



OPEN Variations in blood levels of the anti-fusogenic endogenous retroviral protein suppressyn are associated with fetal growth outcome in preeclampsia

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Suppressyn, a placenta-specific protein characterized by its inhibitory effect on trophoblast cell fusion, is considered to play a direct role in the formation and maintenance of placental villi in humans. Given the potential involvement of aberrant suppressyn expression in the development of placenta-dependent disorders, we focused our analysis on hypertensive disorders of pregnancy, with particular emphasis on preeclampsia (PE). PE is a leading cause of maternal morbidity and mortality worldwide and poses a serious risk to fetal growth and survival. As delivery remains the only definitive treatment and no effective methods for prevention or early diagnosis have been established, there is an urgent need for investigation from novel perspectives. In the present study, we investigated the expression profiles of suppressyn and fusion-associated molecules in human placental villous tissues and maternal blood samples. Our analysis revealed two key findings: (1) suppressyn protein levels are significantly reduced in placentas from pregnancies complicated by PE with fetal growth restriction (FGR), and (2) suppressyn secretion is modulated in an ASCT2 expression-dependent manner, suggesting that the intracellular balance between suppressyn and this transporter may play a critical role in the pathophysiology of PE and in maintaining placental function. Detectable alterations in maternal serum concentrations of secreted suppressyn in pregnancies affected by hypertensive disorders of pregnancy may offer a novel biomarker that monitors placental developmental status in these disease states. These findings may also provide new insights into the molecular underpinnings of PE and a mechanistic link between suppressyn dysregulation and the development of FGR.

Keywords Suppressyn, Pre-eclampsia, Fetal growth restriction, Cell fusion, ASCT2

The placenta is an essential organ for the maintenance of pregnancy and the normal development of the fetus^{1,2}. Its functions include gas exchange and transport of essential nutrients and waste products between mother and fetus, production of essential hormones during pregnancy, and protection of the fetus from harmful blood-borne exposures. This wide variety of functions is primarily carried out by the syncytiotrophoblast, a membranous cell covering the entire placental villous surface that is formed by the fusion and multinucleation of underlying cytotrophoblast cells³. It is widely recognized that alterations in this cell fusion process can have dramatic effects on overall placental and fetal development. Indeed, it has been reported that structural and functional defects in the placenta can lead to perinatal complications like preeclampsia (PE)^{4,5} and fetal growth restriction (FGR)⁶. PE is a pregnancy-specific complication with a prevalence of 5–8% of pregnancies that is characterized by maternal hypertension and proteinuria. The syndrome is classified into two distinct categories: early-onset PE, which occurs before 34 weeks' gestation, and late-onset PE, which occurs after 34 weeks. While the latter is more prevalent, early-onset PE is associated with more severe fetal complications, including FGR.

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The pathogenesis of the syndrome is believed to be the result of abnormal infiltration of the invasive trophoblast sub lineage, the extravillous trophoblast (EVT) cells, into the decidua with associated reductions in essential maternal spiral artery remodeling necessary to allow for adequate placental perfusion^{5,7}. In addition, histological abnormalities and alterations in cellular proliferation can be observed in the floating villi in PE, particularly in its hormone secreting syncytiotrophoblast-covered surface^{8–10}. These morphologic changes are reflected in alterations in maternal serum levels of human chorionic gonadotropin (hCG)^{11,12}, a hormone secreted solely by the syncytiotrophoblast. Increased secretion of soluble fms-like tyrosine kinase-1 (sFlt-1) has also been observed in cases of early-onset PE¹³. Since both cytotrophoblast cells and the syncytiotrophoblast produce sFlt-1, this also suggests impaired syncytiotrophoblast differentiation in disease. Finally, low maternal serum hCG levels during the first trimester have been reported to correlate with an elevated risk of FGR¹⁴, further implicating aberrant syncytiotrophoblast formation in the pathogenesis of FGR. Together, these changes in placental form and function will ultimately manifest as maternal and fetal disease. Due to its complex etiology, efficient prediction and treatment methods for PE are not available and termination of pregnancy is the only effective solution. Therefore, there is an urgent need to elucidate the etiology and to develop biomarkers for early detection that allow therapeutic intervention for this devastating disease¹⁵. Analysis of the dynamics of molecules involved in cell fusion, a key step in placentogenesis, may be useful in determining the cause of this disease^{16,17}. Recently, several molecules involved in cell fusion in placental villous tissue have been identified, including suppressyn¹⁸. A common feature of these molecules is that they are derived from endogenous retroviral sequences that originated from germline infections by retroviruses and that became part of the host genome^{19–24}. The syncytin-1²⁵ and syncytin-2²⁶ proteins, which are responsible for cell fusion, are translation products of HERV sequences located on human chromosome 7q21.2 (*ERVW-1*) and chromosome 6p24.2 (*ERVFRD-1*), respectively. Suppressyn, transcribed from chromosome 21q22.3 (*ERVH48-1*), was the first protein to be isolated and reported by us as a suppressor of cell fusion^{27,28}. Human suppressyn is a secreted protein that is characterized by its placenta-specific expression. It is hypothesized that this protein regulates cell fusion in an inhibitory manner by direct association with ASCT2, the receptor for syncytin-1^{27,29}. Suppressyn protein in the human placenta has been specifically localized to unfused CT and EVT, and regulation of its gene expression is dependent on oxygen concentration^{30–32}. Altered expression of suppressyn may have several effects on placental development that depend on its location and levels^{33,34}. When suppressyn expression is reduced, extravillous trophoblast (EVT) cells may aberrantly fuse with decidual cells, potentially impairing their invasive capacity and proper remodeling of spiral arteries. While low levels of suppressyn in the floating villi may result in syncytiotrophoblast hyperplasia and excessive secretion of human chorionic gonadotropin (hCG), excessive suppressyn expression may inhibit syncytiotrophoblast formation, leading to placental immaturity and insufficient functional development. Various abnormalities in syncytiotrophoblast differentiation have been reported in PE^{8–10,35}, and the dysregulation of suppressyn expression may represent one potential mechanism underlying this complexity.

