Title page**Elevated levels of the secreted Wnt agonist RSPO3 in preeclamptic pregnancies**

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Abstracts and keywords

Abstract

Objective: Preeclampsia is a syndrome characterized by hypertension and poor placental development. The developmental Wnt pathway plays an important role in placental development and we hypothesized that Wnt signaling would be dysregulated in preeclampsia.

Methods: To elucidate aberrations in the Wnt signaling pathway we conducted a pathway analysis on placental mRNA in late-onset preeclampsia and normal pregnancy from the STORK study (n=10 in each group, RNAseq) to identify differentially expressed genes. In addition, we compared circulating levels of secreted Wnt agonists and antagonists at term pregnancy and 6 months post-partum from an acute preeclampsia study (preeclampsia n=34, normal pregnancy n=61).

Results: We found circulating and placental mRNA levels of the secreted Wnt agonist RSPO3 at term elevated in preeclampsia. Increased plasma RSPO3 was associated with high mean arterial pressure. Further, pathway analysis of placental tissue revealed elevated mRNA levels of upstream ligands WNT6 and WNT10A and frizzled receptors 2 and 4 in preeclampsia and downstream activation of the non-canonical Ca^{2+} /NFAT pathway. Finally, plasma DKK3 was decreased in preeclampsia 6 months post-partum.

Conclusion: We identify a potential role for RSPO3 and activation of non-canonical Wnt signaling in preeclampsia.

Keywords: RSPO3, preeclampsia, wnt

Text

Introduction

Up to 10 % of pregnancies suffer from abnormal placental development and represent high risk for pregnancy complications including preeclampsia (PE) (1). PE affects 3–5% of all pregnancies and is characterized by development of hypertension after 20 weeks of gestation with presence of proteinuria or, in its absence, of signs or symptoms reflecting target organ injury (2). The multifactorial etiology of PE includes poor placental development characterized by endothelial dysfunction, inflammation, oxidative stress, dysregulated angiogenic and metabolic changes due to reduced placental perfusion (3). While it previously was thought that PE would resolve quickly after delivery of the placenta, it has become clear that these women have a higher risk of developing hypertension, ischemic heart disease, stroke and venous thromboembolic events within 5-15 years after pregnancy (4).

A number of signaling pathways are involved during placental development, and trophoblast invasion and differentiation are tightly controlled by paracrine and autocrine factors including cytokines, growth factors and other components of the extracellular matrix (ECM) (5). Dysregulation of these factors in the uterine microenvironment may lead to shallow trophoblast invasion resulting in decreased placental perfusion and ischemia/hypoxia followed by subsequent PE (5). Recent studies suggested an important role for Wnt signaling in placental development and an abnormal activation of Wnt signaling pathway in PE (5-8).

The Wnt proteins initiate signaling by binding receptor complexes composed of a Frizzled receptor and a low-density lipoprotein receptor-related protein 5/6, resulting

in different intracellular responses, involving β -catenin (canonical pathway), Ca^{2+} or other second messengers (non-canonical pathways), and these Wnt signaling pathways regulate diverse cellular processes such as proliferation, survival, cell fate determination and migration (7). The majority of Wnt ligands and Frizzled receptor have been detected in first trimester placental tissue (9). The Wnt pathways also consists of a number of endogenous secreted pathway modulators including agonists such as R-spondins (RSPOs) which act synergistically with Wnt ligands to enhance signaling (10) and antagonists such as dickkopf (DKKs) and the secreted frizzled related proteins (sFRPs), that regulate both canonical and non-canonical Wnt signaling by binding various ligands and receptors (11, 12). In the context of PE, increased expression of DKK1 and sFRP4 has been detected in placental tissue (13, 14) leading to decreased β -catenin, emphasizing a role for these secreted Wnt antagonists in dysregulated canonical Wnt signaling in PE (8). However, activation of the non-canonical Ca^{2+} /NFAT pathway during hypoxia modulates the placental expression of several cytokines and growth factors (15) but the role of non-canonical Wnt pathways, which may be equally relevant in the pathogenesis of PE, is less established. Secreted Wnt modulators often circulate at measurable levels and may indirectly reflect activity in the Wnt signaling pathways (12) but have not been evaluated in PE.

In the present study, we aimed to further elucidate aberrations in Wnt signaling in PE. We first conducted a pathway analysis in placental mRNA to compare regulation of canonical and non-canonical Wnt pathways in PE and normal pregnancy (NP). We also hypothesized that secreted Wnt modulators would be dysregulated and represent biomarkers in PE and we evaluated circulating levels of agonists and antagonists at term pregnancy and 6 months post-partum in these groups of women.

