

Impaired Wnt5a signaling in extravillous trophoblasts: Relevance to poor placentation in early gestation and subsequent preeclampsia

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ABSTRACT

Background: Defective decidual endovascular trophoblast invasion and subsequent impaired spiral artery remodeling is highly associated with the pathogenesis of preeclampsia (PE). Since there are scant and conflicting data regarding the function of Wnt5a signaling in extravillous trophoblasts (EVT), the aim of this study was to investigate whether impaired Wnt5a signaling affects the invasive and tube forming capabilities of EVT.

Methods: Expression levels of Wnt ligands were compared between first trimester chorionic villi of women who later developed PE and women with unaffected pregnancies using publicly available microarray data (GSE12767). Wnt5a expression was examined in placentas using quantitative RT-PCR, Western blot analysis and immunohistochemistry. The function of Wnt5a signaling in EVT was investigated in an immortalized first trimester EVT cell line, HTR-8/SVneo, using small-interfering RNAs, recombinant human Wnt5a (rhWnt5a), and inhibitors of JNK or PKC.

Results: Microarray data analysis of the first trimester placentas showed that, among Wnt ligands, Wnt5a expression was significantly lower in women who later developed PE. The mRNA and protein expression levels of Wnt5a were significantly decreased in PE placentas compared with normal term placentas. Wnt5a knockdown significantly suppressed invasion and tube formation of HTR-8/SVneo cells, while the addition of rhWnt5a augmented the cell migration, invasion, and tube formation. Repression of Wnt5a/PKC signaling in HTR-8/SVneo cells inhibited cell invasion, but did not alter cell tube formation. In contrast, inhibition of Wnt5a/JNK signaling attenuated rhWnt5a-induced invasion and tube formation capabilities.

Conclusions: These findings suggest that impaired Wnt5a signaling is associated with poor placentation and subsequent PE.

1. Introduction

Preeclampsia (PE) is estimated to complicate 2–8% of all pregnancies, and is a leading cause of maternal and neonatal morbidity and mortality [1]. It has been postulated that PE occurs in two stages, i.e. that poor placentation typified by insufficient remodeling of spiral arteries (first stage) results in the release of excessive amounts of placental materials that lead to an excessive maternal inflammatory response and endothelial dysfunction (second stage) [2]. Placentation is a highly complex, regulated process that is crucial for normal fetal growth and the maintenance of a healthy pregnancy. During normal placentation, extravillous trophoblasts (EVT) invade into the decidua and the inner one third of the myometrium [3,4]. A subset of EVT, i.e. endovascular trophoblasts, invade maternal vessels, disrupt the

endothelium and the smooth muscle layer, and replace the vascular wall [3,4]. These conversions allow spiral arteries to become widely dilated independently of vasomotor control, thereby providing a sufficient blood supply in intervillous space to meet the requirements of the fetus. On the other hand, defective decidual endovascular trophoblast invasion and subsequent impaired spiral artery remodeling are thought to result in less dilated vessels and a lack of adequate placental perfusion, leading to the development of PE [3,4]. Although early placentation has long been the focus of intense research efforts, the mechanism of impaired spiral artery remodeling has not been fully elucidated.

Accumulating evidence suggests that Wingless (Wnt) signaling plays an important role in placental development and human trophoblast differentiation [5,6]. Wnt proteins comprise a family of 19 secreted

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glycoproteins that act as ligands via 12 putative cell-surface receptors and co-receptors [7]. Wnt signaling is a complex pathway that modulates a number of signal transduction pathways and regulates diverse biological functions in a highly cell- and tissue-dependent manner [7,8,9]. Wnt signaling is broadly divided into two main categories, i.e. beta-catenin-dependent and beta-catenin-independent signaling. The former signaling pathway regulates target gene expression via beta-catenin stabilization and its translocation to the nucleus, and is closely associated with cell proliferation, cell fate determination, and differentiation [7,9]. On the other hand, the latter includes activation of c-Jun NH2-terminal kinase (JNK) and protein kinase C (PKC), which regulate cell functions such as cell movement and polarity [7,8]. Of the 19 known Wnt ligands, 14 have been reported to be expressed in the first trimester placenta [10]. However, the exact role of Wnt ligands in early placentation, especially in spiral artery remodeling, remains controversial. We hypothesized that Wnt signaling is associated with poor placentation and subsequent PE. The aim of this study was therefore to explore the role and underlying mechanisms of Wnt signaling in impaired early placentation and PE.

2. Methods

2.1. Microarray data mining for Wnt ligands in early placentation

Publicly available gene expression data of chorionic villus sampling were obtained from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/gds/>) as series matrix files. There was only one data set (GSE12767) that was composed of the first trimester chorionic villi of women who later developed PE ($n = 4$) and women with unaffected pregnancies ($n = 8$). Expression values of Wnt ligands were log2 transformed, and probe sets whose maximum expression value across samples was less than 5 were removed from further analysis. Data were normalized across all samples by subtracting the mean and then dividing by the standard deviation of the expression value, and those values were converted into a heat map using Python (<https://www.python.org>).

2.2. Placenta samples

Placental villous tissues of the first trimester ($n = 4$) were obtained from normal pregnancies following induced abortion (6, 7, 8, and 11 weeks of gestation). In addition, placentas were collected from normal term pregnancies ($n = 10$) and PE pregnancies ($n = 12$) for quantitative real-time PCR (Table 1). Furthermore, since we have used up placental specimens for quantitative real-time PCR, another sample set of placentas (normal control = 6, PE = 6, respectively, Table 1) were prepared for Western blot analysis of Wnt5a expressions. Placentas were obtained immediately after Cesarean section in the absence of labor at Kyoto University Hospital, Japan. Villous tissues were collected from the central part of the placenta, and were macroscopically free of infarction or calcification. After a brief rinse in saline solution, these tissues were stored in RNeasy lysis buffer (Qiagen) at -80°C . PE was defined as a new onset of hypertension and proteinuria after 20 weeks of gestation with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two occasions at least 6 h apart, and proteinuria ≥ 300 mg/24 h.

