Dysregulated Expression of RPS4Y1 Impairs STAT3 Signaling to Suppress Trophoblast Cell Migration and Invasion in Preeclampsia

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Abstract—Normal placentation and a successful pregnancy depend on appropriate trophoblast cell migration and invasion. Inadequate trophoblast invasion and impaired

spiral artery remodeling may lead to pregnancy-related disorders, such as preeclampsia (PE). Ribosomal protein S4, Y-linked 1(RPS4Y1) is a member of the S4E family of ribosomal proteins. In this study, we found that RPS4Y1 levels were upregulated in placental samples collected from preeclamptic patients, when compared with the normotensive pregnant women. In vitro, inhibition of RPS4Y1 induced trophoblast cell invasion, promoted placental explant outgrowth and increases STAT3 phosphorylation along with elevated expression of N-cadherin and vimentin. Conversely, overexpression of RPS4Y1 results in reduced trophoblast cell invasion and decreased STAT3 phosphorylation. In addition, the suppression of RPS4Y1 promotes trophoblast cell invasion, which could be abolished by the STAT3 knockdown. Meanwhile, we observed reductions of STAT3 phosphorylation expression in preeclampsia patients. Collectively, these results demonstrate that the level of RPS4Y1 expression may be associated with preeclampsia by affecting trophoblast cell migration and invasion via the STAT3/EMT pathway.

Key Words: RPS4Y1, STAT3, trophoblast, migration and invasion, preeclampsia

Short title: Aberrant RPS4Y1 and STAT3 involve in Preeclampsia

A successful pregnancy requires successful placentation. To support the demands of the growing fetus, decidual spiral arteries must be transformed into wide-diameter, non-vasoactive vessels capable of transporting nutrition and oxygen to the fetus[1]. The transformation of the cytotrophoblast (CTB) into the extravillous trophoblast (EVT) is an essential process for remoding the arteries into high-volume conduits that assure an adequate nutrient and oxygen supply for the developing fetus[2]. Compared to those in regulated normal placentae, the differentiation from CTB into EVT and the invasion of placental cells into the uterine milieu are compromised in some pregnancy-related disorders, such as preeclampsia[3,4]. Preeclampsia (PE) is a systemic disease that affects the function and health of multiple organs and leads to a pathophysiology during pregnancy that affects both the mother and the baby[5,6]. PE is characterized by new-onset hypertension and proteinuria at $\geqslant$20 weeks of gestation and affects 5–8% of

pregnant women each year[7-9]; it is a significant cause of maternal and fetal morbidity and mortality worldwide. At present, no effective intervention for preeclampsia exists; the only effective treatment is delivery of the placenta. It has been speculated that inadequate trophoblast invasion and impaired spiral artery remodeling can further contribute to placentation anomalies as the primary cause of the disease[7,8]. However, the molecular mechanism of the trophoblast's reduced invasion capabilities remains elusive.

It has been proposed that the process by which CTB is differentiated into EVT shares similarity with developmental epithelial-mesenchymal transitions (EMTs)[10]. In early placental development, villous CTB undergo a partial EMT differentiating into EVT and gain the capacity for migration and invasion, infiltrating the maternal decidua and vessels[2,10]. Then, the walls of the spiral arteries convert from a high- to a low-resistance system, leading to increased blood volume and flow to the utero-placental unit[11]. Several groups have reported that the transformation of the epithelial-like CTB to the invasive, mensenchymal-like EVT is akin to the EMT that occurs in cancer metastasis[2,10,12]. Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, plays a crucial role in modulating EMT in many cases of cancer metastasis, although the conclusions are paradoxical in different types[13-15]. Intriguingly, several data have implied that STAT3 may function in the placenta as well. It is localized in the first-trimester placental extravillous trophoblasts[16]. Additionally, decreased expression and activation of STAT3 have been detected in preeclamptic placenta[17].