In this study, we aimed to further study the pathogenesis of PE and to identify novel biomarkers of disease by focusing on genes related to trophoblast cell fusion, particularly suppressyn. Our analyses revealed a marked decrease in suppressyn protein levels in the placentas of PE patients compared to controls over the course of gestation, specifically when associated with the presence of fetal growth restriction. We already have developed methods to detect secreted forms of suppressyn in maternal blood and showed that levels of secreted suppressyn can differentiate PE subtypes. Importantly, changes in serum suppressyn levels were closely associated with decreased expression of its receptor, ASCT2, in placental trophoblasts, indicating that maternal blood levels of suppressyn may reflect the degree of receptor-ligand interaction within the placental tissue. These findings suggest that suppressyn is a dynamic protein whose expression levels are associated with pathological conditions such as PE and FGR. As such, suppressyn may serve as a clinically useful biomarker for both disease detection and therapeutic decision-making.

Results

Altered expression of trophoblast cell fusion-related genes in preeclampsia

To investigate the molecular pathogenesis of PE, we performed a quantitative gene expression analysis using placental tissues from 22 gestational age-matched control samples, 17 early-onset PE (EO-PE) samples, and 8 late-onset PE (LO-PE) samples. Compared with controls, both EO-PE and LO-PE groups exhibited significantly elevated maternal blood pressure and marked reductions in placental and fetal weights, confirming the pathological status of the PE samples used in this study (Supplemental Table S1). *Suppressyn* gene expression in control placentas exhibited a gestational age-dependent increase over the course of pregnancy. Placental *suppressyn* expression, however, tended to be elevated in both EO-PE and LO-PE pregnancies relative to controls, although these differences were not statistically significant (Fig. 1a). Transcription of syncytin-1, a key trophoblast fusion-promoting protein, increased until approximately 34 weeks of gestation, followed by a gradual decline toward term in control samples. A similar temporal expression profile was observed in PE samples, with no significant differences between control and PE groups (Fig. 1b). *ASCT2*, a binding partner of suppressyn and a known receptor for syncytin-1^{27,36}, demonstrated a gestational age-associated decrease in expression in all groups. Notably, a significant reduction in *ASCT2* expression was observed specifically in the EO-PE group ($p < 0.01$; Fig. 1c). Other genes exhibiting significant transcriptional downregulation in both EO-PE and LO-PE placentas when compared to controls included *syncytin-2* (Fig. 1e) and *PLGF*³⁷ (Fig. 1h), both of which play key roles in placental function. In contrast, *sFlt-1*³⁸ (Fig. 1g) and *hCGβ*³⁹ (Fig. 1d) were significantly upregulated in EO-PE placentas, consistent with the literature. No significant differences were observed in the expression of *MFSD2*⁴⁰ the receptor for syncytin-2, across any groups (Fig. 1f).