2.3. Cell culture, RNA interference, and reagents

An immortalized first trimester extravillous trophoblast cell line, HTR-8/SVneo, was kindly provided by Dr. C. Graham (Queen's University, Kingston, Canada). The HTR-8/SVneo cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 . In order to examine the effect of Wnt5a on cell function, RNA interference was performed by

transfecting cells with two different small-interfering RNAs (siRNAs) targeting human Wnt5a mRNA or control siRNA (QIAGEN), using the HiPerfect Transfection Reagent (QIAGEN). HTR-8/SVneo cells were treated in the absence or presence of 100 and 200 ng/ml human recombinant Wnt5a (rhWnt5a; R&D Systems). PKC inhibitor GF109203X (5 μM) and the JNK inhibitor SP600125 (5 μM). All were assayed at least in duplicate in each experiment, and each experiment was performed at least three times.

2.4. RNA extraction and quantitative real-time PCR

Total RNA extraction from placental tissues and extravillous trophoblast cells (HTR-8/SVneo cells) was performed using the RNeasy Mini kit (Qiagen) as previously described [11]. Of 19 Wnt ligands, the expression of eight representative ligands in placental villous tissues was examined. The forward and reverse primers used for cDNA amplification are shown in Supplementary Table 1. 18S ribosomal RNA (rRNA) was used as the internal standard (Ribosomal RNA Control Reagents, #4308329, Applied Biosystems). Quantitative real-time PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Gene expression was estimated using the comparative crossing point method for relative quantification. All data were normalized using 18S rRNA as an internal control and expressed relative to controls.

2.5. Western blotting

Western blotting was performed as previously reported [11]. The sources of the antibodies and their concentrations were shown in Supplementary Table 2. Signals were detected by Pierce ECL Plus Western Blotting Substrate (#32132, Thermo Scientific) and visualized by the ChemiDoc system (BioRad). The band intensity of Wnt5a and Tubulin in each sample was quantified by Image J Imaging System Software Version 1.3 (National Institutes of Health).

2.6. Immunohistochemistry

Immunohistochemical staining was conducted by the streptavidin-biotin-peroxidase method as previously reported [12]. Briefly, sections were incubated with mouse monoclonal antibody against Wnt5a or cytokeratin 7 (Supplementary Table 2) overnight at 4°C . Slides were washed and incubated with biotinylated rabbit anti-mouse IgG (Nishirei), followed by incubation with streptavidin-peroxidase complex solution for 30 min at room temperature. Peroxidase activity was visualized by treatment with diaminobenzidine. The nuclei were counterstained with Mayer's hematoxylin and sections were observed under a light microscope (Olympus).

2.7. Immunofluorescent staining

Human placentas were fixed in 10% formaldehyde. After sections (5 μm) were deparaffinized, antigen retrieval was performed by boiling in 10 mM sodium citrate buffer (pH 6.0). Sections were then pre-incubated with 10% normal goat serum (50062Z, Life Technologies) with 0.3% Triton X-100 for 30 min at room temperature. The sources of the antibodies and their concentrations were shown in Supplementary Table 2. Slides were mounted with Prolong Gold Antifade Reagent with 4',6-Diamidino-2-Phenylindole (DAPI, #P36935, Molecular Probes). Images were taken by Leica TCS SP8 confocal microscopy (Leica Microsystems).

2.8. Comparison of Wnt5a expression in placenta using cDNA microarray gene expression data

In order to investigate whether the level of Wnt5a expression in the

Table 1
Clinical characteristics of the study groups.

Quantitative real-time PCR	Control	PE
Number	10	12
Maternal age (year)	34.6 ± 1.1	33.2 ± 1.5
Gestational age at delivery (weeks)	38.2 ± 0.1	33.0 ± 1.2
Nulliparous	3	7
Highest systolic blood pressure (mmHg)	103 ± 3	157 ± 4
Highest diastolic blood pressure (mmHg)	65 ± 3	98 ± 4
Severe hypertension; > 160/110 mmHg (n)	0	4
Proteinuria (n)	0	9
Low platelets and elevated liver enzymes (n)	0	3
Birth weight (z score)	−0.0 ± 0.3	−1.6 ± 0.1
Fetal growth restriction (n)	0	7
Indications for termination of pregnancy and cesarean section	Previous caesarean delivery (n = 6), breech presentation (n = 2), pelvic outlet obstruction due to ovarian tumor (n = 1), previous interstitial pregnancy (n = 1)	Nonreassuring fetal status (n = 6), hemolysis, elevated liver enzymes, and low platelet syndrome (n = 3), severe uncontrolled hypertension (n = 2), placental abruption (n = 1)
Western blot analysis	Control	PE
Number	6	6
Maternal age (year)	36.8 ± 2.4	32.8 ± 1.0
Gestational age at delivery (weeks)	37.7 ± 0.2	33.5 ± 0.9
Nulliparous	2	5
Highest systolic blood pressure (mmHg)	110 ± 3	160 ± 5
Highest diastolic blood pressure (mmHg)	63 ± 3	98 ± 6
Severe hypertension; > 160/110 mmHg (n)	0	4
Proteinuria (n)	0	6
Low platelets and elevated liver enzymes (n)	0	1
Birth weight (z score)	0.3 ± 0.2	−1.6 ± 0.3
Fetal growth restriction (n)	0	3
Indications for termination of pregnancy and cesarean section	Previous caesarean delivery (n = 4), breech presentation (n = 1), placenta previa (n = 1)	Nonreassuring fetal status (n = 2), placental abruption (n = 2) hemolysis, elevated liver enzymes, and low platelet syndrome (n = 1), eclampsia and previous myomectomy (n = 1)

PE, preeclampsia. Values are shown as mean ± SEM.

placenta is influenced by gestational age at delivery, gene expression data (GSE75010) of control and PE placentas were obtained from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/gds/>) as series matrix files. GSE75010 was chosen as it had the largest sample size (n = 157) available at GEO DataSets. There was only one probe corresponding to Wnt5a. Of 157 placentas, 44 women (normotensive controls) had a maximum systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg. 23 women had severe PE and delivered a newborn with birth weight z score < −1.5 before 34 weeks while there were 18 placentas of normotensive pregnancies with delivery at < 34 weeks.

