Reviewer 1：

1. Introduction: The important link between S1P-mediated cytoskeleton polymerisation and regulation of YAP could have been added as a line to the introduction rather than inclusion in results section to help explain this connection to the reviewer.

Response： Thank you for the kind suggestion, we have added a line to introduce the association between S1P-mediated cytoskeleton polymerization and YAP activation.

1. Did the authors measure SPHK1 enzyme activity in placental tissue in addition to mRNA and protein expression. Given that the activity of an enzyme is important rather than expression in a physiological environment?

Response: We totally agree with the reviewer’s comment, enzyme activity plays vital role in enzyme function in addition to expression. Therefore, in the revision, we measured SPHK1 activity using a fluorescence-based assay1, and the SPHK1 enzyme activities were also significantly decreased in preeclamptic placentas, supporting the idea of S1P synthesis is impaired in PE-complicated placentas. These results of activity assay are supplemented into Fig S1B.

1. Were the levels of sphingosine upstream of S1P and SPHK1 additionally altered in their placental samples?

Response: Thank you. Sphingomyelin, ceramide and sphingosine are the major upstream metabolites of S1P, the concentration of sphingomyelin and ceramide have been reported elevated in PE-complicated placentas2. SPHK1 involves in the metabolism of ceramide to S1P, inhibition of which could result in the decreased S1P content but increased ceramide accumulation. Our study focused on the metabolism and function of S1P on trophoblast, which enhance the understanding of role of bioactive sphingolipids in pregnancy.

1. Can the authors comment as the relationship between their evidence of deficiency in placental S1P at term and previous work where S1P was increased in early PE in a metabolite biomarker screen of serum samples (Kenny et al. 2010 Hypertension).

In addition, can the authors comment how this early increase in S1P in PE in serum relates to their demonstrated role for deficient S1P in mediating placentation in early pregnancy?

Response：Thankyou. Professor Kenny has been investigating the pathogenesis of pre-eclampsia for many years and is a collaborator with this work and an author on this publication. Kenny’s pioneering research shielded a light on the early prediction of preeclampsia by using metabolic approach3, which enabled us for the early prediction and intervention of preeclampsia. Preeclamptic serum S1P levels were elevated in Kenny’s study, but our study found out S1P synthesis was impaired in term preeclamptic placentas. It seems that the conclusions are contradictory between two studies, nevertheless, the two studies focused on distinct aspects of S1P metabolism. Erythrocytes, endothelial, platelets are the main sources of circulating S1P, especially erythrocytes4. Preeclampsia is thought to be an ischemia disease5, erythrocytes S1P synthesis and release were induced under hypoxia condition6, which may contribute the elevated serum S1P in PE patients. In our study, we demonstrated that trophoblast itself had a robust S1P synthesis, autocrine could be the major regulation of S1P on trophoblast, especially EVTs, which are isolated from circulation. S1P deficiency impairs trophoblast invasion, led to the inadequate remodeling of spiral artery, hypoxia occurs., which could be the reason of elevated serum S1P level observed in Kenny’s study.

1. Is the timing of PF543 in the murine model at GD7.5 rather late to be having a significant effect on placentation in murine pregnancy? Would placentation already be nearly complete?

Response: Thank you for the comments. The mouse blastocyst implants into the uterus at around embryonic day (E) 4.5. After implantation, the trophectoderm cells continue to differentiate and grow to form extra-​embryonic ectoderm and the ectoplacental cone (EPC), then the amnion, chorion and allantois are formed at around E6.5. Implantation also stimulate the decidualization process at E4.5. At E8.5, Chorio-allantoic fusion allows for mesoderm-derived blood vessels to invaginate into the chorionic trophoblast layer to form the placental labyrinth, which generates the basic structure of mouse placenta7. After a series of differentiation and expansion, mature placenta is formed around E14.58. We chose E7.5 – E12.5 as the manipulation time window to synchronize the placentation process, and intervention at this period resulted in shallow invasion and impaired spiral artery remodeling, along with PE phenotypes in mice, so GD7.5 wouldn’t be late for intervention. What’s more, advance the timing of PF543 treatment may compromise the decidualization and trophoblast differentiation process9,10. Therefore, GD7.5 is the reasonable timing for PF543 treatment.