Gene expression microarray, as a new tool in biotechnology, allows for the simultaneous monitoring of thousands of gene expression levels in diseases[18]. Our group used a cDNA microarray to investigate the genes expressed differently in PE patients' placental tissues as compared to patients with normotensive pregnancies. Among the identified genes expressed differently, a member of the very well-conserved S4E family of ribosomal proteins, ribosomal protein S4, Y-linked 1(RPS4Y1)[19], increased by more than 3 times. It has been reported that trophoblast cells and trophoblast debris, the most important components in the placenta in early pregnancy, express RPS4Y1 protein[20]. RPS4Y1 has functionally equivalent and interchangeable homologous ribosomal protein genes, RPS4X[19,21,22]. Recent reports discovered that a high-level expression of RPS4X is associated with a poor prognosis in intrahepatic cholangiocarcinoma and nonmucinous cancer[23,24].

While trophoblastic cells and cancer cells exhibit their phenotype in the invasive stage similarity, including the mechanism by which they invade adjacent tissues via EMT, it is currently unclear as to whether RPS4Y1 modulates the trophoblast invasion and placental development, as this phenomenon has not yet been intensively investigated. Herein, based on the findings described above, our objective was to investigate the hypothesis that RPS4Y1 is involved in trophoblast migration and invasion. To address this hypothesis, we firstly assessed the expression of RPS4Y1 in the placental villous in the first trimester and full-term pregnancy of normotensive and that of full-term pregnancy of preeclampsic women. Subsequently, we examined the effects of different RPS4Y1 expression levels on the *in vitro* invasive ability of different

trophoblast cell lines, as well as on the expression levels of phosphorylated STAT3, E-cadherin, N-cadherin and vimentin, to determine the role of RPS4Y1 during the EMT process. Finally, we compared the expression levels of phosphorylated STAT3 in preeclampsic and normal placental villous to investigate the possible role of STAT3 in PE pathogenesis.

Materials and Methods

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

Clinical samples

First-trimester human villi and decidua tissues (6-10 weeks gestation) were obtained from normotensive pregnant women who underwent an elective termination of pregnancy for nonmedical reasons. Full-term placenta tissues were obtained from a portion of normotensive pregnancies (38.27±0.95 weeks, n=20) and PE patients (35. 52 ± 2.69 weeks, n=20) after delivery by caesarean section. Informed consent was obtained from all participants. Approval was also granted by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. The clinical characteristics of study subjects are shown in Table S1 in the online-only Data Supplement. More information can be found in the online-only Data Supplement.

Villous explant culture

The placental villous tissues were dissected into explants of 2-5 mm in diameter and explanted as previously described[25]. A 24-well plate was pre-coated with 50 μl of a 1 mg/ml Matrigel matrix solution (BD Biosciences) and incubated at 37°C for four hours to allow for gelling. A serum-free DMEM/F12 (Gibco) medium containing 10% FBS with 500 nM lentiviral vector-based short hairpin RNA (shRNA) (Hanbio) targeting RPS4Y1 or an equal concentration of the scrambled shRNA was added to the wells; the cells were then incubated under low oxygen tension (3% O_2) for 72 hours. The explants with good attachment and outgrowth on the gel were recorded after 24, 48 and 72 hours, respectively, by using inverted phase-contrast microscopy (EVOS™ FL Color Imaging System, Thermo Fisher Scientific) at a magnification of×40. An evaluation of EVT outgrowth was performed as previously described[26]. EVT cell migration distance at individual sites (from the villous tip to the distal edge of the outgrowth sheet) was measured by manual tracing using Photoshop CS5 software. For each explant, four villous tips were randomly chosen, and at least three distances were measured for each tip. The migration distances of the EVT cells from the RPS4Y1 shRNA-transfected and the scrambled shRNA-transfected groups were then used in the statistical analysis. Samples from 11 first-trimester human placentae were used. The explant experiment was performed in quadruplicate and repeated five times.

More detailed Materials and Methods are provided in the online-only Data Supplement.

Statistical analysis

Values are shown as the mean ± SD. All statistical analyses were performed using SPSS 19.0 software (SPSS Statistics, Inc.). The differences between the two groups were analyzed by Student's t test. Statistical significance was considered achieved when $P<0.05$.