Methods

Two study populations (Table 1) were evaluated, each comprising women with normal (NP) and PE pregnancies (16, 17). Plasma levels of secreted Wnt proteins were evaluated in a population consisting of 61 healthy women, collected at term (35-36 weeks), and 34 women diagnosed with PE defined by hypertension (blood pressure >140/90 mmHg) and proteinuria (i.e. urine protein to creatinine ratio above 30 mg/mmol creatinine or at least +1 proteinuria on urine stix on two occasions at least 24 h apart) collected before delivery, as described previously (16). Samples were also collected at six months postpartum. In the second population (Table 1), placental biopsies were collected after vaginal or cesarean delivery from 10 women with NP and 10 with late onset PE (all cases diagnosed after 34 weeks) as described (17). Measurements of brachial arterial systolic and diastolic blood pressure (BP) made with an automated oscillometric technique (Dinamap ProCare 300-Monitor, Criticon, GE Medical Systems,) was performed using a pneumatic cuff on the subject's right upper arm. Triple recordings were obtained, and the mean systolic and diastolic pressures were assessed as the mean of the three recordings.

Exclusion criteria included known pre-gestational diabetes, severe chronic medical conditions (such as lung, cardiac, gastrointestinal or renal diseases); use of immune-suppressive medication and pregnancies complicated by major fetal malformations or aneuploidy. Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Ethics in Medicine. All women received conventional medical treatment at the discretion of the responsible physician. The work described was carried out in accordance with The Code of Ethics

of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Blood sampling and enzyme immune-assays (EIAs)

Peripheral venous blood was drawn into pyrogen-free blood collection tubes with EDTA as anticoagulant. The tubes were immediately immersed in melting ice and centrifuged within 30 min at 2000g for 20 min to obtain platelet-poor plasma. Plasma was stored in multiple aliquots at -80°C and thawed only once.

Plasma levels of secreted Wnt proteins were analyzed in duplicate using commercially available antibodies (R&D Systems, Abingdon, UK) in a 384 format using a combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT, USA) dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an ELISA plate reader (Bio-Rad, Hercules, CA, USA). Intra-/ inter-assay coefficients of variation were $<10\%$. These assays have been validated previously (18).

Collection, storage and RNA extraction of placental biopsies

As previously reported (17), placental biopsies were collected after vaginal or cesarean delivery. Blocks of 2–4 cm were taken from the placental parenchyma, briefly washed in phosphate buffer saline, snap frozen in liquid nitrogen, and stored at -80°C until RNA isolation. Half of the biopsy was homogenized in TRIzol reagent (Invitrogen, Life Technologies) on ice with a tissue grinder (Sigma Aldrich). Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies) and purified with RNeasy microkit columns (Qiagen, Netherlands). Purity and

concentration of isolated total RNA was measured using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA) and RNA integrity number (RIN) was estimated using Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

RNA sequencing

Sequencing libraries were prepared from 500 ng of total RNA using the TruSeq RNA sample preparation reagents (Illumina, San Diego, CA) according to the manufacturer's instructions, with fragmentation for 4 minutes at 94°C. The libraries were sequenced using 125 bp paired-end sequencing on an Illumina HiSeq2500. We recorded an average 22.3 million (range, 20.2–24.6 million) paired reads per sample. Fastq files were generated using bcl2fastq (v1.8.4). Sequence reads were mapped to the reference genome (hg19/GRCh38) using TopHat2 (v2.0.13) and Bowtie2 (v.2.2.3.0). Library sizes and standard deviations for input into TopHat were calculated empirically by aligning 1,000,000 reads to an index built from human cDNA sequence. Sequence alignment was guided using only previously annotated gene models downloaded from Ensembl (Homo_sapiens.GRCh38.79.gtf). On average, there was 73.6% concordant read pair mapping (range 69.6–76.6), with a mean unique mapping of 94.1%. Raw expression counts were calculated per gene using featureCounts and the same gtf file that was used for the read alignment. Differential expressed genes in RNA sequencing (RNAseq) data were tested using DESeq2 (19) package for R dividing the samples between NP and PE groups. Outlier detection (Cook distance cutoff) and filtering out low expressed genes were performed using the default method in DESeq2, as previously published (17).

