices), combined with elevated blood pressure in the intervillous space and increased wall shear stress at the villous syncytiotrophoblast surface, are suggested to be involved in the pathophysiology of pre-eclampsia (*Drewlo* et al., *2020*; *Roth* et al., *2017*). Notably, shear stress has been shown to induce S1PR1 expression in endothelial cells (*Aoki* et al., *2007*; *Josipovic* et al., *2018*), tempting speculation on the impact of onset of uteroplacental blood perfusion on placental S1P receptor subtype expression.

The aim of the present study was to analyse the expression dynamics of placental S1PR across different stages of gestation and to determine their expression in various placental cell types. Because platelets might be the first amongst maternal blood cells able to enter the early intervillous space through interstices of extravillous trophoblast columns (*Guettler* et al., *2021*; *Sato* et al., *2005*), this study investigated whether or not the S1PR subtype repertoire is dysregulated in human trophoblasts in response to platelet-derived factors. Also tested was whether onset of uteroplacental perfusion with maternal blood could affect the placental S1PR repertoire by analysing the receptor expression in regard to different flow rates and oxygen concentrations.

MATERIALS AND METHODS

Human placental tissue samples

The study was approved by the ethical committee of the Medical University of Graz (31-019 ex 18/19, approved 2 December 2022). First trimester placental tissues (n = 10) were obtained with written informed consent from women undergoing legal elective surgical pregnancy terminations between weeks 6 and 12 of gestation. Pre-term (n = 9) and term (n = 10) placental tissue was collected with written informed consent. Pre-term placental tissues were obtained mostly by Caesarean section because of placenta praevia with or without velamentous insertion of the cord and vaginal bleeding or cervical insufficiency from women who had no clinical evidence of infection. Villous tissue from all placentas was fixed in formalin and embedded in paraffin. Furthermore, additional tissue pieces were thoroughly rinsed in phosphate-buffered saline and dissected into small pieces of approximately 5 × 5 mm, before they were snap-frozen in liquid nitrogen and stored at −80°C until further analyses. Characteristics of the study population are shown in TABLE 1.

Culture of BeWo cell line

Trophoblast cell line BeWo was purchased from the European Collection of Cell Cultures (ECACC) and was cultured in DMEM/F12 (1:1, Gibco™, Life Technologies, Paisley, UK) supplemented

TABLE 1 CHARACTERISTICS OF THE STUDY GROUP

Characteristic	First trimester (*n* = 10)	Pre-term (*n* = 9)	Term (*n* = 10)	*P*-value
Maternal age (years)	31.4 (8.7)	34.8 (2.5)	32.0 (2.9)	NS[a]
Maternal BMI (kg/m^2)	24.1 (6.7)	23.8 (4.4)	22.8 (2.8)	NS[a]
Gestational age (days)	53.9 (6.9)	244.7 (6.1)	276.0 (3.4)	<0.0001[b]
Fetal weight (g)	–	2401 (338.3)	3604 (362.3)	<0.0001[b]
Placental weight (g)	–	510.0 (110.5)	633.9 (129.3)	0.0471[b]
Delivery mode (spontaneous/section)	–	1/8	5/5	–
Fetal sex (male/female)	6/4	8/1	6/4	–

Data are presented as mean (SD) or number.

[a] Differences between all groups were calculated using one-way ANOVA, with post-hoc differences by Tukey's multiple comparison test. (Maternal age: first trimester versus pre-term $P = 0.4027$, first trimester versus term $P = 0.9727$, pre-term versus term $P = 0.5107$; BMI: first trimester versus pre-term $P = 0.9845$, first trimester versus term $P = 0.8143$, pre-term versus term $P = 0.9042$)

[b] Differences pre-term versus term were assessed using the unpaired Student's *t*-test.

ANOVA = analysis of variance; BMI = body mass index; NS = not significant.

with 10% FCS (Gibco), 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Gibco) and 1% (v/v) L-glutamine (Gibco; 200 mmol/l 100X) in a humidified atmosphere of 5% CO_2 at 37°C (*Forstner et al., 2020*). Cells between passage 10 and 20 were used for in-vitro experiments. For differentiation, 2×10^5 cells per well were plated in 12-well dishes (Nunc^{TM} Lab-Tek^{TM}, Thermo Scientific, NY, USA) 1 day prior to the start of the experiment in the above-described culture medium. Next day, culture medium was exchanged with medium including 20 μmol/l forskolin (Bio-Techne, Tocris, Abingdon, UK) and cells were incubated for another 48 h. Dimethylsulphoxide (DMSO) served as solvent control for forskolin and was applied at a final concentration of 0.1% (v/v). Thereafter, cells were incubated under hypoxic conditions with 5% CO_2 and either 2.5%, 12% or 21% O_2 concentrations at 37°C for 24 h in a hypoxic workstation (BioSpherix; Redfield, NY, USA). After culture, cell lysates and supernatants were collected for further analysis.

Fluidic flow culture of BeWo cells

BeWo cells were seeded on coverslips in a 12-well dish (Nunc Lab-Tek, Thermo Scientific) with a density of 2×10^5 cells per well in DMEM/F-12 (1:1, Gibco) 1 day prior to the start of the experiment. Next day, cells were treated with forskolin (Bio-Techne, Tocris) with a final concentration of 20 μmol/l for 48 h. Thereafter, cells on coverslips were transferred to flow chambers (Kirkstall; QV500), which were connected to a tubing system (Kirkstall; $2 \times 1/16'$ diameter 22 cm length, $1 \times 3/32'$ diameter 22 cm length) and a 30 ml reservoir bottle. The flow system was placed in a TEB500 flow bioreactor (Ebers Medical Technology, Zaragoza, Spain). Each flow cycle included two chambers, which were perfused with 12 ml fresh media from the reservoir. Pumps were set to a perfusion rate of 1 ml/min and 3 ml/min and culture conditions were set to 2.5% O_2 and 5% CO_2 at 37°C for an experiment duration of 24 h. For static controls, forskolin-differentiated BeWo cells, grown on coverslips, were transferred to 6-well dishes (Nunc Lab-Tek, Thermo Scientific) containing 6 ml fresh culture media. Static controls were cultured in parallel to flow cultures in the TEB500 flow bioreactor. The experiments were performed four times with different cell passages.

Co-incubation of platelet-derived factors with BeWo cells

BeWo cells were seeded in a 12-well dish (Nunc Lab-Tek, Thermo Scientific) with a density of 2×10^5 cells per well in DMEM/F-12 (1:1, Gibco) 1 day prior to the start of the experimental. Next day, cells were treated with forskolin (Bio-Techne; Tocris) to undergo differentiation into multinucleated syncytia at a final concentration of 20 μmol/l for 48 h. After differentiation three different treatments were started for 24 h. For treatment with platelet-derived factors, platelets were first isolated from whole blood of pregnant donors (see Platelet isolation below); 2.25×10^5 to 3.13×10^5 platelets per μl in a total of 500 μl were transferred into a polycarbonate cell culture insert (Nunc Lab-Tek, Thermo Scientific) located above the well containing the differentiated BeWo cells. The insert prevented direct cell contact of platelets and trophoblasts, as platelets also express the S1P receptors and therefore the results would have been compromised by direct contact. Platelets in the insert were activated with 1 IU/ml thrombin directly administered into the culture medium. Control cells cultured in transwell dishes were subjected to either control medium DMEM/F-12, or control medium supplemented with 1 IU/ml thrombin (Sigma-Aldrich, St. Louis, MO, USA). After 24 h culture, cell lysates and supernatants were collected for further analysis.

Isolation of placental primary cells

Primary endothelial cells were isolated from term placentas of healthy pregnancies as previously described by *Lang et al. (2008)*. In brief, arterial and venous chorionic blood vessels were resected and washed with HBSS (Gibco). Arterial endothelial cells (ECA) and venous endothelial cells (ECV) were isolated by separate perfusion of chorionic arteries and veins with collagenase/dispase (0.1/0.8 U/ml, Roche, Vienna, Austria) dissolved in HBSS, pre-warmed to 37°C. The obtained cell suspension was centrifuged and the cell pellet was resuspended in EGM-MV medium (Lonza, Verviers, Belgium) and plated on culture plates. ECA were incubated at 37°C with 12% O_2 and 5% CO_2. ECV were incubated at 37°C, 21% O_2 and 5% CO_2.