Results

RPS4Y1 Was Aberrantly Expressed and Localized in Preeclamptic Placentae

We analyzed RPS4Y1 protein expression and the localization in villous of women with normotensive pregnancy and preeclampsia using immunofluorescence (Fig. 1A), immunohistochemistry (Fig. 1B) and western blot (Fig. 1D). Double-labelling of immunofluorescence and immunohistochemistry demonstrated that trophoblast cells expressed RPS4Y1 protein. As shown in Fig. 1B, RPS4Y1 protein was intensely expressed in cytotrophoblast (CTB) cells, syncytiotrophoblast (STB) cells and trophoblast columns (TC) of first-trimester placental villous (Fig. 1Ba, Fig. 1Bd). In the maternal decidua, RPS4Y1 was expressed in the decidual cells (DC) (Fig. 1Bb) and in some extravillous trophoblast (EVT) cells, which were defined by the positive expression of human leukocyte antigen G (HLA-G). Meanwhile, placental villous from first trimester pregnancies, full-term pregnancies, and preeclampsia (PE) patients were analyzed by immunohistochemistry and dissected and subjected to western blot analysis. As shown in Fig. 1Bc, Fig. 1Bd, Fig. 1Bf and Fig. 1D, expression of RPS4Y1 in term placental villi was significantly lower than that in the first trimester and the RPS4Y1 protein level significantly increased in the preeclampsia group compared to the control group. Furthermore, we examined the expression of RPS4Y1 in four different trophoblast cell lines by western blotting. We found that it was expressed highly in HTR-8/SVneo cells and expressed less in JAR cells (Fig. 1E). RPS4Y1 proteins were also present in JEG3 cells and BeWo cells (Fig. 1E).

RPS4Y1 Knockdown Significantly Enhanced the Invasion Capabilities of HTR8/Svneo Cells

On the basis of the above observations, the expression of RPS4Y1 in trophoblast cell lines suggests a role for RPS4Y1 in the regulation of cell behavior. To investigate the role of RPS4Y1 in the trophoblast, we used a specific shRNA targeting RPS4Y1 to reduce RPS4Y1 expression in human first-trimester extravillous trophoblast cell line HTR-8/SVneo cells.

The effect of RPS4Y1 knockdown on protein expression was verified by western blot (Fig. 2A). ShRNA targeting RPS4Y1 significantly decreased RPS4Y1 protein expression in the knockdown cell as compared to the control knockdown cell (Fig. 2A). We next performed CCK8 assays to determine trophoblast cell proliferation. Significant increase in cell number was observed in the RPS4Y1 knockdown group after 72 hours, which implies that RPS4Y1 has an inhibitory effect on trophoblast cell proliferation (Fig. 2B). After CCK8 assays, we further investigated the effects of RPS4Y1 on

trophoblast cell HTR8/SVneo apoptosis using flow cytometry analysis. The knockdown of RPS4Y1 had no significant effect on cell apoptosis compared to the scrambled shRNA group (Fig. 2C).In addition, the invasion of trophoblast cells was assessed by Matrigel invasion assays. RPS4Y1 knockdown significantly enhanced the invasion (Fig. 2D). Taken together, these results suggest that RPS4Y1 expression is negatively correlated with cell migration and invasion.

Overexpression of RPS4Y1 Suppresses the Invasion Capabilities of JAR Cells

To further confirm the vital role of RPS4Y1 in trophoblast cells, we stably overexpressed RPS4Y1 in the JAR cell line using plasmids transfection (Fig. 3A). As shown in Fig. 3B and Fig. 3C, upregulated RPS4Y1 did not affect JAR cell proliferation and apoptosis, respectively. However, it significantly suppressed the invasion of JAR cells (Fig. 3D). These observations were compatible with the findings with respect to RPS4Y1 knockdown cell lines, thus further supporting the significance of RPS4Y1 in trophoblast cells.

RPS4Y1 ShRNA Promoted the Invasion and Migration of EVTs in an Extravillous Explant Culture Model

To further explore the physiologic significance of RPS4Y1 in placental development, we tested the effect of RPS4Y1 silencing on EVT outgrowths on an *ex vivo* extravillous explant culture model.