1. Is the concentration of S1P (250nM)used in vitro experiments physiologically relevant and can the authors comment on the S1P levels reported in pregnancy?

Response: Thank you for the comments. Yes, the concentration (250nM) used in our study is physiologically relevant, the reasons are listed as follow: 1. Concentrations of S1P in maternal plasma vary from around 100-300 nM2; 2. Whole placental S1P concentration in our study was around 100 nM, our SPHK1 staining indicated that trophoblasts have the most abundance expression of SPHK1, so the concentration in trophoblast would be higher than the whole placenta lysates; 3. 250nM was reported to sufficiently activate all five S1P receptors6. Taken all the factors into consideration, the concentration (250nM) used in our study is logical and physiologically relevant.

1. Can the authors comment on the lack of alteration of the S1PR expression in placental tissue particularly S1PR2 given that other components in the signalling pathway were activated.

Response: Thank you for the comments. Once activated by S1P, S1PR expression on the cell surface is downregulated by receptor internalization, but such effect was transient as the receptor recycle back to the membrane11. However, few factors was reported to regulate the transcription of S1PRs, such as TGF-β and conjugated bile acids12,13. But in our study, no significant change was observed in S1PRs expression, possibly due to the lack of proper stimulation. And S1P works as ligand, its synthesis is impaired in preeclamptic placenta. Even though the S1PR2 doesn’t show any difference between normal and preeclamptic placentas, but the decrease in ligand would also impair the signaling pathway.

1. Figure S3G, labelling of pYAP and CTGF is incorrect, not representative of western blot images (i.e. S1P decreases pYAP1 and increases CTGF on western but graph indicates the opposite. This can be easily corrected.

Response: Great thanks for pointing out the mistake on our graph, we have carefully checked the results and corrected it on the graph, the corrected version was resubmitted along with the manuscript.

1. I would suggest if possible including the Supplementary Figure 9 in the actual manuscript as its an excellent graphic which captures the story of the manuscript.

Response: Thanks for the hints. We rearranged the layout of figure 6, and moved the graphic illustration (Supplementary Figure 9) to figure 6F, which would be more accessible to the readers.

Reviewer 2：

1. the mechanism is very complex and definitely there is a need for a summary slide depicting this mechanism in preeclamptic placentae.

Response: We drew a graphic illustration to summarize our study, but it was in the last supplementary figure of supplementary materials, which could be easily overlooked. In the revised version, we rearranged the layout of figure 6, and moved the graphic illustration (Supplementary Figure 9) to figure 6F, which would be more accessible to the readers.

1. The rational for in vivo study is unclear - although authors intended to show that S1P metabolism is a cause rather than a consequence of preeclampsia, wouldnt it be more translational to create a preeclampsia animal model and then aim to rectify low S1P levels through supplementation and see the effect on preeclampsia symptoms and features?