2.9. Cell proliferation assay

The WST-8 assay was performed using Cell Count Reagent SF (Nacalai). HTR-8/SVneo cells, which were transfected with WNT5A-specific siRNA or control siRNA, were seeded on 96-well plates at a density of 4000 cells/well. Each experiment was repeated five times independently. Cell viability was also determined after treatment with 200 ng/ml rhWnt5a for 24 h, 48 h, and 72 h. Three independent experiments were performed. Cell viability was also determined after transfection with Wnt5A-specific siRNA or control siRNA. The absorbance values at 450 nm were measured using an Emax microplate reader (Molecular Devices).

2.10. Migration assay

HTR-8/SVneo cells were grown to subconfluence in 12-well plates and scratched with a 200 µl pipette tip. After 20 h of incubation, the cells were photographed on an Olympus IX71 microscope. The quantification of cell migration into the wound area was determined using Image J Imaging System Software Version 1.3. Each experiment was repeated six times independently.

2.11. Invasion assay

24-well cell culture inserts (8 µm pore size, Falcon) were used to assess the influence of Wnt5a signaling on the invasive potential of HTR-8/SVneo cells. The upper side of the filter membrane was coated with 0.3 mg/ml Matrigel, then 200 µl of a cell suspension (1,000,000 HTR-8/SVneo cells/ml in RPMI-1640 medium containing 1% FCS) was added to the upper chamber. The lower chamber contained 800 µl of medium with 10% FBS as a chemoattractant. After 20 h, inserts were fixed in methanol and stained with hematoxylin. The invasion rate was determined by counting cells at the bottom of the filter with a microscope (Olympus). The average number of invading cells in five random fields at 200x magnification were calculated for each insert. Invasion assays were repeated independently three times.

2.12. Tube formation assay

The surface of a prechilled 96-well plate was coated with 25 μ l of growth factor reduced Matrigel, and then incubated at 37 °C for 30 min to promote solidification. HTR-8/SVneo cells were plated into the Matrigel-coated wells (1000 cells/well) in RPMI-1640 medium containing 1% FCS in triplicate. After 8 h, tube formation was imaged on an Olympus IX71 microscope. Tube formation assay data were quantified by measuring the total tube length of capillary tubes. The average tube length was calculated in five random fields in each well using Image J Imaging System Software.

2.13. Statistical analysis

The results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were performed according to the Student's *t*-test, the Mann-Whitney *U* test, Pearson correlation coefficients, or one-way analysis of variance followed by Tukey's multiple comparisons test, using Prism 6.0 software (GraphPad Software). Values of *P* < 0.05 were considered statistically significant.

2.14. Ethical approval

The study protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University, and written informed consent was obtained from each patient.

3. Results

3.1. Analysis of microarray data reveals Wnt5a as a plausible candidate gene relevant to poor placentation

In order to identify Wnt ligands potentially relevant to early placentation in PE, a total of 25 probes corresponding to Wnt ligand genes, whose maximum expression value across samples was more than 5, were detected in a microarray data set of chorionic villous sampling (GSE12767) that was composed of first trimester chorionic villi of women who later developed PE (*n* = 4) and women with normal pregnancies (*n* = 8). Among 25 probes, two probes, both of which represent Wnt5a, showed significantly reduced expression in the first trimester placentas of women who later manifested PE (Fig. 1A, Supplementary Table 3). As for other probes of Wnt ligands, no significant differences were observed between placentas in the first trimester of PE and unaffected pregnancies.

3.2. Down-regulation of Wnt5a in placenta is associated with preeclampsia

To validate the result of the microarray data analysis, the mRNA expression of Wnt ligands in placentas (first trimester villi, PE (+) placenta, and PE (−) placenta) was evaluated by qPCR. Wnt5a mRNA alone was expressed at significantly lower levels in PE placentas compared with first trimester and normal term placentas (Fig. 1B). The expression level of eight other Wnt ligands (Wnt2, Wnt2b, Wnt3a, Wnt4, Wnt6, Wnt7a, Wnt7b, and Wnt10a) showed no significant differences between the placentas of normal controls and PE pregnancies (Supplementary Fig. 1). Western blotting analysis also showed that Wnt5a expression was decreased in PE placentas compared to normal placentas (Fig. 1C, *p* = 0.0087). Microarray gene expression data analysis (GSE75010) showed that the expression of Wnt5a was negatively correlated with gestational age at delivery in 44 control placentas (Fig. 1D, *R* = −0.298, *p* = 0.0494). Moreover, the Wnt5a expressions of PE placentas ($\leq$ 34 weeks) with a newborn weight *z*-score < −1.5 (*n* = 23) were lower than those of control placentas ($\leq$ 34 weeks, *n* = 18) (Fig. 1E, *p* = 0.0291). Immunohistochemistry analysis also showed that Wnt5a expression was decreased in PE placentas compared to normal placentas (Fig. 2A). Wnt5a staining was observed in

cytotrophoblasts and syncytiotrophoblasts (Fig. 2B, C). In addition, Wnt5a was expressed in endovascular trophoblasts as well as in interstitial EVT (Fig. 2B, C).

3.3. Wnt5a-knockdown extravillous trophoblasts show decreased invasive activity and tube formation

In order to investigate the possible involvement of impaired Wnt5a signaling in PE placentas, Wnt5a was silenced using siRNA in HTR-8/SVneo cells, which is a well-established EVT cell line. Thereafter, proliferation, migration, invasion, and tube formation assays were conducted. Fig. 3A shows the effectiveness of silencing in two different Wnt5a siRNAs. Since siRNA2 (Qiagen, #SI00051779) effectively decreased Wnt5a at both the mRNA and protein levels at 72 h, siRNA2 was employed in the subsequent experiments. Knockdown of Wnt5a did not affect cell proliferation up to 72 h (Fig. 3B), nor cell migration at 20 h (Fig. 3C). In contrast, Wnt5a knockout HTR-8/SVneo cells significantly reduced cell invasion (Fig. 3D, *p* = 0.0022), and attenuated tube formation (Fig. 3E, *p* = 0.0328), suggesting that suppression of Wnt5a in EVT may be associated with insufficient remodeling of uteroplacental spiral arteries.