In this model, extravillous explants from first-trimester human placental villi were cultured on matrigel. EVT can migrate from the tip of the TC and infiltrate into the matrigel after 24–72 hours of culture. Transfection with RPS4Y1 shRNA stimulated expansion of the EVT outgrowth across and into the matrigel. The area of outgrowth was significantly larger in the RPS4Y1 shRNA-treated group than in the scramble shRNA group (Fig. 4A). Quantitative analysis revealed that knockdown of RPS4Y1 caused a significant increase in the area of outgrowth (Fig. 4B). The immunofluorescence analysis (Fig. 4C) showed that lentiviral vector-based short hairpin RNA (shRNA) successfully transfected into the EVT outgrowth. The efficiency of RPS4Y1 knockdown in this *ex vivo* model was further validated by western blots (Fig. 4D).

STAT3 Is Regulated by RPS4Y1 through Phosphorylation Alteration in Trophoblast Cells and Expression of Phosphorylated STAT3 Was Decreased in PE

Previous studies demonstrated that STAT3 activity was related to the malignant phenotype of different tumor cells and potentially contributed to their invasiveness[27-29]. STAT3 has also been reported to be involved in the functional suppression of trophoblast cells and to be transcribed to a lesser extent in PE[17,30,31], which suggests it may play a vital role in PE. To elucidate the mechanism by which RPS4Y1 affects trophoblast invasion, we evaluated STAT3 signaling in trophoblast cells. We detected the expression of STAT3 and phosphorylated STAT3 (pSTAT3) using western blot in RPS4Y1 knockdown HTR-8/SVneo cells and RPS4Y1 overexpressed JAR cells. Interestingly, we found that the phosphorylated STAT3 significantly increased in

RPS4Y1 knockdown HTR-8/SVneo cells as compared to the control group (Fig. 5Aa). Consistently, we also observed that overexpressed RPS4Y1 resulted in the reduction of STAT3 phosphorylation in JAR cells (Fig. 5Ab).

Next, we measured RPS4Y1, STAT3 and pSTAT3 expression in the placentae of PE patients and normotensive pregnant women using western blot. Placentae from PE patients had lower levels of phosphorylated STAT3 than did those from normotensive pregnant women (Fig. 5B). These clinical findings were consistent with the results shown by RPS4Y1 upregulation and downregulation in two different cultured trophoblast cell lines, respectively. In all, these findings indicate that phosphorylated STAT3 is dysregulated in PE and the phosphorylation of STAT3 is controlled by RPS4Y1 in trophoblast cells.

Decreased Phosphorylation of STAT3 Reversed the Promotion Effects of RPS4Y1 Knockdown on Trophoblast Cell Invasion through the Inhibition of Epithelial-Mesenchymal Transition

Previous studies found that an active STAT3 was necessary for EMT, invasion and metastasis in cancer cell lines[15,32]. To understand the importance of STAT3 as a crucial downstream factor of RPS4Y1, we therefore performed rescue experiments to investigate the effects of differential RPS4Y1 on the expression of N-cadherin and vimentin (mesenchymal makers), and of E-cadherin (epithelial maker) in trophoblast cells. For this purpose, we transfected specific siRNA targeting STAT3 into RPS4Y1-downregulated HTR-8/SVneo cells and then performed cell proliferation and invasion assays. The knockdown effects on STAT3 expression were confirmed using western blot and immunofluorescence analyses. Western blot analyses indicated that downregulation of RPS4Y1 resulted in STAT3 activation by phosphorylation, increasing protein expression of N-cadherin and vimentin (Fig. 6A and 6B). By contrast, the siRNA-mediated knockdown of STAT3 into RPS4Y1-downregulated HTR-8/SVneo cells resulted in the reduced protein expression of N-cadherin and vimentin (Fig. 6A and 6B). The results of the cell proliferation assay showed that decreased STAT3 did not have an apparent influence on the proliferation of HTR-8/SVneo cells (Fig. 6C). On the contrary, STAT3 reduction significant reduced the promotion effects of RPS4Y1 knockdown on trophoblast cell invasion (Fig. 6D). The results of immunofluorescence analysis were consistent with these findings (Fig. 6E). Taken together, these results reveal that RPS4Y1 may regulate trophoblast invasion and migration through the STAT3/EMT pathway.