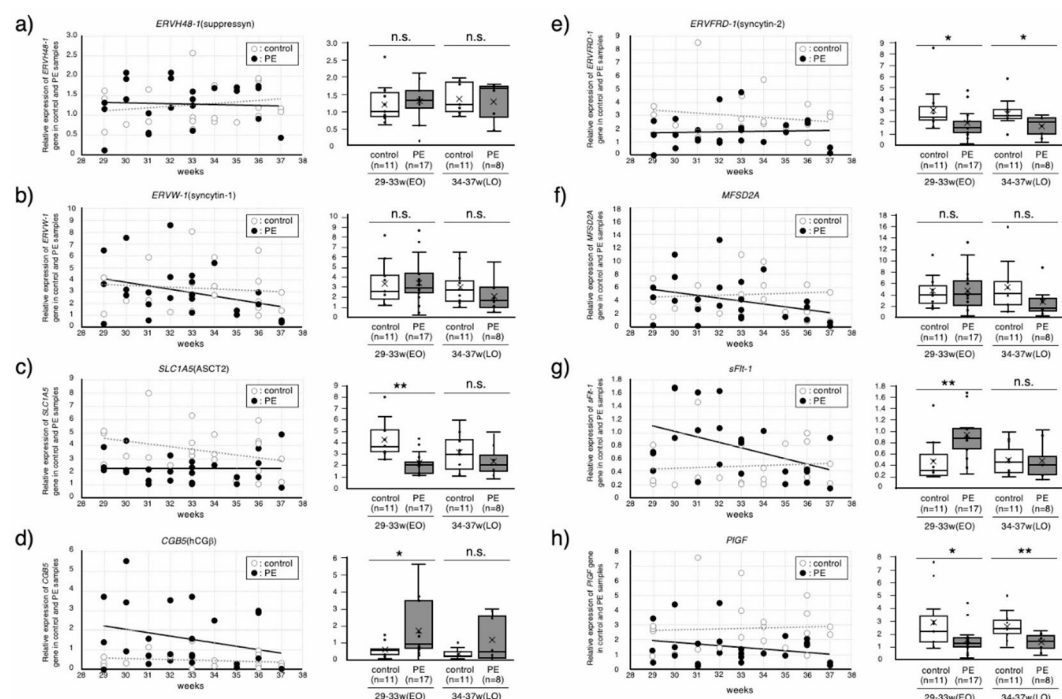


Fig. 1. Gene expression dynamics of cell fusion-related genes in trophoblast tissue from preeclampsia and control pregnancies. Gene expression in placental tissues from preeclampsia (PE) patients (29–37 weeks) was compared with that of matched control placentas over the same gestational period. The gene expression data were plotted according to gestational week (left panel), and further divided into early-onset (EO) PE (29–33 weeks) and late-onset (LO) PE (34–37 weeks) groups for comparison using box plots (right panel). Box plots display the median (horizontal center line), interquartile range (box), mean values (x) and minimum/maximum values (whiskers). The numbers in parentheses indicate the sample sizes used for each group. Statistical significance was assessed using Mann-Whitney U tests. * $p < 0.05$; ** $p < 0.01$. n.s., not significant. (A) to (H) show the gene expression of various proteins involved in trophoblast function, including suppressyn, syncytin-1, ASCT2, hCG β , syncytin-2, MFSD2, sFlt-1, and PlGF. The primers used for gene amplification are listed in Supplementary Table S3.

Altered placental expression of suppressyn protein in preeclamptic patients

Western blot analysis was performed using protein lysates extracted from placental tissues of control and PE pregnancies to examine changes in suppressyn protein levels across gestation (Fig. 2a). Although direct comparison of early and late gestational stages is somewhat limited by use of separate blots, control samples exhibited a general decrease in normalized suppressyn protein levels as gestation progressed. In contrast, PE placentas demonstrated a marked overall reduction in suppressyn protein expression, particularly at later gestational time points (Fig. 2a, lower panel). To quantify this observation, placental samples from PE and control samples were loaded on the same blot and analyzed by gestational age (Supplemental Figure S2). As shown in Fig. 2b, suppressyn expression was significantly reduced in EO-PE cases complicated by FGR ($p < 0.05$), and in LO-PE cases regardless of FGR status ($p < 0.05$). Suppressyn-specific detection revealed two distinct bands corresponding to the intracellular and secreted forms of the protein, presumably due to differential post-translational modifications³³. Notably, in PE placentas, the higher molecular weight band (triangle marker in Fig. 2a)—representing the secreted form—showed a marked reduction in signal intensity (Fig. 2a). This band ratio (secreted form / total cell associated SUPYN protein) was quantitatively compared with gestational age-matched controls to further assess disease-specific decreases in secreted suppressyn (Fig. 2c). The results demonstrated a significant reduction in secreted form of suppressyn in EO-PE, irrespective of FGR status. In LO-PE, the secreted form was significantly decreased only in cases complicated by FGR ($p < 0.05$).

Changes in maternal serum suppressyn levels in normal and preeclampsia patients

As suppressyn is a secreted protein, its concentration in maternal serum was assessed to investigate its potential as a non-invasive biomarker for PE. Serum samples were collected from 13 patients with early-onset PE (EO-PE), subdivided into FGR(–) ($n = 5$) and FGR(+) ($n = 8$), and from 21 patients with late-onset PE (LO-PE), further divided into FGR(–) ($n = 15$) and FGR(+) ($n = 6$). Gestational age-matched serum samples were also obtained from otherwise healthy pregnancies, with 13 samples collected between 29 and 33 weeks for comparison to the EO-PE samples, and 5 samples between 34 and 37 weeks for the LO-PE comparison (Supplemental Table S2, S3). Longitudinal changes in maternal serum suppressyn levels during normal pregnancy were determined using serial or single blood samples collected during routine prenatal visits (Fig. 3a). Suppressyn concentrations