3.4. rhWnt5a promotes cell migration, invasion and tube formation

We further investigated the effect of Wnt5a on cell function using rhWnt5a. rhWnt5a (200 ng/ml) did not alter HTR-8/SVneo cell proliferation up to 72 h (Fig. 4A). In contrast, administration of rhWnt5a promoted cell migration (Fig. 4B, *p* = 0.0007), invasion (Fig. 4C, *p* = 0.0078), and tube formation (Fig. 4D, *p* = 0.0163). Collectively, these data show that Wnt5a is indispensable for the role of EVT in placentation.

3.5. Disruption of JNK signaling but not PKC signaling impairs tube formation

Further experiments were focused on the invasive and tube forming capabilities of EVT. Given that Wnt5a is implicated in the regulation of multiple signaling pathways [13,14], we hypothesized that PKC or JNK signaling would be involved in Wnt5a-induced alteration of phenotypes observed in the invasion and tube formation assays. rhWnt5a elevated PKC and JNK phosphorylation in a dose-dependent manner (Fig. 5A). The phosphorylation of PKC and PKC- β 1 peaked around 60 min after rhWnt5a stimulation. JNK phosphorylation started 5 min following stimulation with rhWnt5a. Inhibition of PKC and JNK signaling resulted in strikingly reduced invasiveness of EVT (Fig. 5B). While impaired PKC signaling did not hamper the increase in tube formation induced by rhWnt5a, the JNK inhibitor significantly reduced the ability of EVT to form vessel-like tubes (Fig. 5C). Taken together, Wnt5a activates the PKC and JNK pathways, and both pathways are involved with invasion of HTR-8/SVneo cells, whereas tube formation is regulated by the JNK pathway but not the PKC pathway.

4. Discussion

In this study, we first showed that Wnt5a expression is suppressed in the early placentas of women before developing PE. Moreover, down-regulated Wnt5a was also observed in placentas of PE compared with normal first and term placentas. Furthermore, Wnt5a via PKC and JNK signaling was involved in EVT cell invasion and tube formation. All these findings suggest that dysregulated Wnt5a signaling can be partly attributed to impaired early placentation and the subsequent development of PE.

The association between Wnt5a expression and PE placentas is controversial. A recently published article demonstrates that Wnt5a is up-regulated in PE placentas [15]. The authors state that the expression of Wnt5a in the first trimester is low in the normal placenta, and that