Primary trophoblasts were isolated from term placentas of healthy pregnancies as previously described by *Loegl et al. (2017)*. In brief, placental villous tissue was minced and digested with trypsin/dispase/DNase (Gibco/Roche/Sigma) solution for 90 min. Cell suspension was centrifuged on a Percoll gradient (Sigma) at 4°C for 30 min at 300*g*. Trophoblast-enriched layers were

purified by immunodepletion of contaminating cells using beads conjugated to MCA-81 antibody (Serotec, Puchheim, Germany) against HLA-A, B and C. Trophoblasts were then seeded in each well of a 6-well plate (Nunc Lab-Tek, Thermo Scientific) in 2 ml DMEM (Gibco) containing 10% FCS (Gibco), 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Gibco) at 37°C and 21% O_2 and 5% CO_2.

Platelet isolation

Citrated whole blood (5 × 3.5 ml VACUETTE® tubes per patient) was collected from healthy term donors before Caesarean section. Donors signed written informed consents. Blood samples were centrifuged at 100*g* for 15 min at room temperature. Afterwards, platelet-rich plasma (PRP) was gently mixed in equal amount with a wash buffer consisting of distilled water with 128 mmol/l NaCl (Supelco®, Merck, Darmstadt, Germany), 11 mmol/l glucose (Sigma), 7.5 mmol/l Na_2HPO_4 (Merck), 4.8 mmol/l sodium citrate (Sigma-Aldrich), 4.3 mmol/l NaH_2PO_4 (Lactan, Graz, Austria), 2.4 mmol/l citric acid (Merck) and 0.35% bovine serum albumin (Biowest, Nuaillé, France) with addition of 2.5 ng/μl prostaglandin (Cayman Chemical Company, Ann Arbor, MI, USA). After centrifugation at 1962*g* for 15 min at room temperature, the pellet was resuspended in 10 ml wash buffer and centrifuged again at 1962*g* for 15 min at room temperature. After centrifugation, platelets were resuspended in DMEM/F12 (1:1, Gibco) supplemented with 0.1 U/ml penicillin and 0.1 μg/ml streptomycin (Gibco) and 1% (v/v) L-glutamine (Gibco; 20 mmol/l 100X) to physiological platelet concentrations. Platelet number was determined using a Sysmex KX-21NTM (Sysmex, Horgen, Switzerland).

qPCR analysis

Total RNA from cell lysates and placental tissue was isolated with an ExtractMe Total RNA Kit (Blirt, Gdansk, Poland) according to the manufacturer's protocol. Quality check and amount calculation by NanoDrop (ND-1000, Peqlab Biotechnology GmbH, Erlangen, Germany) was followed by reverse transcription of 1 μg total RNA per reaction using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. qPCR was performed with SYBR Green (Biozym, Vienna, Austria) using the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the specific primers shown in Supplementary Table 1. Cq values were automatically determined using single thresholds and normalized expression (ΔΔCq analysis, all primers confirmed to have equivalent amplification efficiencies), automatically generated by Bio-Rad CFX Manager 3.1 software. The expression of *GAPDH* or *TBP* was used as reference.

Immunoblotting

Placental primary cells were washed with HBSS and homogenized in RIPA Buffer (Sigma-Aldrich) supplemented with cOmplete™ Mini protease inhibitor (Roche, Mannheim, Germany). Total protein concentration was determined using the Lowry method; 20 μg of total protein were loaded on precast 10% Bis-Tris gel (NuPAGE™, Novex, Life Technologies). Proteins were blotted on a 0.45 μm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK) and blotting efficiency was determined with Ponceau staining (Ponceau S solution, Sigma-Aldrich). Primary antibodies were diluted as described in Supplementary Table 2 and incubated on membranes overnight at 4°C. HRP-conjugated goat anti-mouse and goat anti-rabbit IgG (1:5000, Bio-Rad) were used as secondary antibodies and incubated on membranes for 2 h at room temperature. Immunodetection was performed with a chemiluminescent immunodetection kit (WesternBright Chemilumineszenz Substrat für Film, Biozym) according to the manufacturer's instructions. Images were acquired with an iBright CL 1000 Imaging System (Thermo Fisher Scientific) and band densities were analysed with Image Studio Lite 5.2. Results are presented as a ratio of band densities of target protein and reference protein beta-actin.

Immunohistochemistry

Human formalin-fixed paraffin-embedded (FFPE) first trimester and term placenta tissues were cut into 5 μm slices and mounted on Superfrost Plus slides (Thermo Fisher Scientific). After undergoing standard deparaffinization, sections were subjected to an antigen retrieval in Epitope Retrieval Solution pH 9.0 (Novocostra, Leica) or citrate buffer pH 6.0 for two times 20 min at 150 W in a laboratory microwave (Miele, Guetersloh, Germany). Immunohistochemistry was performed with primary antibodies and IgG-negative controls as indicated in Supplementary Table 2 using the UltraVision Large Volume Detection System HRP Polymer Kit (Thermo Fisher Scientific) as previously described (*Blaschitz* et al., *2015*; *Siwetz* et al., *2015*). The slides were covered with Kaiser's Glycerin Gelatine (Merck) and a cover slip. The negative control for CD42b has previously been verified in *Guettler* et al. *(2021)* Supplementary Figure 1b (CD42b) and 1 h (neg ctrl rabbit)) in FFPE human platelets. Images were obtained with an Olympus microscope (BX3-CBH).

Immunofluorescence staining

For immunofluorescence double staining, sections were deparaffinized and subjected to the appropriate antigen retrieval (Supplementary Table 2). Afterwards, slides were blocked with Ultra V (Thermo Fisher Scientific) for 10 min at room temperature. Primary antibodies and IgG-negative controls were diluted in antibody diluent (Dako) (Supplementary Table 2) and added to the slides for 45 min at room temperature. Slides were washed three times in PBS. Secondary fluorescence-labelled antibodies (Supplementary Table 2) were diluted 1:200 in PBS and slides were incubated for 30 min at room temperature. After washing three times with PBS, DAPI was diluted 1:2000 in PBS and incubated on the slides for 5 min. Slides were washed again three times with PBS, left to dry and mounted with ProLong Gold Antifade reagent (Invitrogen). Images were obtained with an Olympus VS200 Slide Scanner.

Analysis of single-cell RNA sequencing data

Single-cell data from first trimester placenta were obtained from *Vento-Tormo* et al. *(2018)* and procedures for analysing the data were previously described therein. In brief, cells extracted from tissue were counted with a Neubauer haemocytometer and loaded in a 10x-Genomic Chromium to profile gene expression before libraries were sequenced on an Illumina HiSeq 4000. Only cells with more than 300 detected genes and less than 20% total mitochondrial gene expression were used for further analysis (*Vento-Tormo* et al., *2018*). Further bioinformatical analysis was done using R package Seurat (*Butler* et al., *2018*) and RStudio to visualize gene expression data in a dot plot.

Transmission electron microscopy

The membrane was cut from the insert after termination of the cell culture

experiment and transferred into 2% paraformaldehyde (Diapath S.P.A., Martinengo BG, Italy) and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hartfield, USA) in 0.1 mol/l dimethyl arsenic acid sodium buffer (cacodylate buffer) pH 7.4 for 30 min at room temperature. The sample was washed in 0.1 mol/l cacodylate buffer pH 7.4 twice for 15 min at room temperature and afterwards it was post fixed with 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 mol/l cacodylate buffer pH 7.4 for 30 min. After rinsing in 0.1 mol/l cacodylate buffer pH 7.4 the sample was stored in the buffer overnight at 4°C. The next day the membrane was dehydrated in a graded ethanol series (50–96%) for 15 min each and two times 100% ethanol for 7 min. The resin infiltration started with 2:1 100% EtOH/Polybed embedding resin (Polysciences, Inc., Warrington, PA, USA) for 45 min, followed by 1:1 100% EtOH/Polybed embedding resin for 45 min and afterwards 1:2 100% EtOH/Polybed embedding resin overnight at 4°C. The next day pure Polybed resin at room temperature for 90 min was followed by another step of pure Polybed resin but with 1.5% DMP30 for 120 min at 45°C. The sections were embedded in pure Polybed resin with 1.5% DMP30 in silicone forms and polymerized at 60°C for 3 days. For sectioning of semi-thin (500 nm) and ultra-thin (70 nm) sections, an ultra-microtome (Leica, Vienna, Austria) was used. Semi-thin sections were stained with 1% toluidine blue solution (Sigma-Aldrich) for an overview and to identify the region of interest (ROI). The block was trimmed to the ROI and ultra-thin sections were collected on 200-mesh copper grids. Grids were subjected to a staining protocol with lead citrate and platinum blue prior to electron microscope imaging (Ultra-stainer, Leica). Stained grids were examined by a Zeiss 900 TEM (Carl Zeiss Microscopy GmbH, Jena, Germany) operated at 80 kV.