Statistical analyses

For the circulating markers, variables were skewed and log transformed as comparison between groups was performed using linear regression adjusting for age, BMI, parity and gestational age. Paired changes were evaluated within groups with the Wilcoxon signed rank test. Correlation between variables was investigated by Spearman's rho. Logistic regression was used to evaluate the impact of MAP on the association between RSPO3 and having a diagnosis of PE. An á priori power calculation indicated that with $\alpha = 0.05$, a ratio of 1.5 between controls and patients and a standard deviation <45%, we had an 80% chance to detect a difference of 25% between the two group. We considered a two-sided $P < 0.05$ as statistically significant.

Results

Maternal characteristics are summarized in Table 1. In both populations, women with PE had higher MAP at term, and in the plasma population, BMI was higher and gestational age was lower in PE women.

Secreted Wnt proteins in PE at term

When evaluating secreted Wnt proteins at term, several findings were revealed. The secreted Wnt agonist RSPO3 and antagonist SOST was elevated in PE women after adjusting for age, BMI, parity, and gestational age (Figure 1A). No significant difference in the plasma levels of secreted Wnt antagonists, DKK1, DKK3, sFRP3, and WIF1, was detected at term. RSPO3 and SOST were not different during pregnancy from week 14-16 until week 35-36 in controls (Supplemental Figure 1). Figure 1B shows the distribution of MAP in NP and PE. Dichotomizing MAP at 107 mmHg (i.e.

systolic/diastolic blood pressure 140/90) in the plasma population as a whole, revealed markedly elevated plasma RSPO3 levels in women with high MAP (Figure 1C, $p=0.001$). Evaluated as a continuous variable, the correlation between MAP and RSPO3 at baseline was $r=0.38$, $p<0.001$. In univariate logistic regression (Figure 1D), a 1 SD increase in RSPO3 (log) was associated with a 2.53 times higher likelihood of having PE. Further adjustment with age, BMI, parity and gestational age had little impact on this association. However, when including MAP in the adjustment the association between RSPO3 and PE was completely mitigated.

Placental regulation of the Wnt pathway in PE

The expression of Wnt pathway genes in placental tissue are summarized in Figure 2. The Wnt ligands WNT6 and WNT10A and the Wnt receptor FZD2 and FZD4 were increased in PE placentas (Figure 2A/B). Differentially expressed genes in the canonical pathway included some members of the β -catenin destruction complex (CK1 ϵ (CSNK1E), CK2(CSNK2A3), AXIN1 increased in PE), nuclear regulation of β -catenin (SOX17 increased) and increased mRNA levels of the transcriptional targets JUN and CCND3. No differences in the non-canonical planar cell polarity pathway were detected, except an increase in PRICKLE2 mRNA in PE. For the non-canonical Wnt/Ca²⁺ pathway, the major isoform of CaM kinase, CAMK2D belonging to the regulatory subunit of calcineurin (CaN) were increased in PE as was NFAT4C, while a decrease in PPP3CB was observed.

For the secreted Wnt modulators evaluated above in plasma, SOST and WIF1 were not expressed, while a trend for increased RSPO3 ($p=0.056$) mRNA was detected in PE (Figure 2C). To further elucidate placental expression of RSPO3, we investigated

tissue expression in available databases. In the HPA RNAseq dataset, placenta was among the tissues with the highest expression of RSPO3 (Figure 2D). Protein expression data for RSPO3 in placental tissue is available from the Human Protein Atlas and show moderate expression in the majority of placental cells (>75%, Figure 2E).

Finally, we evaluated correlations between RSPO3 and transcriptional targets in the Wnt pathways identified above (i.e. CCND3, CJUN, NFATC4) as well as other reported downstream targets (Figure 2F). No significant association with CCND3 was noted, while a positive correlation with CJUN was observed in NP. In both groups, RSPO3 was strongly correlated with NFATC4. Further, no association with VEGFA was found, while for both RNF43 and LGR5, a strong positive correlation was observed in PE, but not NP. Of relevance, VEGFC mRNA levels were markedly higher in the PE placentas (median counts (25th 75th percentile), 212 (176, 415) vs. 126 (99, 208), $p=0.0003$) and correlated with RSPO3 expression ($r=0.82$, $p<0.001$).

