**Impaired Sphingosine-1-phosphate Synthesis Induces Preeclampsia by Deactivating Trophoblastic YAP through S1PR2 induced Actin Polymerization**

Running title: Impaired S1P synthesis causes preeclampsia

Jiujiang Liao1,2,3#, Yangxi Zheng1,4,5#, Mingyu Hu1,2,3#, Ping Xu1,2,3, Li Lin1,2,3, Xiyao Liu1,2,3, Yue Wu1,2,3, Biao Huang1,2,3, Xuan Ye1,2,3, Sisi Li1,2,3, Ran Duan1,2,3, Huijia Fu1,2,3, Jiayu Huang1,2,3, Li Wen1,2,3, Yong Fu1,2,3, Mark D. Kilby6, Louise C. Kenny7, Philip N. Baker8†, Hongbo Qi1,2,3†\*, Chao Tong1,2,3†\*

1Department of Obstetrics, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400716, China;

2Ministry of Education-International Collaborative Laboratory of Reproduction and Development, Chongqing 400016, China;

3State Key Laboratory of Maternal and Fetal Medicine of Chongqing Municipality, Chongqing 400016, China;

4Department of Biochemistry & Molecular Biology, University of Texas McGovern Medical School at Houston, Houston, Texas 77030, USA;

5Department of Stem Cell Transplantation and Cell Therapy, MD Anderson Cancer Center, Houston, Texas, USA;

6Institute of Metabolism and System Research, College of Medical & Dental Sciences, University of Birmingham and the Fetal Medicine Centre, Birmingham Women’s and Children’s Foundation Trust, Birmingham, B15 2TT, UK;

7 Department of Women’s and Children’s Health, Faculty of Health and Life Sciences, Institute of Translational Medicine, University of Liverpool, Liverpool L8 7SS, UK;

8 College of Life Sciences, University of Leicester, Leicester LE1 7RH, UK.

# These authors contribute equally to this work.

† These authors jointly supervised this work.

\* Correspondence to Chao Tong and Hongbo Qi.

Address: Department of Obstetrics, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Yuzhong District, Chongqing 400016, China (C.T & H.Q)

Tel: +86 23 89011800 (C.T); +86 23 89011101 (H.Q).

Fax: +86 23 89011791(C.T); +86 23 89011102 (H.Q).

Email: chaotongcqmu@163.com (C.T); qihongbo728@163.com (H.Q).

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**Abstract**

Incomplete spiral artery remodeling, caused by impaired extravillous trophoblast invasion, is a fundamental pathogenic process associated with malplacentation and the development of preeclampsia. Nevertheless, the mechanisms controlling this regulation of trophoblast invasion are largely unknown. We report that spingosine-1-phosphate (S1P) synthesis and expression is abundant in healthy trophoblast, whereas in pregnancies complicated by preeclampsia the placentae are associated with reduced sphingosine-1-phosphate and lower sphingosine kinase 1 (SPHK1) expression and activity. *In vivo* inhibition of sphingosine kinase 1 activity during placentation in pregnant mice led to decreased placental sphingosine-1-phosphate production and defective placentation, resulting in a preeclampsia phenotype. Moreover, sphingosine-1-phosphate increased HTR8/SVneo (immortalized trophoblast cells) cell invasion in a Hippo-signaling-dependent transcriptional coactivator Yes-associated protein (YAP) dependent manner, which is activated by sphingosine-1-phosphate receptor-2 (S1PR2)and downstream RhoA/ROCK induced actin polymerization. Mutation-based YAP-5SA demonstrated that sphingosine-1-phosphate activation of YAP could be either dependent or independent of Hippo signaling. Together, these findings suggest a novel pathogenic pathway of preeclampsia via disrupted sphingosine-1-phosphate metabolism and signaling-induced, interrupted actin dynamics and YAP deactivation; this may lead to potential novel intervention targets for the prevention and management of preeclampsia.

**Key words:** preeclampsia, trophoblast, sphingosine-1-phosphate (S1P), Hippo pathway, invasion,cytoskeleton

**Introduction**

Preeclampsia (PE) is a complicated, often multisystem hypertensive disorder that affects about up to 8% of pregnancies worldwide 1. The disease is characterized by new onset hypertension after 20 weeks of gestation, proteinuria and often multiple organ involvement2. The etiology of preeclampsia is not yet been fully defined. Maternal and fetal complications may progress rapidly, leading to the death of fetus, the mother or both 3. In addition, this pregnancy related hypertensive disease, may cause acute end organ injury in the mother, fetal growth restriction or perinatal death and increases the maternal risk of long-term cardiovascular and cerebrovascular disease 4.

PE is associated with malplacentation and abnormal maternal-fetal interaction within the placenta 5. Placental blood supply is controlled by maternal cardiodynamics through the uterine spiral arteries and regulation of this maternal placental perfusion appears to be tightly linked to the extravillous trophoblast (EVT) invasion and remodeling 6. During early stage of pregnancy, EVTs invade decidua and remodel the spiral artery, allowing increased blood flow to the placenta 7. However, EVT invasion is impaired in pregnancies that develop preeclampsia, resulting in inadequate spiral artery remodeling 8, which in turn leads to poor placental perfusion9. The hypoxic placenta produces vasoactive substances, inflammatory factors, etc., which trigger maternal vascular endothelial dysfunction and hypertension 10-12.