Statistical analysis

All data were analysed using GraphPad Prism Version 9.0.0 and are presented as mean with SD or SEM as stated in figure legends. Standard normality was tested by performing the Shapiro–Wilk test and significance was tested with one-way analysis of variance followed by Tukey's multiple comparison test in order to compare groups with one another. Student's *t*-test was used when only two groups were compared.

RESULTS

S1PR2 is the predominant placental S1PR in the first trimester and declines significantly towards term

In order to determine temporal expression of placental S1PR across different stages of human pregnancy, placental tissues from healthy women undergoing either elective termination of pregnancy during first trimester or delivery at pre-term and term were subjected to quantitative gene expression analysis for S1PR subtypes. There were no differences between subjects in terms of maternal age and body mass index; significant differences were seen between pre-term and term cases in gestational age ($P < 0.0001$), fetal weight ($P < 0.0001$) and placental weight ($P = 0.0471$) (TABLE 1). Analysis of *S1PR* in human first trimester placental tissue revealed *S1PR2* as the predominant *S1PR*, which was expressed approximately two-fold higher than *S1PR1* and *S1PR3* ($P = 0.0039$) (FIGURE 1A). Comparison of the placental *S1PR* expression between first trimester samples and pre-term (FIGURE 1B) and term cases (FIGURE 1C) showed a substantial change in the expression pattern over time of gestation. While the expression levels of *S1PR1* (FIGURE 1D) and *S1PR3* (FIGURE 1F) strongly increased by about 20- to 30-fold towards term ($P < 0.0001$ first trimester versus term, both *S1PR1* and *S1PR3*), placental *S1PR2* expression significantly declined in pre-term and almost vanished at term, when compared with first trimester ($P < 0.0001$) (FIGURE 1E).

S1PR are differentially expressed across different placental cell types

Immunohistochemical staining of first trimester as well as term placental tissue showed that S1PR1 expression was restricted to endothelial cells (FIGURE 2A and 2D), while S1PR2 and S1PR3 were predominantly found in the syncytiotrophoblast and cytotrophoblasts (FIGURE 2B, 2C, 2E, 2F). Negative controls using rabbit IgG revealed no staining on term placenta for both antigen retrieval protocols (FIGURE 2G and 2H).

Analysis of publicly available scRNA-Seq data from human first trimester placenta (*Vento-Tormo* et al., *2018*) provided an insight into placental S1PR expression on a single-cell level. A dot plot of single-cell transcriptomes from first trimester placenta samples showed considerable *S1PR2* expression in the syncytiotrophoblast and the villous cytotrophoblast populations, in concordance with the immunohistochemical findings. Moreover, *S1PR2* was expressed in fibroblasts and a small proportion of extravillous trophoblasts. scRNA-Seq data for *S1PR1* confirmed localization of the receptor in fetal–placental endothelial cells, while *S1PR3* was detected mainly in placental fibroblasts, decidual stroma cells and to some extent in villous cytotrophoblasts and decidual natural killer cells (FIGURE 2I).

Double immunofluorescence staining confirmed the expression of S1PR2 in villous syncytiotrophoblast, but did not show its expression on extravillous trophoblasts (FIGURE 2J). Moreover, S1PR2 was expressed on the outer layer of the decidual epithelium (FIGURE 2K). S1PR3 gave immunofluorescence signals in the cytotrophoblast layer and in the proximal parts of the trophoblast cell columns right before these cells developed their extravillous character and started invading into the maternal decidua (FIGURE 2V). A strong staining of S1PR3 was also observed in decidual stroma cells (FIGURE 2M). Immunofluorescence negative controls using rabbit and mouse IgG revealed no staining on first trimester placenta (FIGURE 2N and 2O).

Next, mRNA as well as protein expression levels of the three receptors in primary placental endothelial cells and trophoblasts were analysed (FIGURE 3). *S1PR1* was significantly higher expressed in arterial as well as venous endothelial cells compared with primary trophoblasts ($P < 0.0001$) (FIGURE 3A–C). In contrast, *S1PR2* expression was significantly increased in primary trophoblast cells compared with endothelial cells ($P = 0.0262$) (FIGURE 3D–F). S1PR3 showed somewhat adverse expression levels between qPCR, where it was expressed in all three cell types, and immunoblotting, where it was present in trophoblast cells and to some extent in endothelial cells, especially venous endothelial cells ($P = 0.0182$, $P = 0.0064$, respectively) (FIGURE 3G–I).

S1PR2 and S1PR3 are dysregulated by platelet-derived factors

The next aim was to determine potential effects of fluidic flow on S1PR expression in differentiated BeWo cells, mimicking placental syncytiotrophoblasts exposed to maternal blood flow. For this purpose, BeWo cells were first stimulated with forskolin to undergo differentiation and syncytialization, and were afterwards

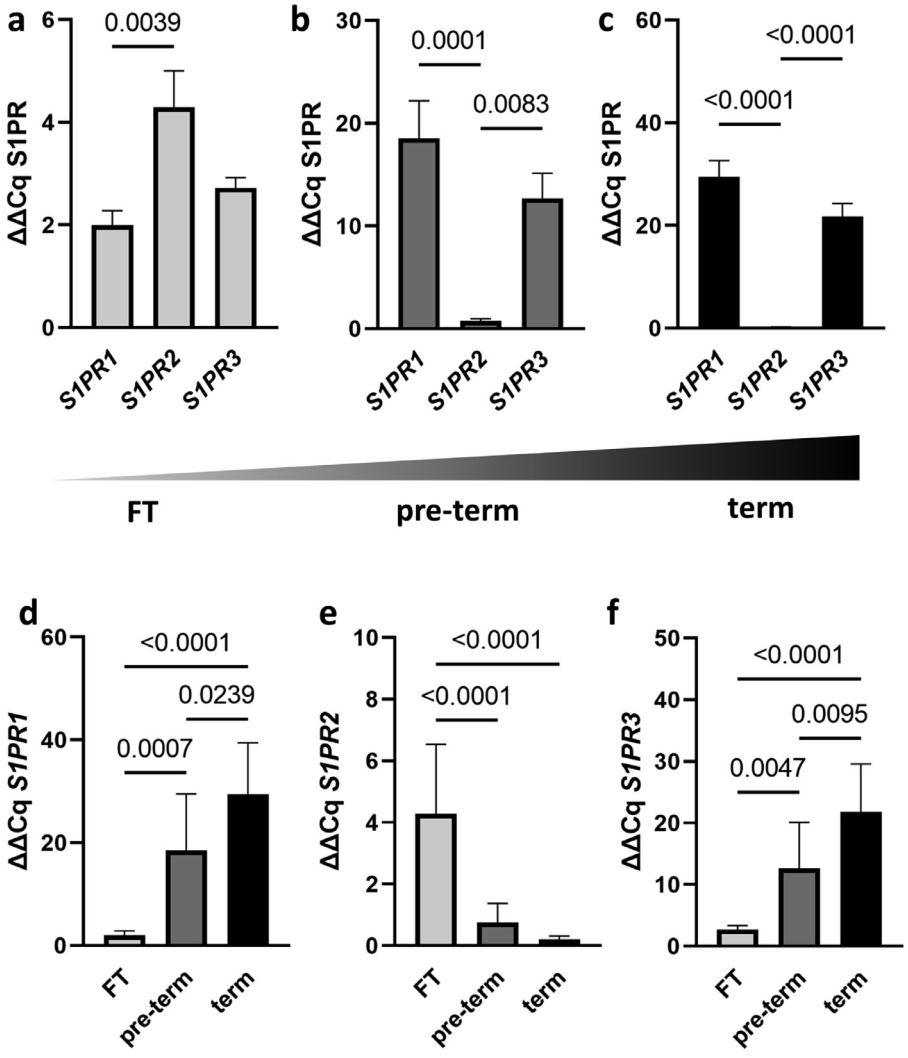

FIGURE 1 Differential placental S1PR expression across human gestation. Human placental tissue was subjected to qPCR analysis of three different S1PR subtypes (*S1PR1*–*S1PR3*) in first trimester (a, $n = 10$), pre-term (b, $n = 9$) and term (c, $n = 10$) samples. Expression levels of *S1PR1* (d), *S1PR2* (e) and *S1PR3* (f) were compared between first trimester, pre-term and term cases using one-way ANOVA. Data are presented as mean ΔΔCq ± SEM, with *GAPDH/TBP* used as reference. ANOVA = analysis of variance; FT = first trimester; qPCR = quantitative polymerase chain reaction.

cultured under fluidic flow. qPCR analysis showed no influence on the expression level of *S1PR2* and *S1PR3* in response to flow rates of 1 ml/min and 3 ml/min, respectively (FIGURE 4A and 4B).