Secreted Wnt proteins in PE at 6 months post-partum

As seen in Table 2, plasma RSPO3 increased in the NP group post-partum, but not in the PE group, giving modestly higher levels in the PE group at 6 months post-partum ($p=0.10$). Nonetheless, RSPO3 remained correlated with MAP at 6 months post-partum in the group as a whole ($r=0.34$, $p=0.002$). In the other secreted Wnt proteins, some significant findings were revealed. First, as for WIF1 and sFRP3 the levels decreased in both PE and NP although not significant for sFRP3 in the NP group. Second, there was a modest increase in SOST in both groups reaching statistical significance in NP. Third, and most notably, whereas there was a large increase in DKK3 in the NP group, a marked decrease in DKK3 in the PE group was observed, resulting in a significant

difference in DKK3 levels at 6 months between PE and NP women. Finally, as for DKK1 there were no changes during follow-up. None of these secreted Wnt proteins correlated with MAP at 6 months

Discussion

We investigated circulating modulators of Wnt signaling and placental mRNA expression of the Wnt pathway in NP and PE. Our main findings can be summarized: i) circulating and placental mRNA levels of RSPO3 at term were elevated in PE, ii) increased plasma RSPO3 was associated with high MAP, iii) pathway analysis of placental tissue revealed elevated mRNA levels of upstream ligands WNT6 and WNT10A and frizzled receptors 2 and 4 in PE and downstream activation of the non-canonical Ca^{2+} /NFAT pathway, iv) plasma DKK3 was quite differently regulated in NP and PE after delivery resulting in decreased levels in PE 6 months post-partum. Taken together, we identify a potential role for RSPO3 and activation of non-canonical Wnt signaling in PE.

In normal pregnancy, adequate trophoblast invasion leads to normal remodeling of the spiral arteries, increasing the vessel's capacity and healthy placental perfusion. Activation of canonical Wnt signaling is critical in promoting trophoblast invasion and disruption of the pathway may be a main cause of shallow trophoblast invasion and disruption of remodeling of the spiral artery (Reviewed in (6-8)). Indeed, incomplete remodeling such as in PE is associated with decreased placental β -catenin mRNA and protein in third trimester placentas, indicating attenuated canonical Wnt signaling probably due to enhanced Wnt antagonism from elevated DKK1 and sFRP4 (14). We did not find alterations in placental or circulating levels of Wnt antagonists, except

higher plasma SOST levels at term in PE. Differences compared to previous publications could be due to placental samples from less severe PE (late onset). Localization where the placenta biopsies have been obtained could also influence expression profiles, which are challenging to interpret, and often more dependent on phosphorylation status than transcription levels (e.g. β -catenin). Although we found little evidence of dysregulated canonical Wnt signaling in our study, the increased CCND3 could reflect attenuated canonical activation since CCND3 in contrast to cyclin D1/D2, is increased in response to inhibition of canonical Wnt signaling (20, 21).

A major finding in our study was increased placental and circulating levels of RSPO3, strongly correlated with MAP, supporting a link between RSPO3 levels, endothelial dysfunction and hypertension in PE. The ubiquitin ligases ZNRF3 and RNF43 promote lysosomal degradation of Wnt receptors and binding of R-spondins to their receptors LGR4/5 clears these ubiquitin ligases from the membranes and stabilizes the binding between Wnt ligand and receptor, promoting Wnt/ β -catenin signaling (10). We speculate that the markedly stronger association between mRNA expression of RSPO3, its receptor LGR5 and the ligase RNF43 in PE placentas indicates activation and crosstalk between the Wnt signaling modifiers in PE. Knockdown of RSPO3 in *Xenopus* embryos induced vascular defects (22). Transgenic mice studies suggest a critical role for RSPO3 in placental development through effects on angiogenesis (22) and vessel remodeling during labyrinth formation, corresponding to chorionic villi in humans, resulting in reduced placental perfusion (23). Together, these studies indicate that impaired RSPO3 function could have a role in placental diseases. Furthermore, RSPO3 promoted proliferation and angiogenesis in endothelial cells *in vitro* by triggering canonical activation of VEGF (22), which plays a crucial role during

placentation and spiral artery remodeling and is dysregulated in PE (24, 25). While we observed no association between placental RSPO3 and VEGF, mRNA levels of VEGFC, which also promotes vascular remodeling in the placenta (26), were elevated in PE and correlated strongly with RSPO3. Indeed, increased circulating VEGFC has been demonstrated in PE and hypothesized to be compensatory enhanced due to edema and hypertension (27).