Emerging evidence indicates that trophoblastic function is at least in part, affected by the metabolism of bioactive sphingolipids 13, 14, composed by ceramide and sphingosine-1-phosphate (S1P). Ceramides are elevated in preeclamptic placentas and lead to excessive autophagy and programmed necrosis of trophoblast cells, thus contributing to the onset of preeclampsia 13. Impaired S1P synthesis and signaling have also been documented in PE vessels 15. However, the correlation between S1P metabolism of EVTs and PE development remains unknown.

Sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK 2) are the main enzymes responsible for the synthesis of S1P from sphingosine (figure S1) 16. SPHK1 is predominantly expressed in cytoplasm, SPHK2, on the other hand is primarily located in the nucleus 17. In contrast, S1P phosphatase 1/2 (SGPP 1/2) converts S1P to sphingosine 18, and S1P can be irreversibly degraded by S1P Lyase (SGPL) into ethanolamine phosphate and hexadecenal18.

S1P metabolism and signaling are thought to be involved in many physiological and pathological process such as senescence 19, immune cell trafficking 20 and oncogenesis 21. S1P is also believed to be closely related to pregnancy processes. Pregnant female mice exhibited defective decidualization when the S1P metabolism was disrupted by interfering *Sphk1*and*Sphk2*, which ultimately lead to early pregnancy loss 22. In addition, it has been reported that S1P is involved in the regulation of differentiation, migration and invasion of trophoblast cells 23. Therefore, understanding the metabolism and signaling of S1P in EVTs may be key to enhanced understanding of the etiology of PE.

YAP is a key effector of the Hippo pathway, a highly conserved pathway in mammals that controls organ growth and homeostasis 24. YAP integrates different signals to regulate its function. The Hippo pathway core component-Large Tumor Suppressor (LATS) mediated phosphorylation plays a major role in this regulation 25. Phosphorylated YAP binds to 14-3-3 proteins and promotes cytoplasmic sequestration, inhibiting its transcriptional activity 26. Other stimulants such as mechanical force 27 and GPCRs signaling pathways 28 can regulate YAP’s subcellular location via cytoskeleton dynamics. YAP activation is closely associated with cell invasion and cancer metastasis 29, while it’s activation was reported to be suppressed in PE 30. Our lab has shown that inactivation of YAP impairs HTR8/SVneo cell invasion 31, implicating YAP inactivation in the pathogenesis of PE. However, the upstream regulatory mechanisms of YAP in the trophoblast have yet to be identified. RhoA-ROCK is the major pathway downstream of S1P signaling via GPCRs mediated Gα12/13 activation, the main function of which is to regulate cytoskeleton dynamics 32. The cytoskeleton’s regulation of Hippo-YAP has been increasingly reported 33, which supports our hypothesis that S1P modulates YAP subcellular distribution through RhoA-ROCK mediated actin polymerization, thus regulating trophoblast invasion and, as a result, potentially involved in PE pathogenesis. In this study, we investigate S1P metabolism in the PE placenta, its regulatory role in EVTs, and the involvement of YAP activation.

**Materials and methods**

All the human sample collecting procedures were in accordance with the principles stated in the Declaration of Helsinki and were approved by the Institutional Ethics Committee of Chongqing Medical University, and all the procedures involved with animals were carried out in accord with the National Institutes of Health guidelines for the use and care of animals and approved by the Institutional Animal Care and Use Committee of Chongqing Medical University.

Materials in the Data Supplement provide details on all methods, including sampling, immunoblotting, experimental animals, quantitative real-time polymerase chain reaction, immunofluorescence, cell culture, SPHK1 activity assay34, S1P measurement, blood pressure monitoring35, high frequency ultrasound examination, Matrigel invasion assay36, GTP-RhoA assay, coimmunoprecipitation (Co-IP), RNA sequencing, transfections, nuclear protein extraction, YAP-5SA plasmid37, transcription factor binding profiling38 and other unlisted procedures. The RNA sequencing (RNA-Seq) data that support the findings of this study are available from the corresponding author upon reasonable request.

**Statistical analysis**

All data were collected using Prism 7 software (GraphPad). Two-tailed Student’s t test was used for the comparison between two groups. One-way analysis of variance (ANOVA) was applied for the comparison among multiple groups, followed by Tukey’s multiple comparisons test. For multiple groups with multiple characteristics, two-way analysis of variance (ANOVA) was used. All data were presented as mean ± SEM, and a p value < 0.05 was considered statistically significant.