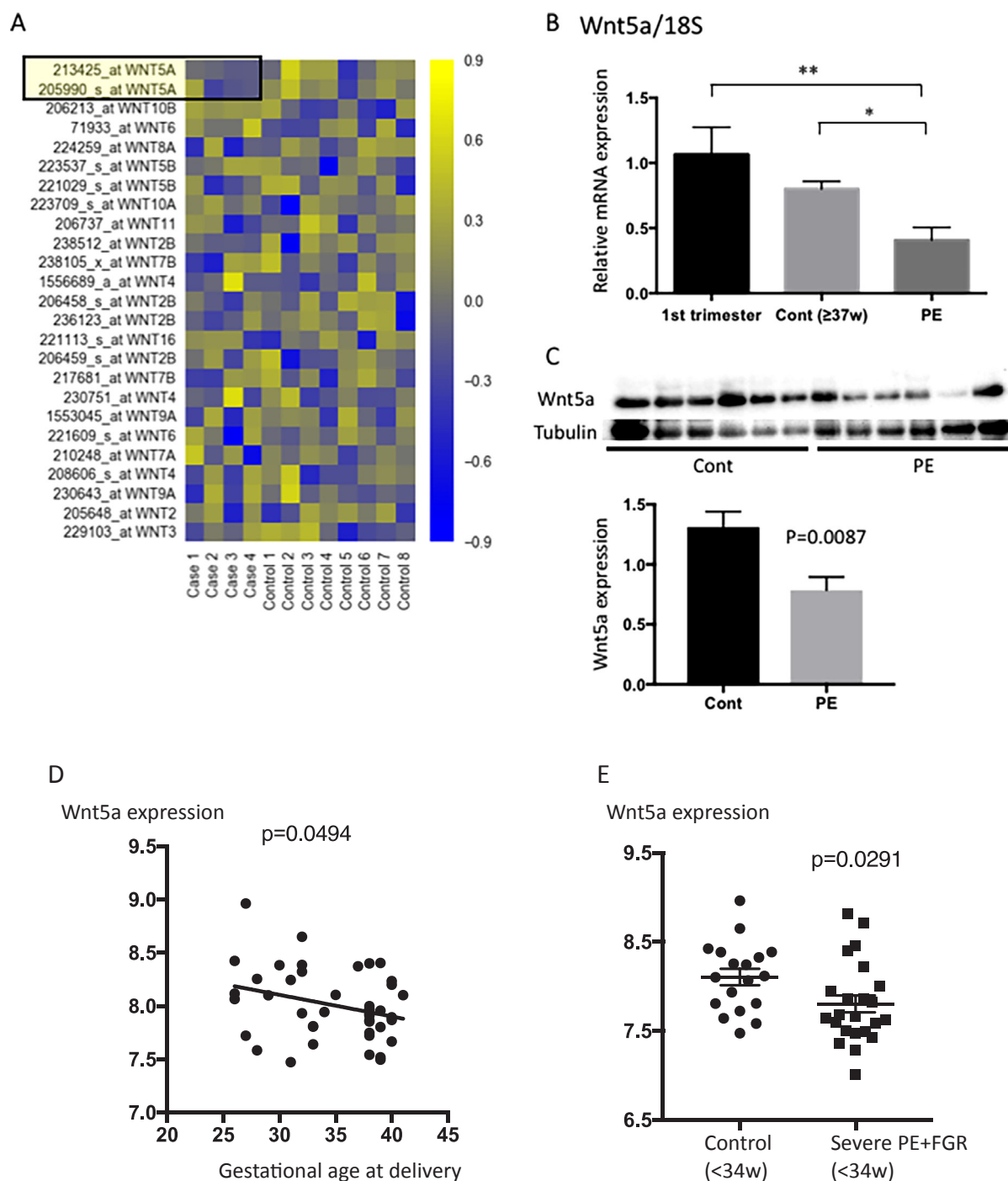


Fig. 1. Expression levels of Wnt ligands. (A) Heat map of differential gene expression patterns of Wnt ligands in placentas in the first trimester of PE (Cases 1–4) and unaffected pregnancies (Controls 1–8). Both of the probes corresponding to Wnt5a were down-regulated in the placentas of women who subsequently developed PE, as shown in the light yellow box. The relative abundance of each gene in the placenta is indicated by the color bar. (B) Wnt5a expression in PE placentas. *Wnt5a* mRNA expression in the placentas of the first trimester ($n = 4$), controls ($n = 10$, ≥ 37 weeks of gestation), and PE pregnancies ($n = 12$) was determined by real-time PCR analysis. (C) Western blot images in PE placentas. The placentas were from normal control ($n = 6$) and PE pregnancies ($n = 6$). (D) Wnt5a expressions in placentas of 44 normotensive control pregnancies. The expression of Wnt5a was negatively correlated with gestational age at delivery. (E) Wnt5a expressions in normotensive control and PE placentas before 34 weeks of gestation. The Wnt5a expressions of PE placentas with a newborn weight z-score < -1.5 ($n = 23$) were lower than those of control placentas ($n = 18$). Statistical analysis was conducted using one-way ANOVA (* $P < 0.05$, ** $P < 0.01$), Pearson correlation coefficients, and Mann-Whitney U test. Data are presented as the mean $\pm$ standard error of the mean (SEM). PE, preeclampsia; FGR, fetal growth restriction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased Wnt5a in early pregnancy may contribute to the pathogenesis of PE with insufficient invasion of trophoblast for placentation [15]. Clearly, this is contrary to our findings. We analyzed Wnt5a expression from two different sources, one is publicly available microarray data from first trimester villi, and another is the placentas taken in our

institutions. Wnt5a expression was repressed in PE placentas in both sources. In the previous report, the authors used term PE placentas. In contrast, we used preterm PE placentas. PE is classified into early-onset and late-onset subtypes according to gestational age of clinical symptoms onset. Accumulating evidence suggests that early-onset PE

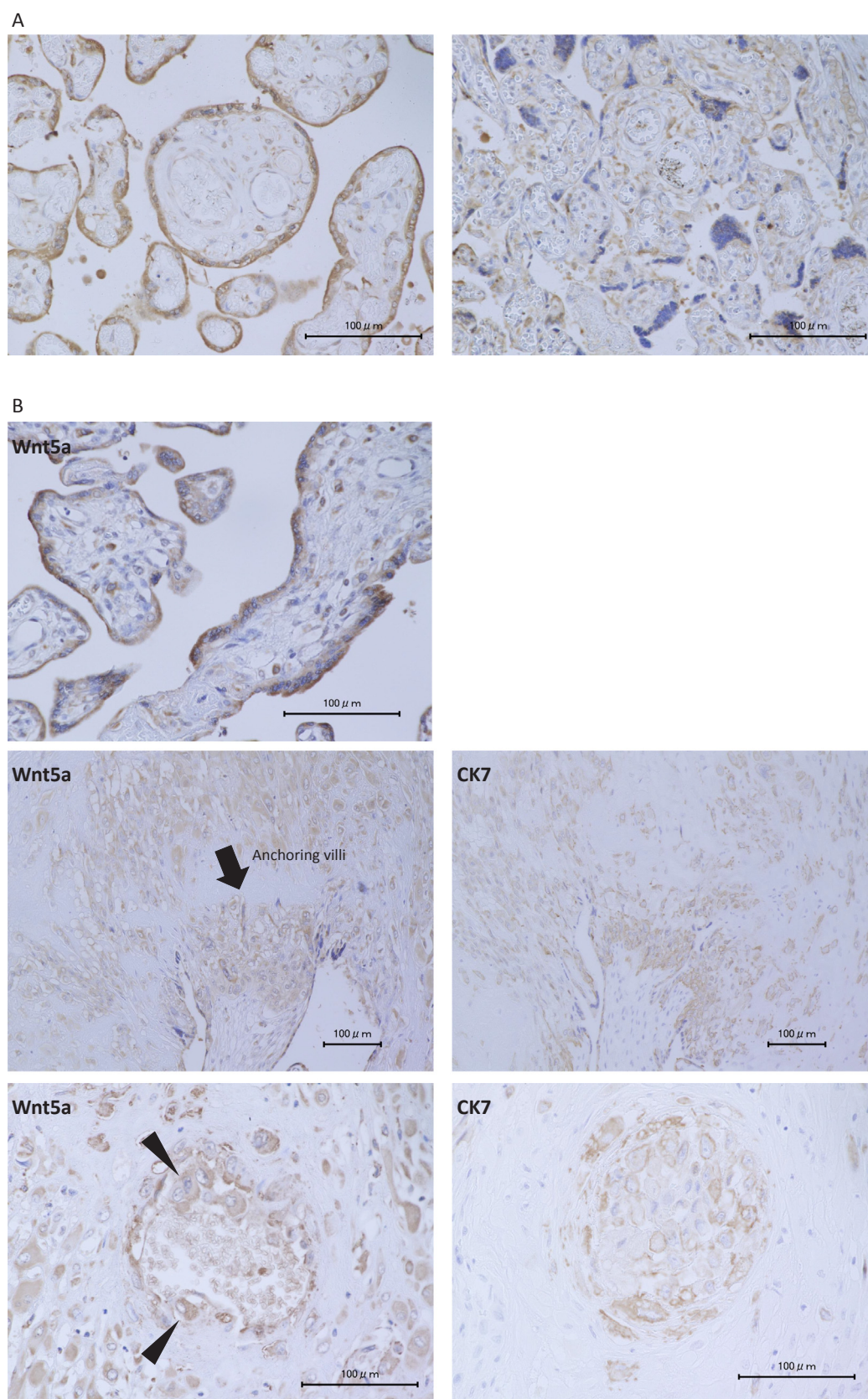


Fig. 2. Immunohistochemical staining of placenta. (A) Normal term (left, 40 weeks) and PE (right, 27 weeks) placentas stained with a Wnt5a antibody. Right image showed accelerated villous maturation and numerous syncytial knots, both of which are characteristic of PE placentas. Decreased Wnt5a expression levels were observed in villous trophoblasts in pregnancies presenting with PE. (B) Villous and extravillous trophoblasts at 15 weeks of gestation in a normal pregnancy. Wnt5a staining was observed not only in cyto- and syncytiotrophoblasts (upper panel), but also in interstitial extravillous trophoblasts at the anchoring villi (middle row, arrow) and decidual endovascular trophoblasts (lower row, arrowhead). Interstitial and endovascular trophoblasts were confirmed by CK7 staining of serial sections (right column). (C) Immunofluorescence staining of Wnt5a and CK7 in trophoblasts. Wnt5a and CK7 were co-localized in cytotrophoblasts (left) and interstitial EVT at the anchoring villi (right). Scale = 50 μm. Images in Fig. 2B represent 3 specimens for each antibody. PE, preeclampsia.