A more evident increase in non-canonical Wnt signaling was observed, as reflected by elevated levels of calcium-sensitive enzymes, including CaN and Ca^{2+} /calmodulin-regulated kinase II (CAMK2), which may activate NFAT signaling (28). Furthermore, NFATC4 mRNA was increased in PE placentas and correlated strongly with RSPO3 mRNA expression. In support of a link between RSPO3 and activation of non-canonical Wnt activation, a recent study identified a role for endothelial RSPO3 driven Wnt/ Ca^{2+} /NFAT signaling in the maintenance of vascular stability (29). To our knowledge, increased expression of the Wnt ligands WNT6/10A or the receptors FZD 2/4 have not been described in PE previously. Interestingly, WNT6 and WNT10A are located immediately adjacent to one another on chromosome 2 suggesting co-regulation of respective promoters (30), and co-expression has also been shown in the placenta (31). While typically related to canonical Wnt activation, their co-expression has also been linked to non-canonical Wnt signaling (32). FZD2 and FZD4 expression are restricted to villous mesenchymal cells, which are important in triggering trophoblast invasion (33). Furthermore, these receptors may influence both canonical and non-canonical Wnt/ Ca^{2+} signaling and activate vascular growth and remodeling (34-36).

Taken together, based on the beneficial effects of RSPO3 in vascular remodeling (22, 23, 29), enhanced RSPO3, as well as several Wnt ligands and receptors that may activate canonical and non-canonical Wnt/Ca²⁺ signaling, could reflect a compensatory response to enhance Wnt signaling and improve placental development in PE. A model showing the potential role of RSPO3 in PE is shown in Figure 3. However, both NFAT (37) and CAMK2 (38) may antagonize the canonical pathway and the prototypical activator of the non-canonical Wnt/Ca²⁺ pathway, Wnt5a, has been shown to contribute to the pathogenesis of PE through insufficient trophoblast invasion and inhibition of canonical Wnt/ β -catenin signaling (9, 39). Conversely, impaired Wnt5a signaling in extravillous trophoblasts has been linked to poor placentation in early gestation and subsequent PE (40). Still, by enhancing non-canonical Wnt/Ca²⁺ pathway activation, RSPO3 could potentially contribute to PE development.

The increase in circulating RSPO3 levels at 6 months post-partum in NP could reflect a natural down regulation during transient hypertension approaching term. The only significant difference between PE and NP at 6 months post-partum was the markedly decreased DKK3 levels in PE. In two recent experimental reports, DKK3 was reported to have anti-atherogenic effects in atherosclerosis, at least partly through its effects on SMC. In these studies, Yu et al. reported that plasma DKK3 was inversely related to carotid artery intima-media thickness and 5-year progression of carotid atherosclerosis (41) and Karamariti et al. showed decreased DKK3 levels in serum from patients with unstable angina (42). As women with previous preeclampsia have a markedly enhanced risk of developing hypertension and ischemic heart disease (4), the dysregulated DKK3 levels are worth further consideration in future studies investigating CVD risk in PE.

Our study supports dysregulated Wnt signaling in PE pregnancies, characterized by increased RSPO3 levels and downstream activation of the non-canonical Wnt/Ca²⁺ pathway. Increased RSPO3 and elevated placental expression of several upstream ligands and frizzled receptors in PE could reflect a compensatory response to enhance vascular function but augmentation of the Wnt/ Ca²⁺ pathway could also contribute to PE by inhibiting canonical Wnt signaling. Further studies are needed to investigate the role of RSPO3 in PE.

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Individual tables and captions

Table 1. Maternal characteristics in normal (NP) and preeclamptic (PE) pregnancies.

	Plasma population			Placenta population		
	NP	PE	P-value	NP	PE	P-value
<i>N</i>	61	34		10	10	
Age (years)	32 ± 5	32 ± 6	0.151	32 ± 4	30 ± 3	0.491
BMI (kg/m ²)	27.0 ± 3.4	30.5 ± 5.7	0.001	26.4 ± 4.0	30.7 ± 5.0*	0.051
Family history CVD	34 (56%)	20 (59%)	0.32	NA	NA	
Previous smoking <i>n</i> (%)	0 (0%)	2 (6%)	0.056	1 (10%)	1 (10%)	1.00
Multiparous (%)	27 (42%)	13 (33%)	0.10	6 (60%)	3 (30%)	0.11
Gestational age (weeks)	36 ± 0	35 ± 5	<0.001	40 ± 1	40 ± 1	1.00
MAP (mmHg)	85 ± 8	116 ± 10	<0.001	86 ± 10	99 ± 13*	0.014

Values are mean ± SD and *n* = (%). NA=Not available. *At gestational week 36-38

Table 2. Secreted Wnt proteins in PE and controls at term and at 6 months post-partum