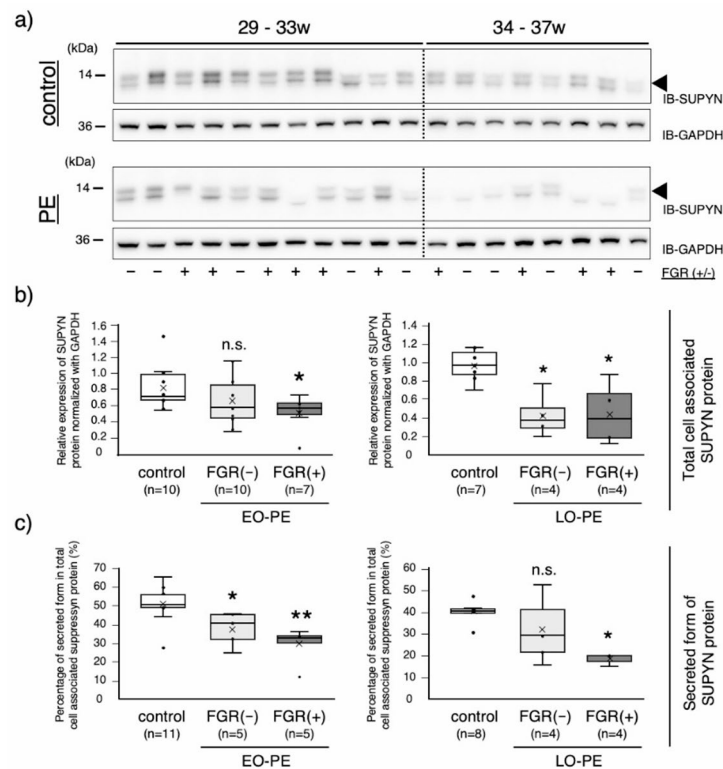


Fig. 2. Suppressyn protein expression dynamics in trophoblast tissue from preeclampsia and control pregnancies (western blot analysis). Changes in suppressyn (SUPYN) protein expression were assessed in placental trophoblast tissues from preeclampsia (PE) patients and matched controls from 29 to 37 weeks of gestation. (a) Changes in suppressyn protein expression were examined in placental trophoblast tissues from PE patients (29–37 weeks), including those with early-onset PE [EO-PE; 29–33 weeks] and late-onset PE [LO-PE; 34–37 weeks], as well as gestational age-matched controls. Due to differences among blotting intensities in control and PE samples, and across gestational weeks (indicated by the dashed lines), direct comparison of band intensity is not possible. The original blot data are shown in Supplementary Figure S1. (b) Suppressyn protein expression levels were compared between control and PE samples, with further sub-grouping based on the presence of fetal growth restriction (FGR) in EO-PE and LO-PE samples. Quantitative analysis of the data was conducted using the blots presented in Supplementary Figure S2, with original blot results shown in Supplementary Figure S3. (c) Both intracellular and secretory forms (indicated by the triangle) of suppressyn protein were present in placental trophoblast tissues. The proportion of secretory forms suppressyn in total suppressyn was calculated for each subgroup, categorized by the presence or absence of FGR (EO-PE-FGR(-), EO-PE-FGR(+), LO-PE-FGR(-), LO-PE-FGR(+)). Quantitative analysis was performed using the blots shown in Supplementary Figure S1. Box plots display the median (horizontal center line), interquartile range (box), mean values (×) and minimum/maximum values (whiskers). The numbers in parentheses indicate the sample sizes used for each group. Statistical significance was assessed using Mann-Whitney U tests. * $p < 0.05$; ** $p < 0.01$; n.s., not significant.

remained very low until approximately 30 weeks of gestation, often approaching the detection limit of the ELISA assay. Levels began to increase gradually after 32 weeks and showed a marked rise from 33 to 34 weeks onward. When compared with these normal secreted suppressyn expression levels, maternal serum suppressyn levels in LO-PE patients exhibited a non-significant trend toward reduction in the FGR(+) group, but no statistically significant differences were observed between any subgroups (Fig. 3b right). At the time of blood collection, the sFlt-1/PIGF ratio in both LO-PE cohorts sub-grouped by the presence or absence of FGR exceeded the diagnostic threshold of 38 (FGR(+): 203 ± 167 ; FGR(-): 246 ± 123), confirming the pathological status of these cases (Supplemental Table S2). In contrast, EO-PE patients demonstrated a distinct pattern. Maternal serum suppressyn levels were significantly elevated in the FGR(-) subgroup ($p < 0.01$), while those in the FGR(+) subgroup remained low and comparable to levels observed in normal pregnancies (Fig. 3b left). Notably, the sFlt-1/PIGF ratio exceeded 38 in both EO-PE subgroups (FGR(+): 977 ± 715 ; FGR(-): 522 ± 584), indicating that both groups met established criteria for PE.

Association between decreased ASCT2 expression and suppressyn secretion

Given the observed downregulation of ASCT2 mRNA expression in PE placentas (Fig. 1c), we next examined whether this decrease in transcription was reflected at the protein level. Western blot analysis revealed a significant reduction in ASCT2 protein expression, particularly in EO-PE and LO-PE cases without FGR