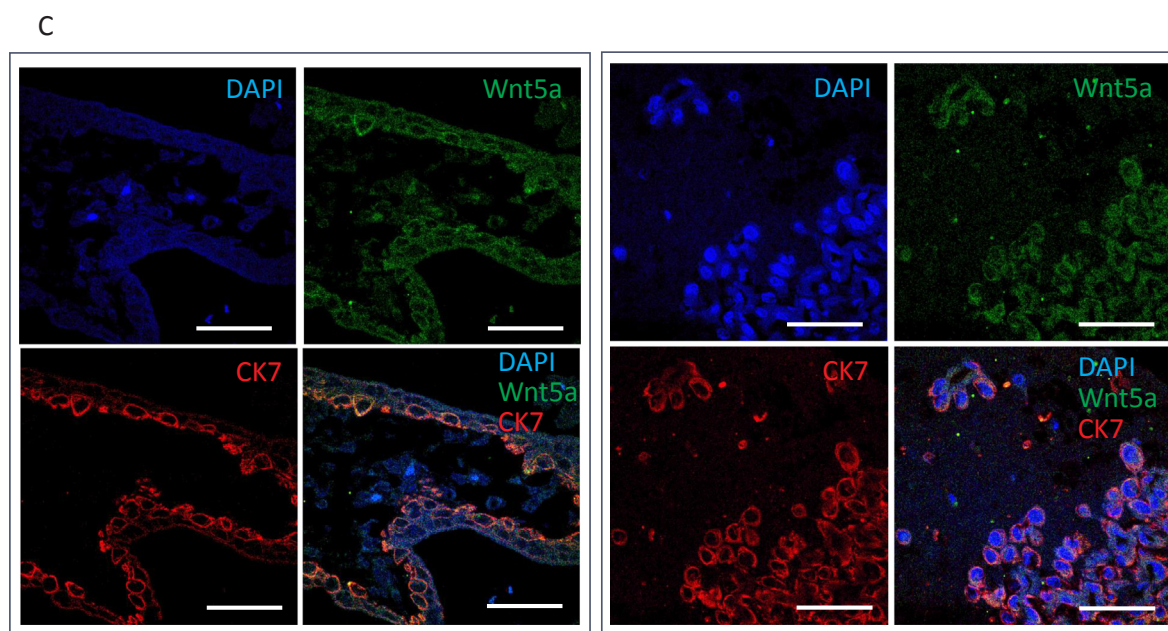


Fig. 2. (continued)

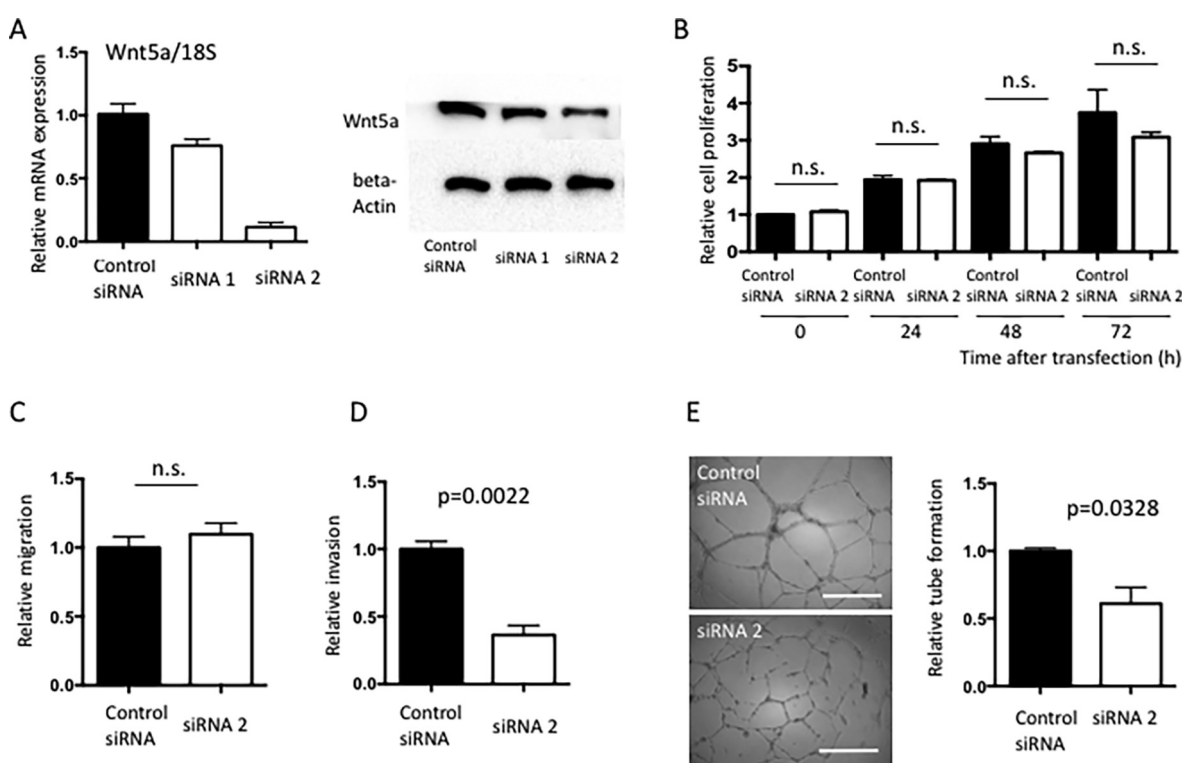


Fig. 3. Effect of Wnt5a knockdown on extravillous trophoblasts. (A) Knockdown efficiency of two different Wnt5a siRNAs in mRNA levels and protein levels of HTR-8/SVneo cells. (B) Proliferation assay, $n = 5$. (C) Migration assay, $n = 3$. (D) Invasion assay, $n = 3$. (E) Tube formation assay, $n = 3$. Scale = 500 μm . Statistical analysis was performed using Student's *t*-test. Data are presented as the mean $\pm$ standard error of the mean (SEM).

develops due to abnormal placentation, while late-onset PE often occurs in women with latent maternal endothelial dysfunction [16]. This might partly explain why the Wnt5a expression in PE placentas was down-regulated rather than up-regulated in the present study. The discrepancy between the previous report and our findings makes further investigation mandatory.