To determine whether the physiological rise in intervillous oxygen tension during the course of pregnancy may cause the observed longitudinal expression changes of placental S1P receptors, the next test was whether different in-vitro oxygen concentrations can affect *S1PR* in differentiated BeWo cells. During the first trimester oxygen concentrations range between 2 and 3% in the intervillous space and increase during the ongoing development to about 8–10% during the second trimester. In order to determine oxygen effects, a rather broad range was tested, including 2.5% O_2 during the first trimester, 12% O_2 to mirror the rise in oxygen concentration and lastly 21% O_2 as a control to depict an atmospheric state. *S1PR2* did not show significant differences in expression at 2.5%, 12% and 21% oxygen (FIGURE 4**c**); *S1PR3* on the other hand showed no significant change from 2.5% to 12% O_2 but from there a significant decrease when cultured under 21% O_2 ($P = 0.0240$) (FIGURE 4D).

As platelets are the first maternal blood cells that establish contact to fetal-derived trophoblast cells, the effect of co-incubation of platelets and trophoblast cells was examined. As platelets express various S1P receptors (Supplementary Figure 1), experiments with direct co-incubation of platelets and trophoblast cells would be difficult to analyse because expression of the cellular origin of a certain receptor cannot be verified. To exclude this possibility, co-incubation experiments were conducted with an insert to keep the trophoblast samples free of platelet RNA (FIGURE 4E–G). However, the platelet-derived factors may well diffuse through the 0.4 μm membrane of the insert and act on underlying BeWo cells. qPCR analysis after co-cultures revealed that *S1PR2* was significantly down-regulated in BeWo cells upon treatment with platelet-derived factors when compared with controls ($P = 0.0055$) (FIGURE 4H). It is worth noting that thrombin itself, used as an agonist to activate platelets, had an impact on the *S1PR2* expression level ($P = 0.0461$). Contrary effects, although not significant, could be seen in the expression of *S1PR3*,

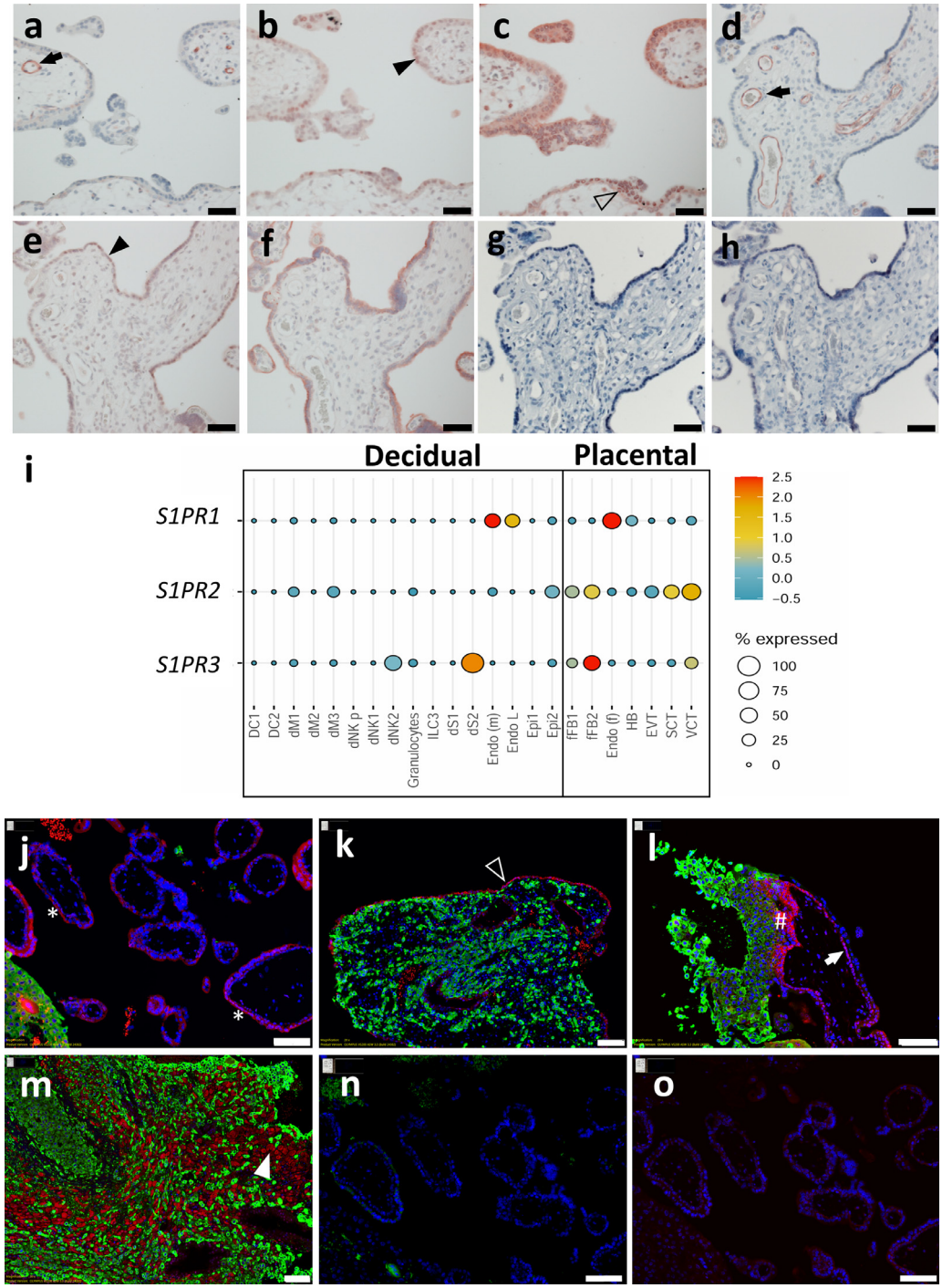

FIGURE 2 Differential expression of S1PR1, S1PR2 and S1PR3 across different cell types of the human placenta. First trimester placenta (a–c) and term placenta (d–f) were stained for antibodies S1PR1 (a and d), S1PR2 (b and e) and S1PR3 (c and f). Fetal–placental endothelial cells: black arrow, cytotrophoblast: open arrowhead, syncytiotrophoblast: black arrowhead. Negative controls rabbit IgG in term placenta for both antigen retrieval protocols (pH6 in g and pH9 in h). Scale bars represent 50 μm. Single-cell RNA-Seq expression levels of S1P receptor subtypes (i). Published data for first trimester placenta from *Vento-Tormo* et al. *(2018)* were analysed. Intensity and colour scale (raw counts) show the gene expression level and dot circumference shows % of cells within a cell type group expressing a gene. DC = dendritic cells; dM = decidual macrophages; dNK = decidual natural killer cells; dS = decidual stromal cells; Endo = endothelial cells; Epi = epithelial glandular cells; EVT = extravillous trophoblast; f = fetal; FB = fibroblasts; HB = Hofbauer cells; ILC = innate lymphocyte cells; L = lymphatic; m = maternal; p = proliferative; SCT = syncytiotrophoblast; VCT = villous cytotrophoblast. First trimester placentas were stained with immunofluorescence double staining for HLA-G (j–m) (FITC – green) and S1PR2 (j and k) or S1PR3 (l and m) (Cy3 – red). Villous syncytiotrophoblast (*), lining at the decidual border (white open arrowhead), cytotrophoblasts (white arrow), the first proximal parts of the trophoblast cell column (#), decidual stroma cells (white arrowhead). Negative control rabbit and mouse IgG on first trimester placenta (mouse IgG – FITC in n and rabbit IgG – Cy3 in o). Scale bars represent 100 μm.