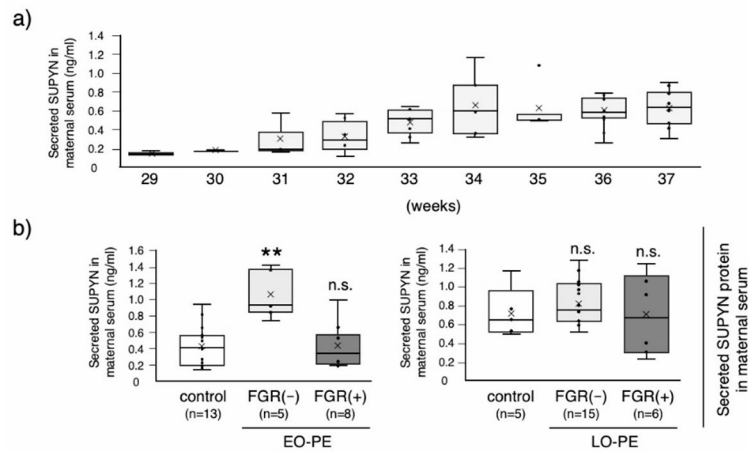


Fig. 3. Expression dynamics of secreted suppressyn protein in maternal blood (ELISA Analysis). Maternal blood concentrations of secreted suppressyn protein were measured in preeclampsia (PE) patients and matched controls from 29 to 37 weeks of gestation. (a) Changes in the expression of secretory suppressyn (SUPYN) protein in maternal blood throughout pregnancy were assessed in normal pregnancies. (b) Maternal blood concentrations of suppressyn protein were compared between control and PE samples, further subdivided by the presence or absence of fetal growth restriction (FGR) in early-onset PE (EO-PE) and late-onset PE (LO-PE) cohorts. Box plots display the median (horizontal center line), interquartile range (box), mean values (×) and minimum/maximum values (whiskers). The numbers in parentheses indicate the sample sizes used for each group. Statistical significance was evaluated using Mann-Whitney U testing. ** $p < 0.01$; n.s., not significant.

(Fig. 4a; Supplemental Figure S2, S3). These results suggested a potential regulatory relationship between ASCT2 and suppressyn secretion. We noted that the maternal serum levels of secreted suppressyn protein (Fig. 3b) exhibited an inverse pattern relative to ASCT2 protein expression, suggesting a receptor-ligand dynamic. Based on this observation, we hypothesized that decreased ASCT2 protein levels may reduce intracellular suppressyn binding, thereby promoting its extracellular release as a secreted protein. To test this hypothesis, we employed the trophoblast-derived choriocarcinoma cell line JAR, as well as a genetically engineered HTR8 cell line stably overexpressing wild-type suppressyn (HTR8-SUPYN). ASCT2 expression was silenced in both cell types using siRNA-mediated knockdown. As shown in Fig. 4b and c, ASCT2 protein levels decreased in a knockdown-dose-dependent manner in both cell lines. Concurrently, intracellular suppressyn protein levels also declined, while the amount of suppressyn secreted into the culture medium increased proportionally. These findings strongly suggest that the intracellular and extracellular distribution of suppressyn is regulated by ASCT2 protein levels. This ASCT2-dependent suppressyn secretion mechanism likely underlies the elevated maternal serum suppressyn levels observed in EO-PE patients without FGR (Fig. 3b, EO-PE-FGR(-)), providing a mechanistic explanation for this specific clinical phenotype.

Discussion

In summary, our analyses revealed three key findings: (1) suppressyn protein expression is markedly reduced in placentas from PE cases complicated by FGR, which may implicate suppressyn in the pathophysiology of fetal growth restriction, (2) significant reductions in ASCT2 protein levels were consistently observed in PE cases without FGR, suggesting a potential association with the pathogenesis of this PE subtype, and (3) aberrant detection of suppressyn in maternal blood—presumably due to disrupted receptor-ligand interactions with ASCT2—may offer clinical utility as a novel placental function-based biomarker.

Initially, we noted that *suppressyn* gene expression demonstrated a general gestational age-dependent increase in normal pregnancies, while both EO-PE and LO-PE samples exhibited slightly elevated expression levels throughout gestation when compared to controls. This pattern likely reflects the oxygen-sensitive regulation of the suppressyn promoter, which contains hypoxia response elements (HREs)³². In contrast, the expression of *syncytin-1*, a well-known promoter of trophoblast fusion, showed no significant difference between PE and control groups. Although previous studies have reported reduced *syncytin-1* expression in PE placentas⁴¹, this discrepancy may stem from our use of intron-spanning primers that selectively detect properly spliced mRNA isoforms. Notably, *ASCT2* gene expression was significantly reduced in PE placentas, consistent with the observed protein-level data. Similar concordance between mRNA and protein expression was confirmed for other key genes, such as *sFlt-1* and *PLGF*¹³. The expression of *syncytin-2*, which is co-expressed with ASCT2 in cytotrophoblast (CT) cells, was also significantly decreased in PE, suggesting that CT cell dysfunction may underlie these gene expression changes. Conversely, *hCGβ* expression was significantly increased in EO-PE, potentially reflecting compensatory syncytiotrophoblast hyperplasia⁴².