**Results**

**Low S1P levels coincide with downregulated SPHK1 in PE-complicated placentas**

The expression of S1P in normal and PE placentas were determined. The results showed that S1P levels were significantly lower in PE placentas (figure 1A), implying PE is associated with impaired S1P metabolism in the placenta. The expression levels of metabolic enzymes of S1P were then determined; our results showed that S1P synthetase *SPHK1*, but not *SPHK2*, was significantly downregulated in PE complicated placentas, while expression of other enzymes involved with S1P degradation – *SGPP 1/2* and *SGPL* were unaltered (figure 1B). The loss of SPHK1 protein and decreased SPHK1 activity were further confirmed in PE placentas (figure 1C, S1B). To further investigate the SPHK1 expression pattern throughout gestation, we then examined the expression of SPHK1 in first-trimester villi and term placenta. The results showed that SPHK1 expression was significantly higher in first-trimester villi (figure 1C), implying that SPHK1 expression might be critical for early stages of pregnancy. Moreover, IF staining suggested that SPHK1 was downregulated in placentas of PE-complicated pregnancies and was mainly expressed in STBs and EVTs (figure 1D, 1E). These facts indicate that SPHK1 is largely expressed and is significantly downregulated in trophoblasts of PE-complicated placentas.

**SPHK1 inhibition during placentation induces a PE phenotype in mice, and impairs placentation**

To further ascertain the regulation of placental S1P by SPHK1 *in vivo* and the involvement of SPHK1 in PE development, we first examined SPHK1 expression in a mouse uteroplacental unit collected at different gestational stages. We found that SPHK1 was mainly expressed in trophoblast giant cells in the placenta during GD8.5 to GD14.5 and started to be expressed in the placental labyrinth layer at GD 18.5 (figure 1F). Next, a specific inhibitor of SPHK1, PF543, was administered to pregnant mice via tail vein injection daily during GD7.5-GD12.5 (figure 2A). Placentae were harvested on GD13.5 and GD18.5. Placental S1P levels measured on GD13.5 were significantly reduced by PF543 treatment (figure S2A); meanwhile, systolic blood pressure was elevated by about 20% (figure 2B). However, such changes in blood pressure induced by PF543 were not observed in non-pregnant mice (figure S2B, S2C).

On GD18.5, the PF543 treatment group demonstrated significant reduction in fetal weight, crown-rump length (CRL), and placental weight (figure 2C), among which fetal weight and placental weight were impaired as early as GD13.5 (figure S2D). Moreover, PF543 administration resulted in glomerular constriction, specifically shrinkage of Bowman's space on GD18.5 (figure 2D), similar to the renal damage reported in PE patients 39. However, such alterations were absent from kidneys collected on GD13.5, indicating that kidney damage occurring in late gestation was a secondary effect rather than the cause of hypertension.Most importantly,morphological examination of the uteroplacental unit showed that the placental labyrinth layer was significantly reduced in PF543-treated mice, along with a decreased labyrinth/placenta ratio (figure 2E). Concurrently, invasion and remodeling of spiral arteries by trophoblast cells were also significantly reduced (figure S2E). Hemodynamic analyses of uterine and umbilical arteries by Doppler (figure S2F) showed that PF543 administration significantly reduced end-diastolic velocity (EDV) but retained peak systolic velocity (PSV) of the uterine artery (figure S2G), which was consistent with the elevation of the resistance index (RI), systolic-to-diastolic ratio (S/D) and pulsatility index (PI) (figure 2F, S2H) following PF543 treatment, indicating an increased blood flow resistance through the uterine artery, likely caused by inadequate remodeling of spiral arteries. Moreover, the RI, S/D and PI of the umbilical artery were significantly increased in dams treated with PF543 (figure 2F, S2I), implying compromised placental function. Taken together, these observations suggest that inhibition of SPHK1 disturbs placentation and induces a PE-like phenotype in mice.

Since S1P is a well-known, anti-apoptotic bioactive metabolite 40, it is plausible that inhibiting S1P synthesis in trophoblasts may lead to excessive apoptosis, ultimately resulting in placental maldevelopment. Nevertheless, although placental apoptosis was significantly increased in PF543 treated dams on GD 18.5 (figure S2K)，there was no observed difference between groups on GD13.5 (figure S2J), which implies that the increased apoptosis in term placentas of PF543 treated dams is more likely a consequence rather than the driving force of impaired placentation.

**S1P increases trophoblasts mobility through RhoA activation**

To investigate the underlying regulatory mechanism of S1P on placentation, next we determined the expression pattern of five known S1P receptors (S1PRs) in human placentas. We found that all are expressed in human placentas (figure S3A). The expression patterns of S1P receptors in various trophoblast cell lines were then determined. Our data suggest that HTR8/SVneo is the only cell line that expresses all *S1PRs* (figure S3B). In addition, the protein levels of S1PRs are higher in HTR8/SVneo except S1PR3 (figure S3C). Thus, HTR8/SVneo cells were subjected to PF543 treatment; this resulted in a significant decrease in S1P levels in both cell lysate and culture medium (figure 3A). Neither PF543 nor S1P influences the viability of HTR8/SVneo cells (figure S3D, S3E). Nonetheless, both invasion and migration of HTR8/SVneo cells were significantly inhibited by PF543, but markedly stimulated in the presence of S1P (figure 3B, S3F).