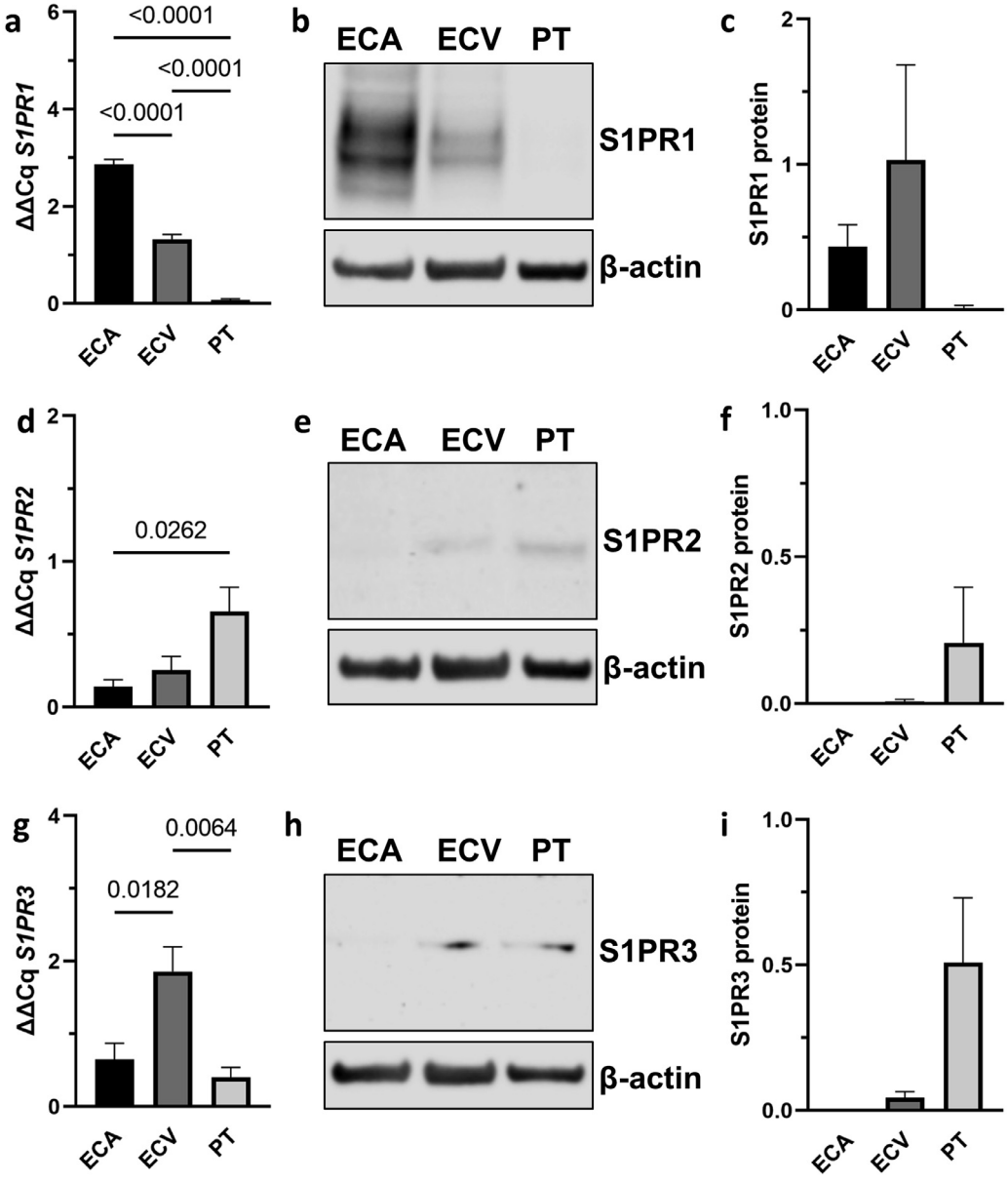

FIGURE 3 Differential expression of S1PR1, S1PR2 and S1PR3 across isolated primary cells of the human placenta. mRNA was investigated in arterial endothelial cells (ECA), venous endothelial cells (ECV) and primary trophoblasts (PT) ($n = 4$ each group) for *S1PR1* (a), *S1PR2* (d), *S1PR3* (g) and protein expression ($n = 3$ each group) for S1PR1 (43 kDa) (b and c), S1PR2 (50–60 kDa) (e and f) and S1PR3 (42 kDa) (h and i). Data are presented as mean ± SEM. *GAPDH/TBP* was used as reference gene for quantitative polymerase chain reaction, and β-actin (42 kDa) as reference protein in densitometry analysis. Differences were assessed by one-way analysis of variance, with post-hoc differences by Tukey's multiple comparison test.

which increased in the presence of both thrombin alone and platelet-derived factors (FIGURE 4I).

DISCUSSION

This study demonstrates the spatiotemporal expression of different S1PR in the human placenta across gestation. While placental *S1PR* subtypes 1 and 3 increase with gestational age, *S1PR2* is predominantly expressed in the first trimester villous trophoblast compartment and sharply declines towards term. In human trophoblasts, S1P action is suggested to inhibit differentiation through G(i)-coupled S1P receptor interaction, leading to the inhibition of adenylate cyclase and reduced production of intracellular cAMP (*Johnstone* et al., *2005*; *Singh* et al., *2012*). However, whether or not this inhibitory effect is mediated through a particular S1PR, e.g. through the prevailing S1PR2 in first trimester placenta, still remains to be determined. Of note, S1P acts as a potent inhibitor of extravillous trophoblast migration, predominantly through S1PR2/Gα12/13 and Rho activation (*Westwood* et al., *2017*). Moreover, S1PR2 is involved in S1P-induced expression and secretion of IL6 in BeWo cells (*Goyal* et al., *2013*). Based on these previous and our own studies, it is tempting to speculate that the predominant S1PR2 expression in human first trimester placenta is involved in regulating trophoblast differentiation and migration/invasion processes, both essential for early placentation. Later on in