Based on the observed protein expression patterns—specifically, the significant reduction of suppressyn in placentas from PE cases with FGR and the marked decrease in ASCT2 in those without FGR—the samples were regrouped, as illustrated in Supplementary Figure S5, to facilitate clearer interpretation. Based on our findings, we propose the following hypothesis: when placental development is perturbed, the tissue may initiate

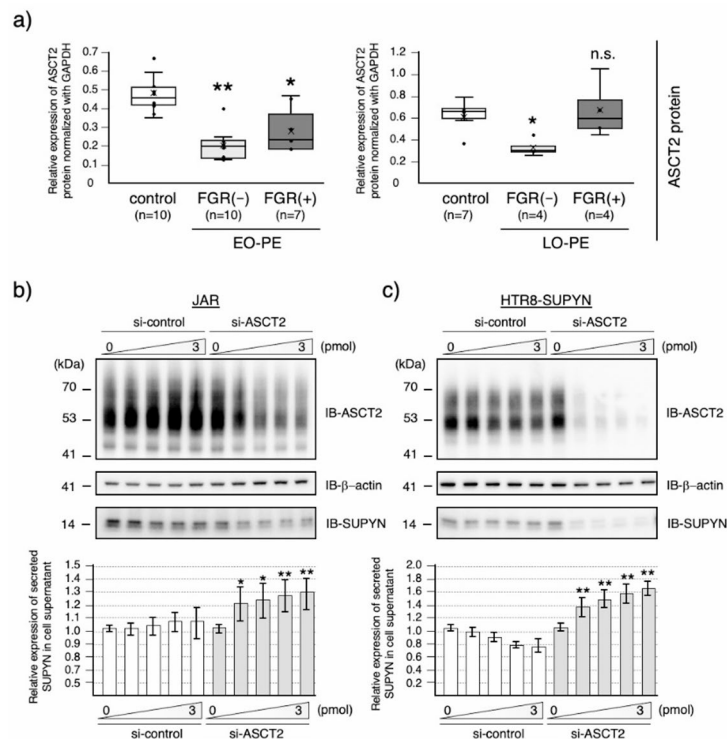


Fig. 4. Changes in the placental expression of the suppressyn-binding protein ASCT2 across pregnancy and a mechanism underlying changes in suppressyn secretion. (a) Translation levels for ASCT2, a binding protein for suppressyn, were compared between controls and preeclampsia (PE) patients throughout pregnancy. Subgroups were created based on the presence or absence of fetal growth restriction (FGR) (EO-PE-FGR(-), EO-PE-FGR(+), LO-PE-FGR(-), LO-PE-FGR(+)). Box plots display the median (horizontal center line), interquartile range (box), mean values (\times) and minimum/maximum values (whiskers). The numbers in parentheses indicate the sample sizes used for each group. Statistical significance was evaluated using Mann-Whitney U tests. * $p < 0.05$; ** $p < 0.01$; n.s., not significant. Quantification was performed based on results from the Supplementary Figure S2 blot, with original blot data shown in Supplementary Figure S3. (b, c) The effects of in vitro ASCT2 knockdown on suppressyn (SUPYN) secretion were examined using trophoblast cell lines: (b) JAR (trophoblast cancer cell line) and (c) HTR8 (SV/neo) - SUPYN (trophoblast-derived cell line stably expressing suppressyn). The upper panels show Western blot analysis of ASCT2 protein levels following siRNA treatment, demonstrating dose-dependent reductions. Middle panels show changes in intracellular suppressyn protein levels. The original blot data are shown in Supplementary Figure S4. The lower panels show the secreted suppressyn protein in the culture medium, as detected by ELISA. Statistical tests were performed in comparison to the 0 pmol treatment group. All experiments were repeated three times in duplicate. Statistical significance was assessed using Mann-Whitney U testing. * $p < 0.05$; ** $p < 0.01$.

a compensatory shift aimed at limiting trophoblast cell fusion in order to preserve placental function. As part of this response, *ASCT2* gene expression is down regulated, while *suppressyn* gene expression is upregulated. However, if this compensatory mechanism fails—due to as yet unidentified factors—the placenta may transition to a more pathological state, as observed in PE with FGR [PE-FGR(+)]. This condition may be characterized by insufficient suppression of ASCT2 protein levels and an abnormal reduction in suppressyn protein, thereby reflecting a breakdown in the adaptive regulatory network. Intracellular suppressyn protein levels were significantly reduced in all PE subtypes except EO-PE without FGR [EO-PE-FGR(-)]. In contrast, ASCT2 protein levels showed a distinct pattern: they were significantly decreased in FGR(-) cases, but remained unchanged or only mildly reduced in FGR(+) cases. These findings suggest a gradient in the strength of trophoblast cell fusion suppression, following the order of: EO-PE-FGR(-) > EO-PE-FGR(+), LO-PE-FGR(-) > LO-PE-FGR(+). This gradient is likely to reflect the underlying severity of placental pathology across these subtypes⁴³. In both PE cases with FGR and in late-onset PE, suppressyn protein levels were reduced despite relatively high mRNA expression, suggesting the presence of post-transcriptional or translational regulatory mechanisms, the nature of which remain to be elucidated. Notably, the strong association between altered ASCT2 expression and the presence of FGR in PE has not been previously reported^{44,45}. This discovery may have been facilitated by our stratified analytical approach based on gestational age and clinical phenotype. Although the involvement of amino acid transporters in PE pathophysiology has been proposed⁴⁶, our results emphasize the need for further investigation into this mechanism.