Discussion

Trophoblast migration, invasion and spiral artery remodeling are affected during placentation by strict spatio-temporally expression regulation[33,34]. It is thought that the dysregulation expression of genes involving placentation is the possible reason for pregnancy-related disorders during this period[7,35]. Through the use of microarray gene

expression profiling, our group found that a member of the very well-conserved S4E family of ribosomal proteins, ribosomal protein S4, Y-linked 1 (RPS4Y1), was aberrantly highly expressed in preeclamptic placentae. Consistent with microarray results, we confirmed that RPS4Y1 are expressed in human placenta and are upregulated in preeclampsia. In the present study, we demonstrated that RPS4Y1 is important for trophoblast cell migration and invasion. The possible molecular mechanism is that overexpression of RPS4Y1 leads to downregulation of STAT3 phosphorylation as well as suppression of the epithelial-mesenchymal transition (EMT) phenotype in trophoblast cells.

It has been reported that RPS4Y1 is ubiquitously expressed in the placenta trophoblast and in trophoblast debris[20]. In addition, the homologous ribosomal protein genes RPS4X were found to be highly expressed in intrahepatic cholangiocarcinoma and nonmucinous cancer[23,24]. In the current study, using IHC analysis, we found that RPS4Y1 was localized in CTBs, STBs as well as the TC of human placenta villi from the first trimester, and can also be seen in EVTs invading into the maternal decidua, indicating their function in regulating trophoblast invasion. However, immunohistochemistry and western blot assays both showed that expression of RPS4Y1 become very low or undetectable in the third trimester, suggesting that strict spatio-temporally expression regulation of RPS4Y1 at this time is important to normal placentation. Factors responsible for such dynamic expression regulation of RPS4Y1 remain to be elucidated. Several studies have indicated that oxygen tension can regulate trophoblast cell proliferation and differentiation in the invasive pathway[36-39]. Intriguingly, we observed that hypoxia could increase RPS4Y1 expression and decrease STAT3 phosphorylation in HTR8/Svneo cells (data were shown in Figure S2 in the online-only Data Supplement). A follow-up study trying to identify the possibility mechanism that hypoxia regulates RPS4Y1 expression is currently being conducted.

In early placental development, the CTB undergo a partial epithelial-to-mesenchymal transition (EMT), losing their organised epithelial phenotype to a migratory and invasive mesenchymal phenotype and, thus, allowing them to migrate to and infiltrate the maternal decidua and vessels[10,35]. With the elaboration, regulation and precise orchestration of EMT, disruption of tight homeostasis is also thought to contribute to a number of pregnancy complications, for example, preeclampsia and placenta accreta[40]. Several groups have reported that the transformation of the epithelial-like CTB into the invasive, mensenchymal-like EVT is akin to the EMT that occurs in cancer metastasis[12]. Signal transducer and activator of transcription 3 (STAT3), as a member of the STAT family, has been demonstrated to play an important role in regulating EMT during cancer metastasis[15,27]. Numerous research has also suggested that STAT3 is a key regulator of trophoblast invasion and that phosphorylated STAT3 enhances the invasiveness of trophoblast cells[30,40-42]. Recent studies have shown that decreased expression and activation of STAT3 has been detected in preeclamptic placenta and that the inhibition of STAT3 signaling induces trophoblast dysfunctions such as suppression of proliferation, migration and invasion[43,44]. In this study, we provide evidence that STAT3 works as a critical factor downstream of RPS4Y1, affecting trophoblast cell migration and invasion via the STAT3/EMT pathway. Using