Increased YAP phosphorylation was associated with PE and suppressed the invasion of HTR8/SVneo cells 31. Intriguingly, S1PR-mediated cytoskeleton polymerization is one of the major regulators of YAP in addition to the Hippo pathway28. Therefore, we then investigated whether S1P regulates trophoblast cell invasion and migration by modulating actin polymerization and YAP activation. RhoA-ROCK pathway is a main regulator of cytoskeletal dynamics, which is also a major downstream target of S1P-S1PRs signaling 32. RhoA belongs to a small GTPase family. Upon activation, it binds and hydrolyzes GTP to increase the kinase activity of ROCK, which further phosphorylates downstream effectors such as myosin phosphatase target subunit 1 (MYPT1) 41. Therefore, GTP-RhoA and p-MYPT1 levels indicate RhoA-ROCK pathway activity. We found that S1P treatment increased GTP-RhoA, and MYPT1 phosphorylation. S1P also significantly diminished YAP phosphorylation in HTR8/SVneo cells, without altering YAP protein levels (figure S3G). The expression of connective tissue growth factor (CTGF), a well-known downstream target of YAP 42, was also upregulated by S1P (figure S3G). In contrast, PF543 not only elevated YAP phosphorylation and suppressed CTGF expression, it inhibited the RhoA-MYPT1 signaling axis (figure S3G). IF staining confirmed that actin polymerization in HTR8/SVneo cells is enhanced by S1P, which consequently shuttles YAP from cytoplasm to the nucleus while PF543 suppresses actin polymerization and leads to sequestration of YAP in the cytoplasm (figure S3H). To further ascertain the role of RhoA signaling and actin polymerization in S1P-induced YAP activation, RhoA and actin polymerization in HTR8/SVneo cells were modulated by specific inhibitors, CCG1423 and Latrunculin B (Lat B), respectively. Lat B acts by sequestering G-actin and preventing F-actin assembly, which is independent of upstream signaling 43. Our data showed that CCG1423 decreased MYPT1 phosphorylation, while both CCG1423 and Lat B increased YAP phosphorylation. Accordingly, expression of the YAP downstream target CTGF was also reduced (figure 3C). Furthermore, both CCG1423 and Lat B disrupted actin polymerization caused cytoplasmic retention of YAP in HTR8/SVneo trophoblasts (figure 3D). S1P also markedly improved invasiveness of HTR8/SVneo cells, but this effect was abrogated in the presence of CCG1423 (figure 3E). Since prolonged treatment with Lat B leads to excessive cell death, Lat B treatment was excluded from the transwell assay.

**S1P induced HTR8/SVneo invasion is dependent on YAP**

To determine whether S1P promoted HTR8/SVneo invasion relay on actin polymerization alone or actin related activation of YAP, YAP expression in HTR8/SVneo cells was downregulated by transfection of si*YAP* (figure S4A). YAP downregulation resulted not only in impaired invasion at basal conditions but also loss of S1P-stimulated invasion (figure 5A). YAP knockdown itself did not alter actin polymerization or actin polymerization promoted by S1P (figure S4B). These results strongly support our hypothesis that S1P promotes cell invasion in a YAP dependent manner.

To further determine the potential effectors underlying the regulatory effects of S1P on trophoblast invasion, vehicle and S1P treated HTR8/SVneo cells were subjected to RNA-seq. In evaluating alterations in the transcriptome (figure 4B), 526 genes were upregulated and 279 genes were downregulated in S1P treated group. Gene Ontology (GO) pathway analysis revealed that the downregulated genes were most associated with the inflammatory response and neutrophil migration (figure S4C), whereas genes in the extracellular matrix pathway were upregulated (figure 4C). Since cell movement and invasion are closely correlated with extracellular matrix dynamics, we examined the expression of several genes enriched in this pathway, including cathepsin D (*CTSD*), elastin microfibril interfacer 1 (*EMILIN1*) and collagen type IX alpha 3 chain (*COL9A3*), all of which were upregulated by S1P treatment (figure 4D).

It is well known that the co-transcriptional factor, YAP, mainly interacts with TEA domain transcription factor 4 (TEAD4) to promote its transcriptional activity 44. Intriguingly, *CTSD*, *EMILIN1* and *COL9A3* all contain multiple predicted binding sites with TEAD4 (figure S4D), indicating these genes could be direct downstream targets of YAP-TEAD4 complex. Consistent with transcription, CTSD and EMILIN1 protein levels were augmented by S1P but repressed by PF543(figure 4E). However, such promotive effects of S1P on CTSD and EMILIN1 protein levels was largely blunted in si*YAP* transfected cells (figure 4F). These results implied that S1P promotes cell invasion through YAP dependent transcription of genes correlated with extracellular matrix dynamics.

**S1P activates YAP** **in both hippo-dependent and hippo-independent ways**

To further determine the underlying mechanism of S1P-dependent actin polymerization regulating YAP activity in trophoblast, we performed reciprocal CoIP to detect the interaction between actin and components of the Hippo pathway, namely MST1, LATS1 and YAP. Our data showed that F-actin bonds to LATS1 and YAP rather than MST1 in both human placenta tissue and HTR8/SVneo cells (figure 5A - C), while G-actin monomer binds to none of these proteins (figure S5A)

Accordingly, phosphorylation of LATS1 and YAP was enhanced by PF543 but suppressed by S1P (figure 5D). Since YAP phosphorylation is tightly regulated by LATS1, these results indicated that LATS1 and the Hippo pathway are involved in the regulation of S1P in YAP phosphorylation and activation. Furthermore, to determine whether S1P-induced dephosphorylation of YAP is exclusively dependent on LATS1, a LATS1-insensitive YAP mutant (YAP-5SA) was introduced into HTR8/SVneo cells (figure S5B), in which five serine phosphorylation sites by LATS were mutated to alanine. We found that the mutation did not affect the phosphorylation of MYPT1, LATS1 (figure 5D) or the actin polymerization, but S1P and PF543 maintained their ability to regulate the subcellular location of mutated YAP-5SA (figure 5E). Most importantly, the invasion of YAP-5SA cells was still inhibited by PF543 or promoted by S1P (figure 5F). These results suggest that the regulation of YAP by S1P can be dependent or independent of the Hippo pathway.