Taken together, these findings suggest that an imbalance between ASCT2 and suppressyn may contribute to the pathogenesis of preeclampsia. Importantly, this study demonstrates that the relative levels of these two proteins

can be readily assessed in maternal blood, highlighting their potential utility as accessible biomarkers for placental dysfunction. Maternal serum levels of suppressyn showed no substantial changes in most subgroups, with the exception of the EO-PE-FGR(−) subjects. This may reflect a compensatory effect: any decreased suppressyn secretion due to lower tissue expression may have been offset by reduced suppressyn/ASCT2 complex formation secondary to ASCT2 downregulation. Conversely, in EO-PE-FGR(−) cases, decreased ASCT2 expression may have led to excess unbound suppressyn being secreted into the mother's blood. This hypothesis was supported by *in vitro* analyses, which demonstrated that suppressyn secretion levels are dependent on ASCT2 expression. In early-onset preeclampsia (PE), suppressyn gene expression may be upregulated by hypoxic conditions. However, a pathological decrease in ASCT2 expression may disrupt suppressyn/ASCT2 complex formation, leading to increased secretion of unbound suppressyn. This ASCT2-dependent mechanism of suppressyn secretion could serve as a diagnostic marker. When combined with established biomarkers (e.g., the sFlt-1/PlGF ratio), it may enable the detection and prediction of EO-PE in patients who do not present with FGR.

In this study, we demonstrated an association between suppressyn expression and PE/FGR status. However, we acknowledge there are several limitations to our study as presented. More specifically: (1) the sample size was relatively small; (2) all disease samples were obtained from placental tissue and maternal blood collected at the time of delivery; and (3) key clinical parameters—including fetal sex, placental and fetal weight, mode of delivery, presence or absence of treatment, and gross or microscopic morphological features of the placenta—were not included in the analysis. These limitations were all introduced during the study design and institutional study approval process. Moreover, to validate the potential for suppressyn to be used as a clinical biomarker, it would be essential to perform analyses using maternal blood samples collected during early pregnancy, particularly around the time of disease onset. Since PE is typically not diagnosed until mid-to late gestation, such collections are inherently problematic. Still, these analyses, using additional patients who are sampled during early pregnancy but may or may not develop these diseases, will be critical to further clarify the clinical relevance of suppressyn as a disease biomarker.

Additionally, given that strong suppressyn protein localization was observed in EVT cells³⁰, it remains unclear whether suppressyn contributes to disease pathogenesis additionally, or even primarily, by affecting EVT-mediated spiral artery remodeling within the decidua, leading to abnormal placental development. To address this distinction, future studies should include (i) the analysis of patient samples from early pregnancy and (ii) the use of trophoblast stem cell (TSC)-, embryonic stem cell (ESC)-, induced pluripotent stem cell (iPSC)-derived *in vitro* models, and/or trophoblast organoids (TO), to study villous and extravillous trophoblast development^{47,48} as they relate to PE and FGR.

Despite these limitations, our findings do suggest that the fusion-associated proteins, suppressyn and ASCT2, may contribute to the pathogenesis of PE and FGR, and may represent potential biomarkers for evaluating placental function and dysfunction.

Methods

Serum and placenta samples

Clinical samples were collected from otherwise discarded placentas from vaginal or cesarean deliveries necessitated to treat pre-eclampsia. Gestational age-matched control samples were obtained from pregnancies affected by pPROM (preterm premature rupture of membrane) (see Supplemental Table S1). Maternal serum was obtained from patients and/or healthy pregnant donors. All collections were approved by the Ethical Committee for Epidemiological Research (E2018, E1782, E2016 for Hiroshima University) or (E2020 for Tokyo University). Appropriate informed consent was obtained from all participants or through approved opt-out methods. All research was performed in accordance with relevant guidelines/regulations.

RNA extraction from placental tissue

Approximately 25 mg of placental tissue, stored at −80 °C, was homogenized in 350 µl of lysis buffer containing β-mercaptoethanol using a pestle. The homogenate was further disrupted using a QIAshredder column (79654: Qiagen, Valencia, CA, USA), and total RNA was extracted from the resulting lysate according to the manufacturer's instructions of the RNeasy kit (74104: Qiagen, Valencia, CA, USA). An on-column DNase digestion step was performed during the extraction process at room temperature for 15 min following the manufacturer's protocol (79254: Qiagen, Valencia, CA, USA). RNA was eluted in 50 µl of DEPC-treated water. RNA concentration and purity were assessed by spectrophotometry, and 1 µg of total RNA was used for reverse transcription.

Quantitative RT-PCR

One microgram of total RNA was subjected to reverse transcription using a ReverTraAce kit (FSK-101: Takara, Shiga, Japan) at 30 °C for 10 min, 42 °C for 60 min and 95 °C for 5 min. Six microliters of a twenty-fold diluted reverse transcription product was used for real time RT-PCR with SYBR Green I in 25 µl total volume (Applied Biosystems *Power SYBR Green Master Mix*: 4368577: Thermo Fisher Scientific, Waltham, MA, USA). Each sample was analyzed using technical duplicates. Quantification was achieved by generating a standard curve from a serial dilution of a reference sample. Real-time PCR was performed using the CrontoSTAR™ 96 Real-Time PCR System (WN0232: Takara, Shiga, Japan). Gene expression levels were normalized to an internal *GAPDH* control, and relative expression was calculated using one of the control samples as a reference. The *suppressyn* and other fusion-related gene primers used for real-time PCR are listed in supplementary Table S3⁴⁹.