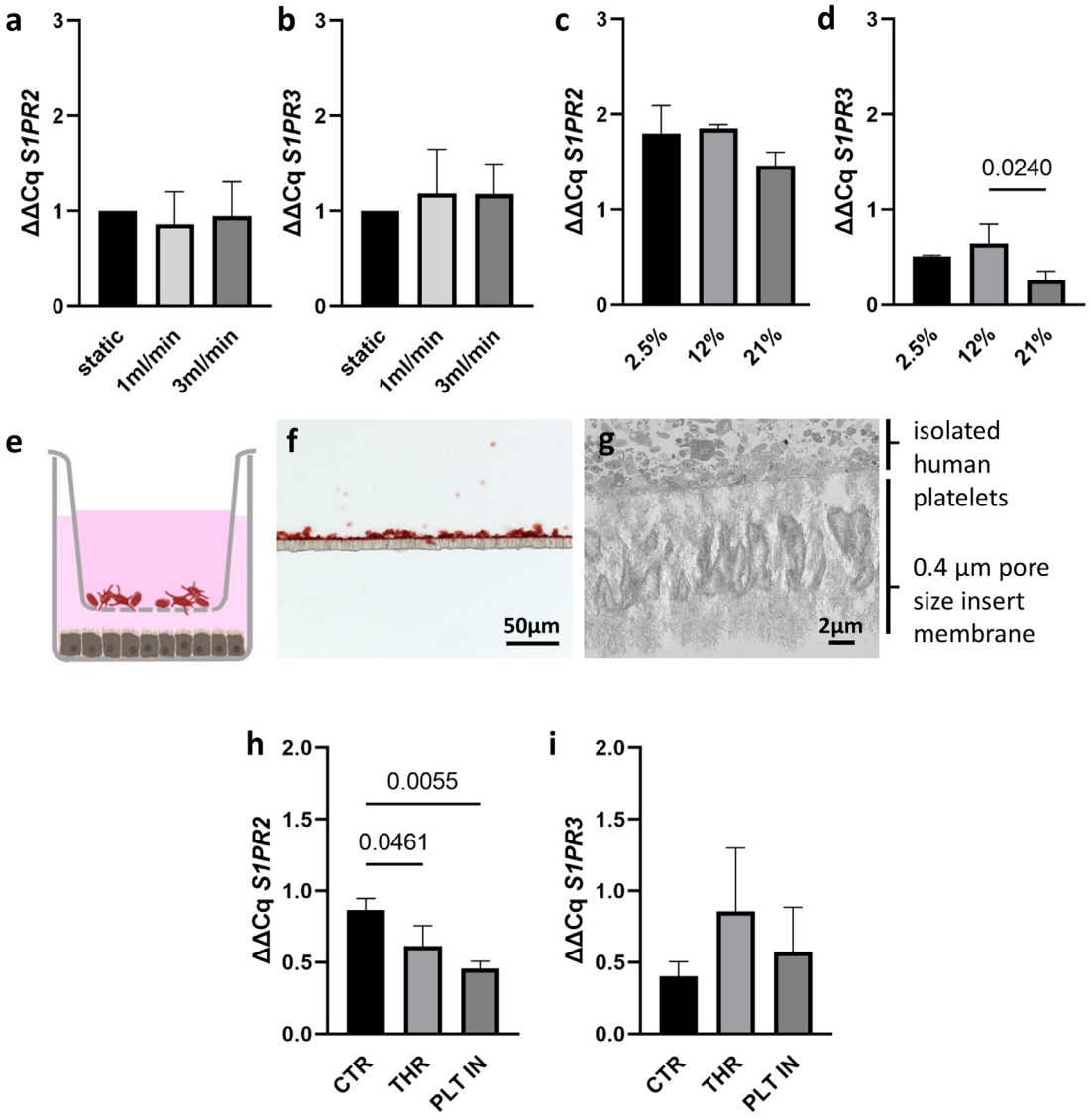

FIGURE 4 Effect of fluidic flow, different oxygen concentrations and platelet-derived factors on S1PR2 and S1PR3 expression in trophoblast cell line BeWo. BeWo cells were stimulated with forskolin (20 μmol/l) for 48 h to undergo differentiation under static conditions. Thereafter, cells were cultured under fluidic flow culture with indicated flow rates of 1 ml/min and 3 ml/min, respectively, for 24 h. Expression levels of *S1PR2* (a) and *S1PR3* (b) were compared with quantitative polymerase chain reaction (qPCR) between cells cultured under static control conditions and different flow rates (n = 4 independent experiments). After 48 h of differentiation, BeWo cells were cultured under three different oxygen concentrations (2.5%, 12% and 21% O_2) for 24 h. Expression levels of *S1PR2* (c) and *S1PR3* (d) were compared with qPCR between the different oxygen concentrations (n = 3 independent experiments). After 48 h of differentiation BeWo cells were co-incubated with freshly isolated human platelets from pregnant donors. 2.25 $\times$ 10^5 to 3.13 $\times$ 10^5 platelets per μl in a total of 500 μl were loaded into an insert and activated with thrombin (1 IU/ml). Released platelet cargo could penetrate through the 0.4 μm insert membrane to get in contact with the underlying layer of trophoblast cells (e). The insert membrane was embedded for immunohistochemistry after the experiment and stained for platelet marker CD42b (f) scale bar represents 50 μm. The membrane was also embedded for transmission electron microscopy to further visualize the membrane and the incubated platelets on one side of the membrane (g). Scale bar represents 2 μm. Expression of *S1PR2* (h) and *S1PR3* (i) were compared with qPCR between the control (CTR), thrombin control (THR) and platelet-derived factors (PLT IN) (n = 3 independent experiments). All data are presented as mean $\Delta\Delta$Cq $\pm$ SD, *GAPDH/TBP* was used as reference gene for qPCR and differences assessed by one-way analysis of variance with post-hoc differences by Tukey's multiple comparison test.

pregnancy, when the initial phase of rapid cell mass expansion and invasion is replaced by metabolic processes, expression of the placental S1PR repertoire may shift to meet these demands. Significantly reduced levels of *S1PR1* and *S1PR3* in non-severe as well as severe pre-eclampsia cases are suggested to be responsible for impaired vasculogenesis/angiogenesis observed during this syndrome (*Dobierzewska* et al., *2016*), and may be a sign for an insufficient shift in the placental S1PR repertoire.

This study confirms the expression of S1PR1 in endothelial cells, which is in agreement with previous studies showing the expression of this receptor subtype in the endothelium of various other organs (*Panetti, 2002*; *Wang* et al., *1999*) and to be strongly induced by shear stress in the vascular network of flow-positive regions (*Josipovic* et al., *2018*; *Jung* et al., *2012*). S1PR2 and S1PR3 were mainly found in

trophoblasts, which is in accordance with the speculations that these two receptor subtypes play a significant role in trophoblast development and remodelling of spiral arteries. This assumption is substantiated by recent studies highlighting the involvement of S1PR2 in trophoblast mobility (*Liao* et al., *2022*).

S1PR are increasingly acknowledged for their role as cellular mechanosensors that convert various physical signals, such as laminar and turbulent flow, into biochemical signals, coupling them with downstream signalling pathways (*Hu* et al., *2022*). In vascular endothelial cells, S1PR1 is described as one of the predominant mechanosensors, which is up-regulated by laminar shear stress itself, as shown in HUVEC cultured under laminar shear stress of 20 dyne/cm^2 (*Aoki* et al., *2007*; *Josipovic* et al., *2018*). During human gestation, uteroplacental blood flow is fully established with transition to the second trimester, causing fluid shear stress that is produced by haemodynamic forces across different trophoblast subtypes (*Brugger* et al., *2020*). Whether onset of maternal blood flow is triggering the observed (up) regulation of placental S1PR towards term remains speculative. Our data from flow culture experiments rather argue against this speculation, because neither *S1PR2* nor *S1PR3* were up-regulated in differentiated BeWo cells in response to fluid flow.

Low oxygen concentrations of about 2–3% in the early developing placenta are a special attribute described for the first couple of weeks during pregnancy, while with ongoing placentation and remodelling of the spiral arteries the oxygen concentration increases to about 8–10% (*Zhao* et al., *2021*). Previous studies have shown a significant up-regulation of SPHK1 under low oxygen tension, but a decrease in expression levels of receptors *S1PR2* and *S1PR3* in pre-eclamptic placentas (*Dobierzewska* et al., *2016*). Because this study found no significant difference in the expression level of *S1PR2* or *S1PR3* when cells were cultured under 2.5% or 12% oxygen, the tremendous difference in the expression pattern of the receptors from first trimester to term is most likely not due to the rise in oxygen concentrations over gestation. The controls incubated at atmospheric conditions (21% oxygen) showed a slight decrease in the expression level of *S1PR2* and even a significant difference in the expression of *S1PR3*, but it can be assumed that this condition is considered rather hyperoxic and therefore not physiologically present in the human placenta (*Burton* et al., *2021*).

During uncomplicated pregnancy, the count of maternal platelets decreases gradually over time of gestation, reaching an overall decrease of about 10% by the end of pregnancy (*Michelson* et al., *2019*). This decrease in platelet count can be explained mainly by haemodilution, and to some extent by accelerated sequestration and consumption of platelets in the utero-placental circulation. Increased mean platelet volume (MPV), as a measure of platelet activation, has been associated with inflammatory pregnancy pathologies, including pre-eclampsia and IUGR (*Guettler* et al., *2022*; *Moser* et al., *2019*). Before haemochorial placentation is fully established, extravillous trophoblasts form plugs within the lumen of spiral arteries to prevent maternal blood cells from entering the intervillous space. However, by the middle of the first trimester these trophoblast plugs become loosely cohesive, forming narrow capillary-shaped channels (*Roberts* et al., *2017*). We and others have found platelets within trophoblast columns and assume that they can enter the channels due to their small diameter of 2–3 μm (*Forstner* et al., *2020*; *Sato* et al., *2005*). Electron microscopy confirmed that these platelets are in an activated state, tempting speculation as to whether released factors can impact the expression of the S1P receptors (*Guettler* et al., *2021*). The extensive reports of the involvement of S1P in inflammatory processes and the fact that a link between a dysfunction in the sphingolipid signature and pre-eclampsia has been discussed before, makes it an obligation to investigate the dysregulation of the receptors upon contact with platelet-derived factors (*Del Gaudio* et al., *2020*; *Dobierzewska* et al., *2016*; *Spiegel and Milstien, 2011*). The approach of incubating platelets from pregnant women in cell culture inserts above trophoblasts without any direct contact provides a perfect model to study the effect of platelet-derived factors without having to deal with the bias that platelets themselves express S1PR.

The effect observed on *S1PR2* and *S1PR3* subtypes upon treatment with thrombin alone can be drawn back to the fact that thrombin is a versatile enzyme, thereby able to bind to numerous substrates and thus affecting the molecular basis of cells in many ways (*Davie and Kulman, 2006*). However, because the difference in expression of *S1PR2* upon treatment with platelet-derived factors is even higher compared with the difference seen with cells treated with thrombin alone, it can be argued that a considerable part of the effect is solely due to factors released from platelets. Interestingly, platelet-derived factors seem to have contrary effects on the expression levels of *S1PR2* and *S1PR3*. The significant down-regulation of *S1PR2* and the tendency of up-regulation of *S1PR3* demonstrated here could be speculated to be in concordance with the opening of the spiral arteries and the influx of maternal blood into the intervillous space at the end of the first trimester of pregnancy. Here, one could speculate that an earlier influx of maternal blood, and thus potential activation of maternal platelets and release of their cargo, would dysregulate the expression pattern of the S1PR too soon and therefore could take part in pregnancy complications with insufficient infiltration and migration of extravillous trophoblasts into the maternal decidua. Further research is necessary to evaluate the timeline of S1PR pattern change and the influence this could have on the developing human placenta. Although the underlying mechanisms of S1PR2 and S1PR3 dysregulation are not widely discussed in the literature, it has been shown that S1PR1 expression on the cell surface is in fact down-regulated by its activation of agonists, like small-molecule FTY720-P, HDL-S1P or albumin-S1P (*Cartier and Hla, 2019*). We suggest a very similar response of S1PR2 due to the fact that it is down-regulated upon excessive release of platelet cargo, which includes S1P.