Response: We appreciate your comments and advice. The ideal model to investigate preeclampsia should duplicate the pathogenesis, pathophysiology and phenotypes of preeclampsia in human, such as early immune response, shallow invasion of trophoblast, inadequate spiral artery remodeling, reduced placental perfusion, systemic inflammation, endothelial dysfunction, maternal organ damage, high blood pressure and fetal growth restriction. However, none of the present models exhibit all the features of preeclampsia. sFlt-1 overexpression model and reduced uterine perfusion pressure (RUPP) model are the most widely used PE model, but neither of which are suitable for our study, as sFlt-1 overexpression model induces PE phenotypes by impairing the angiogenesis14, and RUPP model reduces placenta perfusion by physical ligation of the artery15. Other genetically modified mouse models of preeclampsia may mimic the shallow invasion of trophoblast such as galectin-7 overexpression16, but they are focused on their specific factors, S1P deficiency may not manifest in such models. To specify the influence of S1P deficiency on trophoblast invasion and preeclampsia, we interfered the S1P synthesis by inhibiting SPHK1 activity during placentation, which resulted in shallow invasion of trophoblast and impaired spiral artery remodeling, along with PE phenotypes in mice, indicating S1P deficiency could be one of the pathogenesis of preeclampsia. However, lack of extensive validation is one of the limitations of our study, further validations are needed to make it translational when a better PE model is established.

1. apart from the fact that this new mechanism is important in the pathogenesis of preeclampsia, what type of therapeutic intervention should be explored to target the aberrant sphingolipid metabolism in preeclampsia?

Response: We identified placental S1P deficiency contributes to the pathogenesis of PE, S1P supplementation or activate SPHK1 with specific agonist - defensamide could be the therapeutic intervention targets. Since S1P is an endogenous metabolite, S1P supplementation may be the preferable approach considering the safety. Extra caution is needed when treating the pregnant women, in future study, nanotech based placenta specific delivery system would come in handy to specifically delivery of S1P to placenta.

1. there was no discussion of different phenotypes of preeclampsia, early and late onset and how this mechanism relates to either.

Response: Thank you for the comments. Preeclampsia is divided into two sub-types: early and late onset pre-eclampsia, early onset preeclampsia arises owing to shallow invasion of EVTs and defective placentation, whilst late onset pre-eclampsia may center around endothelial dysfunction17. In our study, we demonstrated that S1P deficiency caused by downregulated SPHK1 impaired trophoblast invasion, thus contributing to the pathogenesis of preeclampsia. In addition to what we observed in our study, impaired endothelial S1P signaling in placental chorionic arteries was reported by other group18, disruption of S1P metabolism also led to the endothelial dysfunction. So, to our knowledge, S1P is involved in both early and late onset PE, which could be a promising target of PE intervention.

1. Since S1P is shown to activate Yap, can the authors explain why phosphorylation of Yap is downregulated by S1P (Figure 3C)?

Response: YAP is a co-transcription factor, subcellular location controls its activation. YAP is phosphorylated when Hippo pathway is activated, phosphorylated YAP binds to 14-3-3 protein, which increases YAP cytoplasm retention, thus restricts its nuclear translocation and activation. S1P treatment inhibits Hippo pathway as shown by decreased LATS1 and YAP phosphorylation, decreased YAP phosphorylation, on the contrary, increases YAP nuclear localization and activation. Therefore, it’s not contradictory that S1P decreases YAP phosphorylation and activates YAP at the same time.

1. I strongly encourage the authors to summarize their proposed pathogenic mechanism in a graphical format and suggest targeting options for in vivo scenario.

Response: Thank you for your suggestion. In the revised version, the graphic summary of this work has been moved into figure 6 from Figure S9 of previous submission. It depicts the proposed mechanism that In trophoblasts from normal placenta, S1P produced by SPHK1 interacts with S1PR2 to activate downstream RhoA-ROCK signaling, which promotes F-actin formation. F-actin increases YAP activity and downstream gene (CTSD, EMILIN1) transcription to promote trophoblast invasion to maintain proper placental function and successful pregnancy. S1P synthesis is impaired in preeclampsia placentas; due to SPHK1 deficiency, RhoA-ROCK pathway and F-actin formation are downregulated, leading to the sequestering of YAP in the cytoplasm and inhibited CTSD, EMILIN1 expression. Trophoblast invasion is thus inhibited, resulting in inadequate spiral artery remodeling and PE. Based on our findings, S1P supplementation could be a promising target for PE intervention, but further validation in better or multiple preeclampsia models is essential.

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