**S1P regulates actin polymerization and YAP activation through S1PR2**

To determine the receptors that mediate the effects of S1P on actin polymerization and YAP activation, *S1PR1-5* in HTR8/SVneo cells were individually manipulated (figure S6A-J). *S1PRs* knockdown cells and control si*NC* cells were treated with S1P or vehicle. Western blotting demonstrated that S1P failed to phosphorylate MYPT1 and dephosphorylate YAP when *S1PR2* was downregulated (figure S7A). IF staining further confirmed that S1P-induced actin polymerization was only abolished in *S1PR2* knockdown cells (figure S7B). S1P-dependent YAP cytoplasm-to-nucleus translocation was consistently abrogated (figure S7C). Furthermore, S1P failed to increase the invasion of HTR8/SVneo cells in *S1PR2*-KD cells (figure S7D). These results demonstrated that S1PR2 mediates S1P-induced actin polymerization and YAP activation in the trophoblast.

**RhoA-ROCK pathway and YAP activation are impaired in PF543 treated mice placentae and PE placentae**

Our *in vitro* data demonstrated that S1P-promoted actin polymerization and YAP activation through S1PR2, which promoted the invasion of HTR8/SVneo cells. To further validate this regulatory pathway *in vivo*, we examined the RhoA-ROCK pathway and YAP in mouse placentes treated with PF543 during placentation. S1PR2 exhibited a similar expression pattern with SPHK1 (figure 1L) in uteroplacental units throughout gestation (figure S8A). Consistent with the findings in HTR8/SVneo cells, PF543 treatment increased phosphorylation of LATS1 and YAP (figure 6A) and reduced RhoA activation, MYPT1 phosphorylation, YAP nucleation (figure 6B) and YAP downstream CTSD, EMILIN1 expression. These changes indicate that S1P regulation in the RhoA-ROCK pathway and YAP activation also exist *in vivo*. To address the pathological relevance, we then assessed RhoA-ROCK pathways and YAP activation in normal and PE human placentas. Firstly, we determined the expression of S1PR2 in human trophoblasts. IF staining demonstrated that S1PR2 was also expressed in STB and EVTs (figure S8B, S8C). S1PR expression levels did not show any change between normal and PE placentas (figure 6C, S8D - E). Similar to the observation in SPHK1-inhibited mouse placentas, the phosphorylation of LATS1 and YAP were significantly increased (figure 6C) in PE placentas. Additionally, RhoA activity (figure 6D), MYPT1 phosphorylation and nuclear YAP retention were compromised (figure 6E) and expression levels of CTSD, EMILIN1 were decreased. Taken together, these findings indicate that the compromised RhoA-ROCK pathway and LATS1-YAP axis are associated with the PE placenta via S1P deficiency, connected with SPHK1 and responsible for downregulation of genes related to the extracellular matrix pathway.

**Discussion**

The pathogenesis of PE is multifactorial and is incompletely understood, but appears to be a consequence of maternal uteroplacental malperfusion and abnormal adaptive placentation. Sphingolipid metabolism is involved in multiple biological functions and disease processes. Recent evidence suggests that the sphingolipid metabolic pathway is highly active during pregnancy and that maternal S1P deficiency leads to defective decidualization and fetal loss 22, 45. Several reports highlighted the importance of sphingolipids in other aspects of pregnancy such as placental trophoblast differentiation 23, 46, placental angiogenesis 47, trophoblast autophagy 13 and necroptosis 14. Circulating S1P has been reported to be elevated in preeclampsia pregnancy as early as 15 weeks, which could be a promising “biomarker” for early prediction48. EVTs play a vital role in the process of pregnancy by invading decidua and remodeling the spiral artery, yet little is known about S1P metabolism and function in EVTs. Here, we report that S1P synthesis is highly active in EVTs, as shown by robust expression of SPHK1, which indicates a potential physiological function for S1P in EVTs. We also observed a correlation between PE and downregulated SPHK1 expression and activity, along with its product S1P.

Increased SPHK1 expression and S1P are associated with tumor progression and negative prognoses 21. Whether decreased S1P content is associated with abnormal EVT function and the onset of PE still needs to be clarified. Similar to EVTs in humans, mice trophoblast giant cells are instrumental in promoting blood flow towards the mouse embryo by invading the uterine endometrium 49. We demonstrated that SPHK1 was highly expressed in mice trophoblast giant cells during placentation. Moreover, S1PR2 had the same expression pattern as SPHK1, suggesting that the S1P-S1PR2 axis is involved in the placentation process. Our *in vivo* evidence showed that SPHK1 inhibition impaired placentation and induced PE-like phenotypes in mice, implicating downregulated SPHK1 the pathogenesis of PE. To further validate the function of SPHK1 on trophoblasts, a placenta-specific, nanoparticle drug delivery system 50 or trophoblast-specific, cre-based loxp-cre placenta specific knockout transgenic mice is warranted to manipulate SPHK1 51, 52.