The lack of data for placental S1PR subtypes 4 and 5 at various stages of pregnancy is a clear limitation of the current study, but may be justified by the fact that accurate detection of endogenous S1PR proteins is *per se* a challenge and specific antibodies have either been discontinued or simply were not available (*Talmont* et al., *2019*). However, comparative S1PR expression data from first trimester, pre-term and term placenta samples, and furthermore the use of platelets from pregnant women, can be considered a strength of this study. This is the first report on S1PR expression analysis in a trophoblast cell line cultured under fluidic flow in combination with low oxygen conditions. Moreover, to our knowledge this is the first paper to discuss the impact of platelet-derived factors on the expression pattern of the S1P receptors in the human trophoblast.

In summary, this study suggests that the placental S1PR repertoire is differently expressed across gestation. The trophoblastic S1PR expression pattern depends on differentiation status of the cells and may be dysregulated in response to platelet-derived factors, including S1P.

DATA AVAILABILITY

Data will be made available on request.

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AUTHOR CONTRIBUTIONS

FL, JG and MG conceived and designed the study, analysed data and drafted the manuscript; FL, JG, BAB, DF, SW, CF and ON performed experiments and analysed data; CS and BH provided material and all authors revised the manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.04.006.

REFERENCES

Aoki, S., Osada, M., Kaneko, M., Ozaki, Y., Yatomi, Y., 2007. Fluid shear stress enhances the sphingosine 1-phosphate responses in cell-cell interactions between platelets and endothelial cells. Biochem. Biophys. Res. Commun. 358, 1054–1057. https://doi.org/10.1016/j.bbrc.2007.05.028.

Blaschitz, A., Siwetz, M., Schlenke, P., Gauster, M., 2015. Adhering maternal platelets can contribute to the cytokine and chemokine cocktail released by human first trimester villous placenta. Placenta 36, 1333–1336. https://doi.org/10.1016/j.placenta.2015.09.002.

Brugger, B.A., Guettler, J., Gauster, M., 2020. Go with the flow – trophoblasts in flow culture. Int. J. Mol. Sci. 21, E4666. https://doi.org/10.3390/ijms21134666.

Bryan, A.M., Del Poeta, M., 2018. Sphingosine-1-phosphate receptors and innate immunity. Cell. Microbiol. 20, e12836. https://doi.org/10.1111/cmi.12836.

Burton, G.J., Cindrova-Davies, T., Yung, H.W., Jauniaux, E., 2021. HYPOXIA AND REPRODUCTIVE HEALTH: Oxygen and development of the human placenta. Reprod. Camb. Engl. 161, F53–F65. https://doi.org/10.1530/REP-20-0153.

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., Satija, R., 2018. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. 36, 411–420. https://doi.org/10.1038/nbt.4096.

Cartier, A., Hla, T., 2019. Sphingosine 1-phosphate: lipid signaling in pathology and therapy. Science 366, eaar5551. https://doi.org/10.1126/science.aar5551.

Charkiewicz, K., Goscik, J., Blachnio-Zabielska, A., Raba, G., Sakowicz, A., Kalinka, J., Chabowski, A., Laudanski, P., 2017. Sphingolipids as a new factor in the pathomechanism of preeclampsia – mass spectrometry analysis. PloS One 12, e0177601. https://doi.org/10.1371/journal.pone.0177601.

Davie, E.W., Kulman, J.D., 2006. An overview of the structure and function of thrombin. Semin. Thromb. Hemost. 32 (Suppl 1), 3–15. https://doi.org/10.1055/s-2006-939550.

Del Gaudio, I., Sasset, L., Di Lorenzo, A., Wadsack, C., 2020. Sphingolipid signature of human feto-placental vasculature in preeclampsia. Int. J. Mol. Sci. 21, 1019. https://doi.org/10.3390/ijms21031019.

Dobierzewska, A., Palominos, M., Sanchez, M., Dyhr, M., Helgert, K., Venegas-Araneda, P., Tong, S., Illanes, S.E., 2016. Impairment of angiogenic sphingosine kinase-1/sphingosine-1-phosphate receptors pathway in preeclampsia. PloS One 11, e0157221. https://doi.org/10.1371/journal.pone.0157221.

Drewlo, S., Johnson, E., Kilburn, B.A., Kadam, L., Armistead, B., Kohan-Ghadr, H.-R., 2020. Irisin induces trophoblast differentiation via AMPK activation in the human placenta. J. Cell. Physiol. 235, 7146–7158. https://doi.org/10.1002/jcp.29613.

Fakhr, Y., Brindley, D.N., Hemmings, D.G., 2021. Physiological and pathological functions of sphingolipids in pregnancy. Cell. Signal. 85, 110041. https://doi.org/10.1016/j.cellsig.2021.110041.

Forstner, D., Maninger, S., Nonn, O., Guettler, J., Moser, G., Leitinger, G., Pritz, E., Strunk, D., Schallmoser, K., Marsche, G., Heinemann, A., Huppertz, B., Gauster, M., 2020. Platelet-derived factors impair placental chorionic gonadotropin beta-subunit synthesis. J. Mol. Med. Berl. Ger. 98, 193–207. https://doi.org/10.1007/s00109-019-01866-x.

Goyal, P., Brünnert, D., Ehrhardt, J., Bredow, M., Piccenini, S., Zygmunt, M., 2013. Cytokine IL-6 secretion by trophoblasts regulated via sphingosine-1-phosphate receptor 2 involving Rho/Rho-kinase and Rac1 signaling pathways. Mol. Hum. Reprod. 19, 528–538. https://doi.org/10.1093/molehr/gat023.

Guettler, J., Forstner, D., Cvirn, G., Maninger, S., Brugger, B.A., Nonn, O., Kupper, N., Pritz, E., Wernitznig, S., Dohr, G., Hutter, H., Juch, H., Isermann, B., Kohli, S., Gauster, M., 2021. Maternal platelets pass interstices of trophoblast columns and are not activated by HLA-G in early human pregnancy. J. Reprod. Immunol. 144, 103280. https://doi.org/10.1016/j.jri.2021.103280.

Guettler, J., Forstner, D., Gauster, M., 2022. Maternal platelets at the first trimester maternal-placental interface – small players with great impact on placenta development. Placenta 125, 61–67. https://doi.org/10.1016/j.placenta.2021.12.009.

Hu, Y., Chen, M., Wang, M., Li, X., 2022. Flow-mediated vasodilation through mechanosensitive G protein-coupled receptors in endothelial cells. Trends Cardiovasc. Med. 32, 61–70. https://doi.org/10.1016/j.tcm.2020.12.010.

Johnstone, E.D., Chan, G., Sibley, C.P., Davidge, S.T., Lowen, B., Guilbert, L.J., 2005. Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G(i)-coupled receptor response. J. Lipid Res. 46, 1833–1839. https://doi.org/10.1194/jlr.M500095-JLR200.

Josipovic, I., Pflüger, B., Fork, C., Vasconez, A.E., Oo, J.A., Hitzel, J., Seredinski, S., Gamen, E., Heringdorf, D.M.Z., Chen, W., Looso, M., Pullamsetti, S.S., Brandes, R.P., Leisegang, M.S., 2018. Long noncoding RNA LISPR1 is required for S1P signaling and endothelial cell function. J. Mol. Cell. Cardiol. 116, 57–68. https://doi.org/10.1016/j.yjmcc.2018.01.015.

Jung, B., Obinata, H., Galvani, S., Mendelson, K., Ding, B., Skoura, A., Kinzel, B., Brinkmann, V., Rafii, S., Evans, T., Hla, T., 2012. Flow-regulated endothelial S1P receptor-1 signaling sustains vascular development. Dev. Cell 23, 600–610. https://doi.org/10.1016/j.devcel.2012.07.015.

Kerage, D., Brindley, D.N., Hemmings, D.G., 2014. Review: novel insights into the regulation of vascular tone by sphingosine 1-phosphate. Placenta 35 (Suppl), S86–S92. https://doi.org/10.1016/j.placenta.2013.12.006.