a human trophoblast cell line, HTR8/SVneo, we found that inhibition of RPS4Y1 induced trophoblast cell invasion and increases STAT3 phosphorylation along with upregulation of N-cadherin and vimentin. Besides, silencing RPS4Y1 significantly enhanced the outgrowth capacity of EVTs in an ex vivo extravillous culture model. Similar to the results obtained in JAR cells, overexpression of RPS4Y1 significantly compromised the invasion capacity of JAR cells and was accompanied by the inhibition of STAT3 phosphorylation. Since RPS4Y1 inhibits trophoblast cell proliferation, it was essential to rule out the possibility that these effects also caused by the decrease in the number of cells that have invaded through the transwell. We found that in the presence of mitomycin C (a proliferation inhibitor), RPS4Y1 still inhibited cell invasion (data were shown in Figure S3 in the online-only Data Supplement). Furthermore, our results are consistent with the preceding result that the protein phosphorylation of STAT3 is downregulated in the placentae of PE patients. Taken together, these finding strongly substantiates the significance of RPS4Y1 and STAT3 in human placental trophoblast invasion.

In summary, this study suggests that dysregulated expression of RPS4Y1 impairs STAT3 signaling to suppress trophoblast cell migration and invasion. However, the effects of RPS4Y1 expression on trophoblast cell behavior and its implication in pregnancy-related diseases, such as PE, still require in vivo investigation for further verification.

Perspectives

In this study, we uncovered aberrant RPS4Y1 and STAT3 phosphorylation in the placenta of preeclamptic patients. Our data provide strong evidence that STAT3/EMT pathway partially mediates the effect of RPS4Y1 on trophoblast cell migration/invasion. These findings offers valuable insight into the pathogenesis of PE. In view of significant upregulation of RPS4Y1 in PE, further studies are required to comprehensively validate the potential use of antibodies or specific inhibitors to block the RPS4Y1 in preeclampsia.

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Conflict of interest

The authors declare no competing financial interests.

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Novelty and Significance

What Is New?

- Our study demonstrated that RPS4Y1 is aberrantly highly expressed in preeclamptic placentae.
- We provide the first evidence that RPS4Y1 restricted the migration and invasion of trophoblast cells via the downregulation of STAT3 phosphorylation
- Our data indicate that the RPS4Y1－STAT3 axis influences the downstream EMT pathway in trophoblast.

- This is the first report revealing dysregulation of RPS4Y1 in preeclampsia.

What Is Relevant?

- Aberrant RPS4Y1 and STAT3 phosphorylation are correlated with preeclampsia.

Summary

Dysregulation of RPS4Y1 and STAT3 phosphorylation is associated with preeclampsia.

Figure 1. Aberrant expression and localization of RPS4Y1 was found in preeclamptic placenta. **A.** Double-labelling the immunofluorescence analysis of RPS4Y1 protein expression and localization in the placentae from full-term normotensive pregnancies and preeclampsia (PE) patients, Bar = 50μm. **B.** Immunohistochemistry analysis of RPS4Y1 protein expression and localization in the placentae at different stages of pregnancy. **(a, d)** first-trimester villous immunostaining for cytokeratin 7(CK7), which serves as a marker for CTB and TC and RPS4Y1, respectively, **(b， e)** first-trimester decidua immunostaining for human leukocyte antigen G (HLA-G), which serves as a marker of EVT in the maternal decidua and RPS4Y1, respectively, **(c, f)** full-term normotensive pregnancies and preeclampsia (PE) patients placental immunostaining for RPS4Y1. Bar = 50μm. **C.** The total percentages of trophoblast cells positive for RPS4Y1 staining were counted. The statistical data were analyzed by Student's t-test. Data were means±SD of 3 pairs of independent samples. **D. (a, c)** Western blot analysis of RPS4Y1 protein expression in the placentas from first trimester normotensive pregnancies, full-term normotensive pregnancies, and full-term preeclampsia (PE) patients. **(b, d)** Statistical analysis of protein densitometry quantification in Western

blot **(a, c)** by Student's t test, respectively. Data are means±SD. **E.** Expression of RPS4Y1 in different trophoblast cell lines determined by western blotting. $^{**}P<0.01$; $^{***}P<0.001$; NS, nonsignificance.