Extracellular S1P functions through G protein-coupled receptors, S1PR1-S1PR5. Each receptor possesses a specific physiological function and participates in different pathophysiology by coupling with different G proteins 53. Cell migration and invasion are fundamental processes of embryogenesis, organ development, immune response and cancer progression. S1P was reported to possessed dual regulatory effects on cell migration in different cell types 54. We reported that S1P supplementation enhanced invasion of HTR8/SVneo cells through RhoA/ROCK-mediated cytoskeleton polymerization; furthermore, the effect was blocked by knockdown of S1PR2. However, a previous study indicated that S1P promotes HTR8/SVneo cell invasion in a S1PR1-dependent manner 55. Each S1PR exhibits a different binding affinity to S1P. The dose of S1P in Yang’s study (0-10 nM) was much lower than that of this study (250 nM), potentially resulting in preferential activation of specific S1PRs as well as distinctive cell signaling and function. 250 nM exogenous S1P used in our study is high enough to activate all five S1PRs 53, with unique observations resulting from the combination of numerous activations. The promoting effect of S1P on invasion was only abolished when S1PR2 was downregulated, indicating that S1PR2 is the predominant receptor in the regulation of cell motility. Another study demonstrated that S1P inhibits EVT migration through S1PR2 activation 56. Different cell lines possess distinct S1PR composition and signaling, and altered condition of cell treatment may also contribute to disparate observations in each study. Culture media serum contains multiple factors that promote actin polymerization and YAP activation, including LPA and S1P 28. In our study, to rule out the influence of bioactive factors in serum, HTR8/SVneo cells were serum starved for 2 hours before each treatment, and all treatments were supplied in a serum-free medium, which differs from the previous report56. Supplementary S1P may result in enhanced cell rigidity, which inhibits cell migration of EVTs when cells were already stimulated by factors in serum but restores cells motility when actin polymerization and YAP activation is hampered through serum starvation. S1PR2’s major intracellular downstream signaling pathway, RhoA/ROCK, regulates the polymerization of cytoskeleton, which is critical for EVT invasion but impaired in PE 57. Furthermore, RhoA/ROCK inhibition by either Rho inhibitor C3 exoenzyme or the ROCK inhibitor Y-27632 inhibited cytotrophoblast migration in a dose-dependent manner 58. Here, we report that suppressing RhoA activity by a novel inhibitor CCG4123 or inhibiting actin polymerization by Lat B abolished the effects of S1P on HTR8/SVneo invasion, further confirming that S1P promotes HTR8/SVneo invasion through RhoA/ROCK-mediated actin polymerization.

Accumulating evidence indicates that actin polymerization is a major determinant of YAP activation, which regulates trophoblast migration and invasion 31. There have been conflicting reports of YAP expression in trophoblasts. Meinhardt’s group showed that YAP was predominantly expressed in cytotrophoblasts (CTBs), with weak EVT expression and YAP absent from STBs in the first-trimester placenta 44. Liu’s study reported that YAP was expressed in both EVTs and villous trophoblast (VTs) in term placenta 30. The present study confirms that YAP is highly expressed in CTBs and absent in STBs in the first-trimester villi, reappearing in STBs in term placentas (figure S8F); EVT YAP expression was also detected throughout gestation (figure S8G), suggesting a temporal and spatial regulation of placental YAP expression during pregnancy.

A previous study proposed that the Hippo-YAP pathway is a downstream branch of GPCR signaling 28. Rho GTPases and actin polymerization appear to be located between GPCR signaling and YAP activation, but the precise mechanism by which actin polymerization controls YAP activation has yet to be elucidated. A recent study revealed that mechanical force could trigger YAP nuclear entry by stretched nuclear poles 42. Here, we determined that polymerized F-actin rather than G-actin interacts with both LATS1 and YAP. G-actin and F-actin are two types of structural forms of actin. Soluble globular G-actin monomers polymerize to form insoluble double-helix F-actin filament. During this process, negative regulatory proteins of F-actin, such as Gelsolin and CapZ, were identified as key regulators of actin polymerization and YAP activation by preventing ﬁlament annealing and polymerization 59. The presence of these F-actin proteins suggests that they play a role in mediating the connection between actin dynamics and YAP activity, though detailed mechanistic studies are required. LATS1 was reported to be the downstream of actin polymerization to regulate YAP activity 28, and inactivation of LATS1 by decreasing phosphorylation and subsequently decreasing YAP phosphorylation was observed by S1P treatment in our study. However, the LATS1-insensitive YAP mutant (YAP-5SA) still respond to S1P treatment, indicating that S1P-induced actin polymerization not only acts through LATS1-mediated phosphorylation, but also functions independently to regulate YAP activity in HTR8/SVneo cells. This dual-regulation is also supported by genetic evidence in human mammary epithelial cells (MEC) and Drosophila 59, 60.