Little is known regarding the function of Wnt5a in trophoblasts, especially regarding placentation. Wnt5a knockout mice exhibit multiple deformities and perinatal lethality, and the fetus is smaller in size

[17]. On the other hand, Wnt5a-inducible transgenic mice also show a reduction in size [18]. These conflicting previous reports focused on embryonic development, and there have been few investigations into the role of Wnt5a during pregnancy and placentation. To date, the putative role of Wnt5a in placentation has not been elucidated *in vivo*. Moreover, there are scant and conflicting data regarding the function of Wnt5a signaling in trophoblasts. Wnt5a protein represses proliferation and induces apoptosis in JAR choriocarcinoma cells [19]. Another recent report shows that Wnt5a increases proliferation in primary

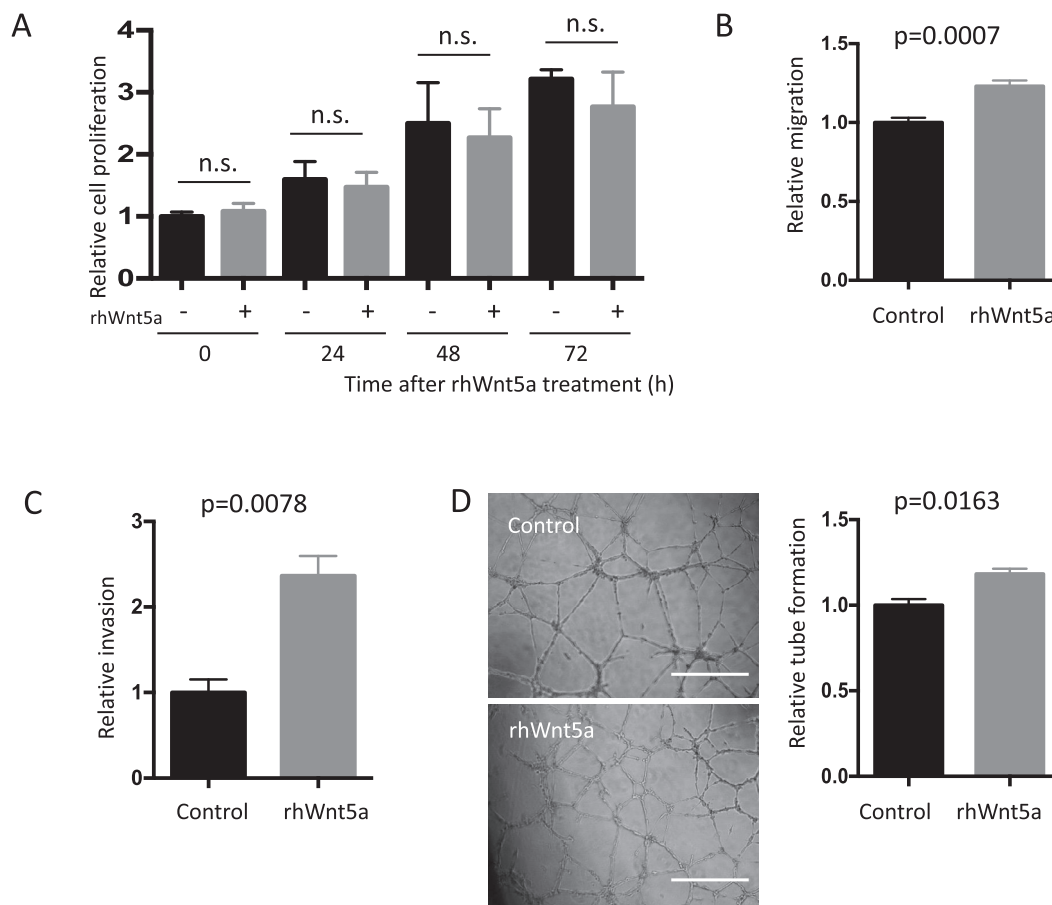


Fig. 4. Influence of recombinant human Wnt5a on extravillous trophoblasts. HTR-8/SVneo cells were treated with recombinant human rhWnt5a (200 ng/ml). (A) Proliferation assay, $n = 3$. (B) Migration assay, $n = 3$. (C) Invasion assay, $n = 3$. (D) Tube formation assay, $n = 3$. Scale = 200 μm . Statistical analysis was conducted using Student's t -test. The results are shown as means $\pm$ standard error of the mean (SEM).

cytotrophoblasts and in SGHPL-5 cells, a first trimester EVT cell line [20]. Furthermore, there is a report showing that rhWnt5a diminishes cell migration and invasion in HTR-8/SVneo cells [15]. However, van Zuylen et al. and Takahashi et al. argued against the findings by demonstrating that Wnt5a promotes cell migration and invasion [21,22]. The present study also showed that Wnt5a promoted EVT cell invasion and tube formation. These discrepancies may be explained by different cell lines and different assay conditions. Indeed, Wnt5a stimulates cell invasion in some types of cancer, but inhibits it in other types [14,23]. In addition, aberrant activation or inhibition of Wnt5a signaling exerts opposite effects on cell proliferation, migration, and invasion, depending on cell type or receptor availability [14,23]. Further research including *in vivo* experiments is warranted in order to clarify the role of Wnt5a signaling in early placentation.

Wnt5a regulates multiple intracellular signaling pathways; two of the best characterized pathways are the planar cell polarity (PCP) pathway and the Wnt/ Ca^{2+} pathway [23]. PCP refers to the polarization of cells within the plane of an epithelial cell layer; Wnt-induced JNK (PCP pathway) activation regulates cell morphology and migration. On the other hand, Wnt5a causes an increase in intracellular calcium, and the released Ca^{2+} can activate PKC [24]. The present study showed that both JNK and PKC signaling play essential roles in EVT cell invasion. Intriguingly, Wnt5a/JNK signaling rather than Wnt5a/PKC signaling affected tube formation. The modulation of Wnt5a/PKC signaling and/or Wnt5a/JNK signaling may be a promising therapeutic strategy for the prevention of PE.