Książek, M., Chacińska, M., Chabowski, A., Baranowski, M., 2015. Sources, metabolism, and regulation of circulating sphingosine-1-phosphate. J. Lipid Res. 56, 1271–1281. https://doi.org/10.1194/jlr.R059543.

Lang, I., Schweizer, A., Hiden, U., Ghaffari-Tabrizi, N., Hagendorfer, G., Bilban, M., Pabst, M.A., Korgun, E.T., Dohr, G., Desoye, G., 2008. Human fetal placental endothelial cells have a mature arterial and a juvenile venous phenotype with adipogenic and osteogenic differentiation potential. Differ. Res. Biol. Divers. 76, 1031–1043. https://doi.org/10.1111/j.1432-0436.2008.00302.x.

Li, Q., Pan, Z., Wang, X., Gao, Z., Ren, C., Yang, W., 2014. miR-125b-1-3p inhibits trophoblast cell invasion by targeting sphingosine-1-phosphate

receptor 1 in preeclampsia. Biochem. Biophys. Res. Commun. 453, 57–63. https://doi.org/10.1016/j.bbrc.2014.09.059.

Liao, J., Zheng, Y., Hu, M., Xu, P., Lin, L., Liu, X., Wu, Y., Huang, B., Ye, X., Li, S., Duan, R., Fu, H., Huang, J., Wen, L., Fu, Y., Kilby, M.D., Kenny, L.C., Baker, P.N., Qi, H., Tong, C., 2022. Impaired sphingosine-1-phosphate synthesis induces preeclampsia by deactivating trophoblastic YAP (yes-associated protein) through S1PR2 (sphingosine-1-phosphate receptor-2)-induced actin polymerizations. Hypertension 79, 399–412. https://doi.org/10.1161/HYPERTENSIONAHA.121.18363.

Loegl, J., Nussbaumer, E., Cvitic, S., Huppertz, B., Desoye, G., Hiden, U., 2017. GDM alters paracrine regulation of feto-placental angiogenesis via the trophoblast. Lab. Investig. J. Tech. Methods Pathol. 97, 409–418. https://doi.org/10.1038/labinvest.2016.149.

Maceyka, M., Harikumar, K.B., Milstien, S., Spiegel, S., 2012. Sphingosine-1-phosphate signaling and its role in disease. Trends Cell Biol 22, 50–60. https://doi.org/10.1016/j.tcb.2011.09.003.

Michelson, A.D., Cattaneo, M., Frelinger, A.L., Newman, P.J, 2019. Platelets, fourth ed. Elsevier/Academic Press, London.

Moser, G., Guettler, J., Forstner, D., Gauster, M., 2019. Maternal platelets — friend or foe of the human placenta? Int. J. Mol. Sci. 20, E5639. https://doi.org/10.3390/ijms20225639.

Nagamatsu, T., Iwasawa-Kawai, Y., Ichikawa, M., Kawana, K., Yamashita, T., Osuga, Y., Fujii, T., Schust, D.J., 2014. Emerging roles for lysophospholipid mediators in pregnancy. Am. J. Reprod. Immunol. 72, 182–191. https://doi.org/10.1111/aji.12239.

Obinata, H., Hla, T., 2019. Sphingosine 1-phosphate and inflammation. Int. Immunol. 31, 617–625. https://doi.org/10.1093/intimm/dxz037.

Panetti, T., 2002. Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. Biochim. Biophys. Acta 1582, 190–196. https://doi.org/10.1016/S1388-1981(02)00155-5.

Patanapirunhakit, P., Karlsson, H., Mulder, M., Ljunggren, S., Graham, D., Freeman, D., 2021. Sphingolipids in HDL — potential markers for adaptation to pregnancy? Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1866, 158955. https://doi.org/10.1016/j.bbalip.2021.158955.

Roberts, V.H.J., Morgan, T.K., Bednarek, P., Morita, M., Burton, G.J., Lo, J.O., Frias, A.E., 2017. Early first trimester uteroplacental flow and the progressive disintegration of spiral artery plugs: new insights from contrast-enhanced ultrasound and tissue histopathology. Hum. Reprod. 32, 2382–2393. https://doi.org/10.1093/humrep/dex301.

Roth, C.J., Haeussner, E., Ruebelmann, T., Koch, F.V., Schmitz, C., Frank, H.-G., Wall, W.A., 2017. Dynamic modeling of uteroplacental blood flow in IUGR indicates vortices and elevated pressure in the intervillous space—a pilot study. Sci. Rep. 7, 40771. https://doi.org/10.1038/srep40771.

Sato, Y., Fujiwara, H., Zeng, B.-X., Higuchi, T., Yoshioka, S., Fujii, S., 2005. 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, 428–435. https://doi.org/10.1182/blood-2005-02-0491.

Singh, A.T., Dharmarajan, A., Aye, I.L.M.H., Keelan, J.A., 2012. Sphingosine—sphingosine-1-phosphate pathway regulates trophoblast differentiation and syncytialization. Reprod. Biomed. Online 24, 224–234. https://doi.org/10.1016/j.rbmo.2011.10.012.

Siwetz, M., Sundl, M., Kolb, D., Hiden, U., Herse, F., Huppertz, B., Gauster, M., 2015. Placental fractalkine mediates adhesion of THP-1 monocytes to villous trophoblast. Histochem. Cell Biol. 143, 565–574. https://doi.org/10.1007/s00418-014-1304-0.

Spiegel, S., Milstien, S., 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell Biol. 4, 397–407. https://doi.org/10.1038/nrm1103.

Spiegel, S., Milstien, S., 2011. The outs and the ins of sphingosine-1-phosphate in immunity. Nat. Rev. Immunol. 11, 403–415. https://doi.org/10.1038/nri2974.

Takabe, K., Paugh, S.W., Milstien, S., Spiegel, S., 2008. Inside-out' signaling of sphingosine-1-phosphate: therapeutic targets. Pharmacol. Rev. 60, 181–195. https://doi.org/10.1124/pr.107.07113.

Talmont, F., Moulédous, L., Baranger, M., Gomez-Brouchet, A., Zajac, J.-M., Deffaud, C., Cuvillier, O., Hatzoglou, A., 2019. Development and characterization of sphingosine 1-phosphate receptor 1 monoclonal antibody suitable for cell imaging and biochemical studies of endogenous receptors. PloS One 14, e0213203. https://doi.org/10.1371/journal.pone.0213203.

Tukijan, F., Chandrakanthan, M., Nguyen, L.N., 2018. The signalling roles of sphingosine-1-phosphate derived from red blood cells and platelets. Br. J. Pharmacol. 175, 3741–3746. https://doi.org/10.1111/bph.14451.

Vento-Tormo, R., Efremova, M., Botting, R.A., Turco, M.Y., Vento-Tormo, M., Meyer, K.B., Park, J.-E., Stephenson, E., Polański, K., Goncalves, A., Gardner, L., Holmqvist, S., Henriksson, J., Zou, A., Sharkey, A.M., Millar, B., Innes, B., Wood, L., Wilbrey-Clark, A., Payne, R.P., Ivarsson, M.A., Lisgo, S., Filby, A., Rowitch, D.H., Bulmer, J.N., Wright, G.J., Stubbington, M.J.T., Haniffa, M., Moffett, A., Teichmann, S.A., 2018. Single-cell reconstruction of the early maternal-fetal interface in humans. Nature 563, 347–353. https://doi.org/10.1038/s41586-018-0698-6.

Wang, F., Van Brocklyn, J.R., Hobson, J.P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., Spiegel, S., 1999. Sphingosine 1-phosphate stimulates cell migration through a Gi-coupled cell surface receptor. J. Biol. Chem. 274, 35343–35350. https://doi.org/10.1074/jbc.274.50.35343.

Westwood, M., Al-Saghir, K., Finn-Sell, S., Tan, C., Cowley, E., Berneau, S., Adlam, D., Johnstone, E.D., 2017. Vitamin D attenuates sphingosine-1-phosphate (S1P)-mediated inhibition of extravillous trophoblast migration. Placenta 60, 1–8. https://doi.org/10.1016/j.placenta.2017.09.009.

Yamamoto, Y., Olson, D.M., van Bennekom, M., Brindley, D.N., Hemmings, D.G., 2010. Increased expression of enzymes for sphingosine 1-phosphate turnover and signaling in human decidua during late pregnancy. Biol. Reprod. 82, 628–635. https://doi.org/10.1095/biolreprod.109.081497.

Zhao, H., Wong, R.J., Stevenson, D.K., 2021. The impact of hypoxia in early pregnancy on placental cells. Int. J. Mol. Sci. 22, 9675. https://doi.org/10.3390/ijms22189675.

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