In our study, S1P-induced actin polymerization increased YAP activation and ultimately HTR8/SVneo invasion, but YAP knockdown significantly reduced HTR8/SVneo cell invasion without affecting actin polymerization, suggesting that YAP rather than actin polymerization is the key regulator of HTR8/SVneo motility. As a co-transcription factor, YAP increases the expression of downstream genes by promoting the transcriptional activity of TEAD4 44. RNA-sequencing revealed that S1P increased Cathepsin D (CTSD) and EMILIN1 expression, both of which are predicted to be downstream targets of YAP-TEAD4. CTSD is a broadly expressed peptidase, which belongs to the family of lysosomal aspartic protease and is involved in the degradation of intracellular protein. This degradation is tightly linked to the autophagy process but compromised in PE 61. Secreted CTSD could also work extracellularly on cleavage proteins such as SPARC to promote cell invasion 62 and tumor metastasis 63. While EMILIN1 is a connective tissue glycoprotein associated with elastic ﬁbers that exerts a crucial role in promoting EVT migration and/or invasion by increasing expression of membrane type I-matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase 2 (MMP-2) 64. Increased transcription of CTSD and EMILIN1 may mediate the promoting effect of S1P on HTR8/SVneo invasion.

In summary, we found that the SPHK1-S1P-S1PR2 axis is downregulated in PE placentas, which impairs the RhoA/ROCK signaling pathway and actin polymerization, and that YAP activation mediates genes transcription and trophoblast invasion (figure 6F). Our novel findings highlight the importance of SPHK1-mediated S1P synthesis in PE pathogenesis, thus providing an in-depth insight into the etiology of PE and potential intervention targets in the sphingolipid metabolism.

**Perspectives**

Altered sphingolipid metabolism has been reported to take part in the pathogenesis of multiple cardiovascular disease65. However, in the field of pregnancy related hypertension, little is known about the sphingolipid metabolism and signaling. A previous study focused on the role of sphingolipids in the pathophysiology of placental blood vessels in PE, and suggested that impaired S1P signaling in the endothelium might contribute to the vascular maldevelopment15. Here we demonstrates that S1P metabolism and signaling are active in trophoblasts. Furthermore, S1P promotes trophoblast in a YAP dependent manner. Mechanistically, YAP mediated gene transcription is enhanced by S1P induced actin polymerization, in both hippo dependent and independent ways. Our findings not only highlight the importance of SPHK1-mediated S1P synthesis in PE pathogenesis, but also broaden our understanding of the regulation of YAP activity by GPCR mediated cytoskeleton dynamics.

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**Disclosure**

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**Novelty and Significance**

**What Is New?**

* S1P is metabolized throughout pregnancy in placental trophoblast.
* PE pregnancies are associated with S1P and SPHK1deficiency, and inhibition of SPHK1 during placentation induces a PE-phenotype in mice.
* S1P promotes trophoblast invasion and migration in a YAP-dependent manner, which is mediated by S1PR2 and requires downstream RhoA/ROCK activation as well as actin polymerization.

**What Is Relevant?**

* Previous reports highlight that impaired S1P signaling in the endothelium may contribute to the vascular maldevelopment of PE.
* Our findings highlight the importance of SPHK1-mediated S1P synthesis in trophoblast for PE pathogenesis, thus providing an in-depth insight into the etiology of PE and potential interventions targeting sphingolipid metabolism.

**Summary**

Our study demonstrates that the SPHK1-S1P-S1PR2 axis is downregulated in PE placentas; this impairs the RhoA/ROCK signaling pathway and actin polymerization. YAP activation mediates gene transcription and trophoblast invasion. Together, our findings suggest that disrupted S1P metabolism and signaling contribute to the pathogenesis of PE.

Figure 1. **Low S1P levels were associated with downregulated SPHK1 in PE placentas.**

(A) S1P levels in normal and PE complicated placentas, n=6, two-tailed t-test, \*p<0.05; (B) mRNA levels of *SPHK1*, *SPHK2*, *SGPP1*, *SGPP2* and *SGPL*, n=12, two-tailed t-test, \*p<0.05; (C) Western blotting of SPHK1 in normal and PE term placentas, first-term villi and term placentas, n=6, two-tailed t-test, \*p<0.05; IF staining of SPHK1 in different gestations of human placentas (D); decidual (E) or mouse uteroplacental unit at different gestations (F), scale bar 200 µm (D,E) and 20000 µm (F). Data presented as means ± SEM.

Figure 2. **SPHK1 inhibition during placentation induced a PE phenotype in mice and impaired placentation.**

(A) Schematic illustration for experimental design; (B) Systolic blood pressure of pregnant mice treated with PF543, saline and blank, n=6, two-way ANOVA and Tukey’s multiple comparison test, \*p<0.05; (C) Fetal weight, fetal crown-rump length, and placenta weight measured on GD 18.5, n=63-74, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. saline; (D) H&E staining of maternal kidney on GD13.5 and GD 18.5, and measurement of Bowman 's space, n=3, two-tailed t-test, \*p<0.05 vs. saline; (E) IHC staining of CK7 in uteroplacental unit and labyrinth to placenta ratio, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. saline, scale bar 20000 µm; (F) Hemodynamics of uterus and umbilical artery, RI and S/D, two-tailed t-test, \*p<0.05. Data presented as means ± SEM.