Western Immunoblotting

A 25 mg placental tissue sample was homogenized in RIPA buffer (50mM Tris-HCl, pH8.0, 150mM Sodium Chloride, 0.5w/v% Sodium Deoxycholate, 0.1w/v% Sodium Dodecyl Sulfate, 1.0w/v% NP-40) substitute with protease inhibitor (165-26021: Fuji film, Osaka, Japan) using a pestle, followed by vortexing at 4 °C for 30 min.

The homogenate was then centrifuged at 15,000 rpm for 10 min, and the supernatant was collected. Protein concentrations were determined using the Bradford assay. A total of 25 µg of protein was analyzed by standard PAGE followed by Western immunoblotting. NuPAGE Bis-Tris 12% or 4–12% (for SUPYN and ASCT2, respectively) precast gels (NP0343, NW04127; Thermo Fisher Scientific, Waltham, MA, USA) were used for gel electrophoresis according to the instructions. Immunoblotting was performed by using the iBlot2 dry blotting system (IB21001, IB24002; Thermo Fisher Scientific, Waltham, MA, USA). HRP-labeled monoclonal anti-suppressyn antibody (1/2000 dilution)³⁴ or polyclonal anti-ASCT2 antibody (1/3000 dilution, 8057; Cell signaling technology, MA, USA), monoclonal anti-β-actin (1/10000 dilution, A5441; Sigma-Aldrich, St. Louis, MO, USA), and monoclonal anti-GAPDH antibody (1/5000 dilution, sc-32233; Santa Cruz, Dallas, TX, USA) were used for detection. Blot signals were detected using the Ez-Capture MG luminescence imaging system (ATTO, AE-9300; ATTO Corporation, Tokyo, Japan). The intensity of the resulting bands was quantified using CS Analyzer software, with signal normalization performed against GAPDH as an internal control.

ELISA

A suppressyn-specific monoclonal antibody (clone: 2J16) was dissolved in carbonate buffer (0.5 µg/ml) and used as the capture antibody. 100 µl of the capture antibody solution was placed into wells on a 96-well plate and incubated overnight at 4 °C. Plates were washed 5 times with PBST (PBS with 0.05% Tween 20) using a microplate washer (ImmunoWash 1575; BioRad, Hercules, CA, USA) and blocked with 0.5% BSA / PBST for 1 h at room temperature. After washing, 50 µl of sample was diluted with 10 µl of HAMA blocker (ab193969; abcam, Cambridge, UK) and 40 µl of PBS and 100 µl was dispensed into each well and overnight at 4 °C. Plates were washed five times and HRP labeled anti-suppressyn monoclonal antibody (clone: 3H6) was used as a detection antibody at a concentration of 0.2 µg/ml. After 1 h at RT, plates were washed 5 times and incubated with 100 µl of POD (ELISA POD Substrate TMB kit: 05298-80; nakalai tesque, Kyoto, Japan) for 7 min at RT. Color development was stopped with 100 µl of H₂SO₄ and absorbance measured at a wavelength of 450 nm by a microplate reader (MPR-A100; ASONE, Osaka, Japan). ELISAs were performed in duplicate for each sample.

Transient gene knockdown by siRNA

Aliquots of 2.5×10^4 cell / well of the choriocarcinoma cell line, JAR, was cultured in RPMI with 10% FBS and of HTR8-SUPYN (as previously described²⁷ in DMEM medium with 10% FBS, 1 µg/ml puromycin (540411; Sigma-Aldrich, St. Louis, MO, USA) in 24 well plates at 37 °C in 5% CO₂. Cells were transfected using 1 µl of RNAiMAX transfection reagent (13778100; Thermo Fisher Scientific, Waltham, MA, USA) and 0–3 pmol ASCT2 siRNA (sc-60210; Santa Cruz, Dallas, TX, USA) in 250 µl of Opti-MEM medium. After 24 h, medium was changed, and incubation continued for 24 h and samples were collected in 150 µl of RIPA buffer (see Western Immunoblotting section). The concentration of suppressyn in 10 µl of the collected culture supernatants was determined using a suppressyn-specific ELISA assay (see ELISA section). Each assay was performed in duplicate and independently repeated three times.

Statistical analysis

Statistical analysis was performed using the Mann Whitney U test. A *p* value below 0.05 (* : *p* < 0.05) or 0.01 (** : *p* < 0.01) was considered significantly different.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

J.S., D.J.S., and Y.K. designed experiments. J.S., and M.S. performed the experiments and analyzed the data. T.N., E.Y. T.Y. and Y.K. collected clinical samples. J.S., D.J.S., T.N. and Y.K. interpreted the data. J.S. and D.J.S. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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