There are still unanswered questions regarding the role of Wnt5a in early placentation. First, it remains unknown as to whether impaired

Wnt5a signaling in trophoblasts *in vivo* causes poor placentation and subsequent PE. Placental-specific Wnt5a knockout mice may answer this question, but this was not performed due to technical challenges and is beyond the scope of the present study. Second, we did not examine the role of any receptors through which Wnt5a signaling is mediated. Recent studies have shown that Wnt5a signaling is inherently complex, and exerts various cellular functions by activating multiple signaling through binding to different members of the Frizzled (Fzd) family and Ror family receptors [13,14]. For instance, Wnt5a signaling can be transmitted through Fz3, Fz4, Fz5, Fz7, and Fz8. In addition, Wnt5a competes with Wnt3a for binding to Fzd2, and inhibits Wnt3a-mediated beta-catenin-dependent signaling [25]. The complex cross-talk between Wnt5a, its receptors, and its co-receptors in early placentation should be elucidated in future research.

5. Conclusion

Impaired Wnt5a signaling may be relevant to poor placentation and subsequent PE, and inhibition of Wnt5a/JNK signaling attenuated rhWnt5a-induced invasion and tube formation capabilities. Therefore, modulation of Wnt5a signaling can be a promising therapeutic strategy for the prevention of PE.

Author's roles

Kondoh E, Matsumura N, and Konishi I designed this study and supervised the project. Ujita M, Chigusa Y, Mogami H, Kawasaki K, Kiyokawa H, Takai H, Sato M, and Kawamura Y performed the *in vitro*

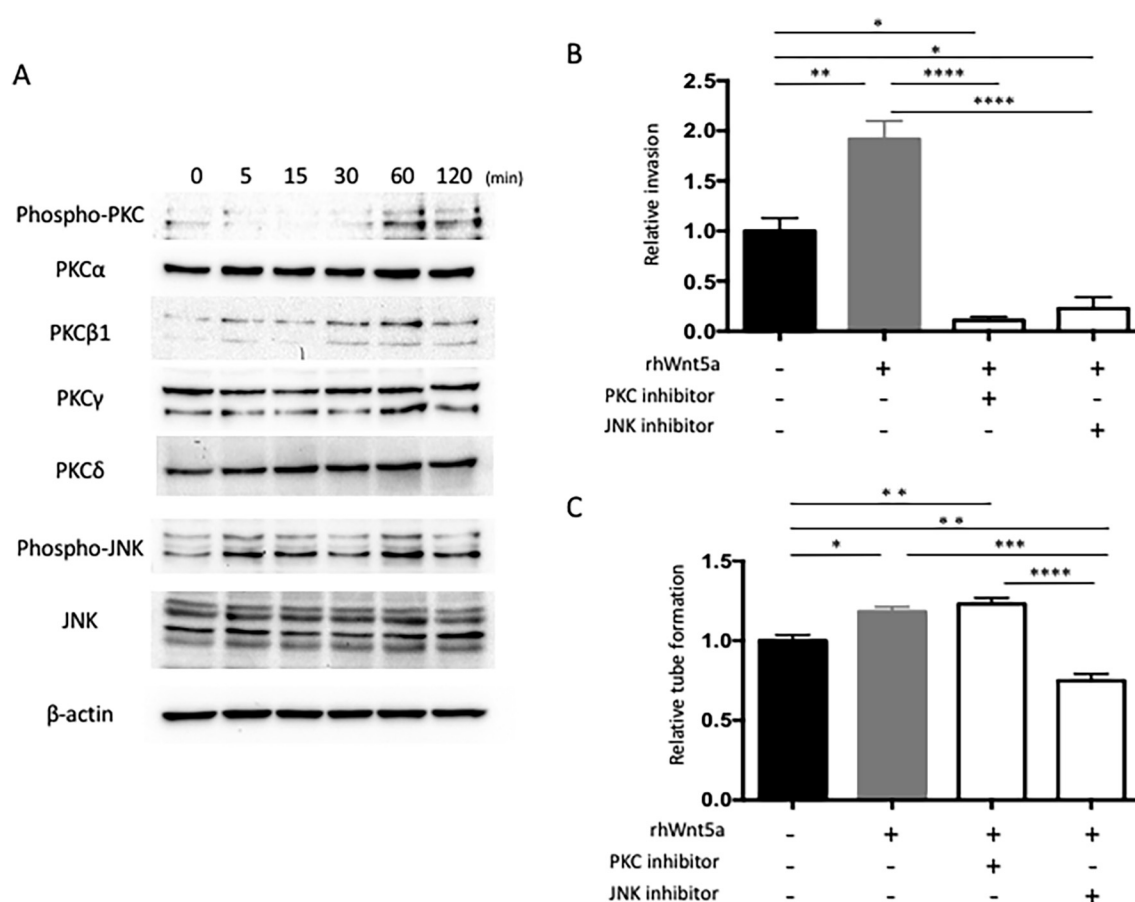


Fig. 5. Role of Wnt5a signaling via PKC or JNK in invasion and tube formation of extravillous trophoblasts. (A) Promotion of PKC and JNK phosphorylation. Western blots analysis shows that the phosphorylation of PKC and PKC-beta 1 peaked around 60 min after rhWnt5a stimulation. JNK phosphorylation was detected by 5 min following stimulation with rhWnt5a. (B) and tube formation assay (C) of HTR-8/SVneo cells following treatment with rhWnt5a, a PKC inhibitor (5 μ M), and a JNK inhibitor (5 μ M) for 20 h or 8 h, respectively (n = 3). Western blot analysis on EVT, extravillous trophoblasts. Statistical analysis was performed using one-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

experiments. Ujita M and Kondoh E analyzed data and wrote the paper. Chigusa Y, Mogami H, Horie A, and Baba T edited the manuscript. Mandai M finally approved the version to be published. All authors discussed the results and implications and commented on the manuscript at all stages.

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Conflict of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.preghy.2018.06.022>.

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