Figure 3. **S1P increased trophoblasts mobility through RhoA activation**

(A) S1P levels in HTR8/SVneo cell lysate and culture medium after vehicle (methanol) or PF543 (100 μM) treatment, n=3, two-tailed t-test, \*p<0.05; (B) Representative images and statistics of Matrigel-based invasion assays on HTR8/SVneo cells given vehicle and S1P treatments, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle, scale bar 400 µm; (C) Western blotting of GTP-RhoA, RhoA, p-MYPT1(Thr696), MYPT1, p-YAP(Ser127), YAP and CTGF of HTR8/SVneo cells after 1 hour of incubation with 20 μM CCG1423, 10 μM latrunculin B (Lat B) or 250 nM S1P, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle (DMSO); (D) IF staining of HTR8/SVneo cell after aforementioned treatment, YAP (red) and phalloidin (green), n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle (DMSO), scale bar 20 µm; (E) The representative images and statistics of Matrigel-based invasion of HTR8/SVneo after 24 hours incubation with 20 μM CCG1423 or 250 nM S1P, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle, scale bar 400 µm. Data presented as means ± SEM.

Figure 4. **S1P induced HTR8/SVneo invasion is dependent on YAP**

(A) HTR8/SVneo cells were transfected with si*NC* or si*YAP* for 6 hours followed by culturing in fresh medium for another 48 hours. Cells subjected to Matrigel transwell assay in the presence of vehicle or 250 nM S1P, n=3, two-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. si*NC* vehicle, scale bar 400 µm; (B) Volcano plot of gene expression of vehicle and S1P treated HTR8/SVneocells; (C) Enriched gene ontology (GO) analysis of biological process of genes upregulated in S1P treatment group; (D) mRNA levels of *COL9A3*, *EMILIN1* and *CTSD* after 24h of S1P treatment, n=3, two-tailed t-test, \*p<0.05; (E) Western blotting of CTSD, EMILIN1 in HTR8/SVneocells after 24 hour of incubation with 100 µM PF543 or 250 nM S1P, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle (methanol); (F) Western blotting of CTSD, EMILIN1 in si*YAP* or si*NC* transfected HTR8/SVneocells followed by 24h of incubation with 250 nM S1P, n=3, two-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. si*NC*. Data presented as means ± SEM.

Figure 5. **S1P activated YAP in both Hippo-dependent and -independent ways.**

Co-IP of F-actin with Hippo pathway components in (A) HTR8/SVneo cells and human placenta tissue; Co-IP of LATS1 and F-actin in (B) HTR8/SVneo cells and human placenta tissue; Co- IP of YAP and F-actin in (C) HTR8/SVneo cells and human placenta tissue; (D) Western blotting of p-MYPT1(Thr696), MYPT1, p-LATS1, LATS1, p-YAP(Ser127), YAP and CTGF in WT and YAP-5SA cells after 1 hour of incubation with 100 µM PF543 or 250 nM S1P, n=3, two-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. WT vehicle (methanol); #p<0.05 vs. YAP-5SA vehicle (methanol); (E) IF staining of phalloidin (green), YAP (red, for WT) and MYC-tag (red, for YAP-5SA) after 1 hour incubation with 100 µM PF543 or 250 nM S1P, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. vehicle (methanol), scale bar 100 µm; (F) Matrigel transwell assay of WT and YAP-5SA cells been treated with 100 µM PF543 or 250 nM S1P for 1h, n=3, two-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. WT vehicle (methanol); #p<0.05 vs. YAP-5SA vehicle (methanol), scale bar 400 µm. Data presented as means ± SEM.

Figure 6. **RhoA-ROCK pathway and YAP activation were impaired in PF543 treated mice placentas and PE placentas.**

(A) Western blotting of S1PR2, GTP-RhoA, RhoA, p-MYPT1(Thr696), MYPT1, p-LATS1, LATS1, p-YAP(Ser127), YAP, CTSD and EMILIN1 in mouse placentas treated with blank, saline or PF543 (1mg/kg) at GD 13.5, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. saline; (B) Western blotting of nucleic and cytoplasmic YAP of blank, saline or PF543 (1mg/kg) treated mouse placentas at GD 13.5, n=3, one-way ANOVA and Tukey’s multiple comparison test, \*p<0.05 vs. saline; (C) Western blotting of S1PR2, p-MYPT1(Thr696), MYPT1, p-LATS1, LATS1, p-YAP(Ser127), YAP, CTSD and EMILIN1 in normal and PE placentas, n=6, two-tailed t-test, \*p<0.05; (D) GTP- RhoA to RhoA ratio in normal and PE placentas, n=3, two-tailed t-test, \*p<0.05; (E) Western blotting of nucleic and cytoplasmic YAP of normal and PE human placentas, n=6, two-tailed t-test, \*p<0.05; (F) Proposed working model of S1P-regulated YAP activation through S1PR2-RhoA-ROCK pathway in normal and PE placental trophoblasts. Data presented as means ± SEM.