Figure 2. Downregulated RPS4Y1 promoted HTR8/SVneo cell invasion. **A.** Stable HTR8/SVneo cell lines were established by lentivirus infection of scramble shRNA and specific shRNA targeting RPS4Y1. Confirmation of RPS4Y1 knockdown by RNA interference is shown by western blot. **B.** HTR8/SVneo cells were transfected with the indicated shRNA over three days in culture. Proliferation of the HTR-8/SVneo stable cell lines was detected by CCK8 assay. **C.** The ratio of apoptotic cells in a population of HTR8/SVneo cells transfected with the indicated shRNA, as confirmed by flow cytometric analysis. **D.** Invasion of HTR-8/SVneo stable cell lines was determined by Matrigel invasion assays. Representative images are shown. Bar = 50μm. The relative fold changes in cell invasion were counted. All the statistical data were analyzed by Student's t test. All data are means±SD of three independent experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.01$.

Figure 3. Upregulated RPS4Y1 suppressed JAR cell invasion. The JAR cell line stably overexpressing RPS4Y1 or empty vector was established using plasmid transfection. **A.** The lysates were subjected to western blot analysis with the indicated antibodies. **B.** JAR cells were transfected with the indicated plasmids over three days in culture. Proliferation of the JAR stable cell lines was detected by CCK8 assay. **C.** The ratio of

apoptotic cells in a population of JAR cells transfected with the indicated plasmids, as confirmed by flow cytometric analysis. **D.** Invasion of the JAR stable cell lines was determined by Matrigel invasion assays. Representative images are shown. Bar = 50μm. The relative fold changes in cell invasion were counted. All the statistical data were analyzed by Student's t test. All data are means±SD of three independent experiments performed in triplicate. *$P<0.05$.

Figure 4. Silencing of RPS4Y1 promoted the outgrowth of induced EVTs in the placental villous explant culture model. **A.** Migration (dotted line circled area) of induced EVTs from the placental villi treated with scrambled or RPS4Y1 shRNA for indicated time duration. Bar = 200μm. **B.** Statistical analysis of the outgrowth distance of induced EVTs treated with scrambled or RPS4Y1 shRNA for 72h. **C.** Immunofluorescence analysis of the explant post-transfection with a lentiviral vector-based short hairpin RNA (shRNA) targeting RPS4Y1 after 24 hours. Representative images are shown. Bar=50μm. **D.** Confirmation of RNA interference of RPS4Y1 shown by western blot. All the statistical data were analyzed by Student's t-test. All data are means±SD of three independent experiments performed in triplicate. **$P<0.01$.

Figure 5. RPS4Y1 operated STAT3 through changing its phosphorylation in trophoblast cells and Phosphorylated STAT3 was dysregulated in preeclampsia (PE). **A. (a)** The lysates of HTR8/SVneo cells after transfection with the indicated shRNA were subjected to western blot analysis with the indicated antibodies. **(b)** The lysates of

JAR cells after transfection with the empty vector or RPS4Y1 expression plasmids were subjected to western blot analysis with the indicated antibodies. **B.** Western blot analysis of phosphorylated STAT3 (pSTAT3) expression in placentae from normotensive pregnant women (n=20) and PE patients (n=20). Densitometry quantification of pSTAT3/STAT3 levels are shown. All the statistical data were analyzed by Student's t test. All data are means±SD. $^{**}P<0.01$; $^{***}P<0.001$.

Figure 6. RPS4Y1 knockdown-induced cell invasion was dependent on the STAT3/EMT pathway. An HTR-8/SVneo stable cell line with/without RPS4Y1 knockdown was transfected with scramble or STAT3 siRNA for 48 hours. **A.** and **B.** Western blot and statistical analyses of STAT3/RPS4Y1 protein levels in the stable cell lines. **C.** Proliferation of the stable cell lines was detected by CCK8 assay. **D.** Invasion of the stable cell lines was determined by Matrigel invasion assays. Representative images are shown. Bar = 50μm. The relative fold changes in cell invasion were calculated. **E.** Immunofluorescence analysis of the stable cell lines. Representative images are shown. Bar = 50μm. All the statistical data were analyzed by Student's t test (two groups). All data are means±SD of three independent experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS, nonsignificance.