Protein	Grp	Term	6 months postpartum
RSPO3	NP	0.47 (0.42 - 0.53)	0.55 (0.49 - 0.62)**
	PE	0.69 (0.59 - 0.81) †††	0.62 (0.53 - 0.73)
DKK1	NP	0.27 (0.22 - 0.32)	0.28 (0.22 - 0.35)
	PE	0.33 (0.25 - 0.43)	0.33 (0.24 - 0.45)
DKK3	NP	24.3 (16.5 - 35.8)	38.6 (29.3 - 50.8)***
	PE	31.1 (23.1 - 41.8)	20.7 (14.4 - 29.6) ††**
sFRP3	NP	1.03 (0.86 - 1.24)	0.96 (0.77 - 1.18)
	PE	1.21 (0.94 - 1.55)	0.77 (0.57 - 1.03)**
WIF1	NP	1.13 (1.07 - 1.2)	0.89 (0.84 - 0.94)***
	PE	1.05 (0.98 - 1.13)	0.83 (0.77 - 0.89)***
SOST	NP	0.16 (0.14 - 0.19)	0.21 (0.19 - 0.24)***
	PE	0.21 (0.17 - 0.26)†	0.22 (0.18 - 0.26)

Data are given as back-transformed estimated marginal means adjusted for BMI, parity and gestational age. † *p*<0.05 †† *p*<0.01, ††† *p*<0.001 between NP and PE. ***p*<0.01, ****p*<0.001 between term and 6 months postpartum.

Figure Legends

Figure 1. Secreted Wnt proteins at term. A) Circulating secreted Wnt proteins (ng/mL) at term (week 35-36) in normal- (NP) and preeclamptic pregnancy (PE). B) Histogram of mean arterial pressure (MAP) in NP and PE at the time of plasma isolation. C) Plasma RSPO3 at term pregnancy according to dichotomized MAP. D) Logistic regression analysis showing association between RSPO3 and PE (expressed as normalized log RSPO3, i.e. divided by SD) in univariate analysis, adjusted with maternal and gestational age, BMI, parity (RSPO3^{M1}) and further with MAP (RSPO3^{M2}). Odds ratios (OR) and 95% confidence intervals (CI) and p-value are presented. Data are presented as Tukey plots with outliers and p-values are adjusted for age, BMI, parity and gestational age.

Figure 2. Differences in Wnt pathway gene expression between women with normal (NP) and preeclamptic (PE) pregnancies. Significant results (unadjusted p-values) are indicated in red/blue (for increased/decreased mRNA expression). The figure summarizes relevant genes for the **A.** canonical and **B.** non-canonical Wnt/Ca²⁺ pathway. The figure is based on the Wnt signaling pathway in the KEGG database (Table S1). **C.** Bar graphs (median, 25th/75th percentile) focusing on placental mRNA expression of secreted Wnt modulators. **D.** Tissues with the highest (median levels) mRNA expression of RSPO3. Data source: GTEx Analysis Release V6p (dbGaP Accession phs000424.v6.p1). Expression values are shown in RPKM (Reads Per Kilobase of transcript per Million mapped reads), calculated from a gene model with isoforms collapsed to a single gene. **E.** Protein expression of RSPO3 in placental tissue. Data source: The Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000146374-RSPO3/tissue/placenta>). **F.** Correlations (spearman) between placental mRNA expression of RSPO3 and various targets in NP (blue) and PE (red). *p<0.05, **p<0.01.

Figure 3. Model showing the potential role of RSPO3 in preeclamptic (PE) pregnancies. **1.** In normal pregnancy, adequate trophoblast invasion leads to normal remodeling of the spiral arteries causing an increase in the vessel's capacity and healthy placental perfusion. Incomplete remodeling such as in PE

leads to increased resistance to maternal blood flow. **2.** Attenuated Canonical Wnt signaling in PE may contribute to vascular dysfunction. **3.** We hypothesize that increased levels of some Wnt ligands, Fzd receptors and the Wnt agonist RSPO3 in PE may reflect a compensatory response to enhance vascular remodeling and increase blood flow. **4.** Endothelial RSPO3 may also enhance vascular remodeling through activation of non-canonical Wnt/Ca²⁺ signaling. **5.** Alternatively, RSPO3 augmented Wnt/Ca²⁺ signaling contributes to PE through insufficient trophoblast invasion and inhibition of canonical Wnt signaling.