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Human Placental Expression of SLIT/ROBO Signaling Cues: Effects of Preeclampsia and Hypoxia[1]

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ABSTRACT

Preeclampsia is characterized by dysfunctional endothelium and impaired angiogenesis. Recent studies suggest that the neuronal guidance SLIT/ROBO system regulates tumor angiogenesis. This study investigated if SLIT and ROBO are differentially expressed in healthy term and preeclamptic placentas and if hypoxia regulates SLIT and ROBO expression in placental trophoblast and endothelial cells. Total RNA and protein were extracted from placental tissues of healthy term (n = 5) and preeclamptic (n = 6) pregnancies and used for SLIT/ROBO expression analyses with reverse transcription-polymerase chain reaction (RT-PCR), real-time quantitative-PCR, and immunoblotting. Paraffin-embedded tissues were processed to localize SLIT/ROBO proteins in placental villi by immunohistochemistry. BeWo choriocarcinoma cells and human umbilical vein endothelial cells (HUVEC) were treated with 2% or 10% oxygen or the hypoxia mimetic deferoxamine mesylate (100 μM) to test if hypoxia regulates SLIT/ROBO expression. SLIT2, SLIT3, ROBO1, and ROBO4 mRNA and proteins were detected in the placenta. SLIT2 and ROBO1 proteins localized in the syncytiotrophoblast, and SLIT3, ROBO1, and ROBO4 in capillary endothelium of the placental villi. Levels of ROBO1 and ROBO4 as well as sFLT1 (soluble *fms*-like tyrosine kinase-1) proteins were significantly greater in preeclamptic placentas compared to normal controls. Hypoxia significantly increased both mRNA and protein levels of SLIT2 in BeWo cells and of SLIT3, ROBO1, and ROBB4 in HUVEC. Thus, trophoblast and endothelial coexpression of SLIT/ROBO suggests an autocrine/paracrine regulatory system for regulating placental function. Differential expression of SLITs and ROBOs in healthy term and preeclamptic placentas and hypoxia regulation of their expressions in placental cells implicate a potential pathophysiological role for this system in preeclampsia.

hypoxia, placenta, preeclampsia, ROBO, SLIT

INTRODUCTION

Preeclampsia (PE) is a human pregnancy-specific disease affecting approximately 5%–7% of all pregnancies and is a leading cause of maternal and fetal morbidity/mortality [1]. Its manifestations include vasospasm, hypertension, and proteinuria as well as fetal growth restriction; in severe cases, the disease can necessitate premature delivery. Clinical manifestations of PE typically arise in the second half of gestation (after 20 wk) and rapidly resolve postpartum. The presence of a placenta, but not a fetus, is a prerequisite of the disease, which hints that the disease is of placental origin [2]. Although the precise etiology of PE remains unknown, the consensus is that the disease is associated with derangement of the maternal-fetal interface vasculature, which in turn leads to placental/fetal ischemia, hypoxia and/or reperfusion injury, and dysfunctional endothelium [1, 2].

During pregnancy, a low-resistance and high-capacitance circulatory system must form at the maternal-fetal interface during placentation and further expands onward to execute the bidirectional maternal-fetal exchanges essential for fetal development and survival [2]. Similar to vasculatures in any organ, the formation, development, and expansion of the vasculature at the maternal-fetal interface rely on a deliberate balance between various pro- and antiangiogenic factors. During the last decade, the search for the pathogenesis and etiology of the endothelial dysfunction seen in PE has particularly focused on the shift in this balance toward an antiangiogenic state. For example, several placenta-derived antiangiogenic factors, including soluble *fms*-like tyrosine kinase-1 (sFLT1) [3] and soluble endoglin [4] as well as autoantibodies of angiotensin-II receptors [5], have been linked to placental damage and maternal endothelial dysfunction, although their role in the development of PE remains to be determined.

The SLIT/ROBO signaling system was initially identified in 1999 as an extracellular cue to guide axon pathfinding, to promote axon branching, and to control neuronal migration [6]. SLIT proteins are secreted as ligands, and ROBO (roundabout) proteins function as their specific single-pass transmembrane receptors. Three SLITs (1, 2 and 3) and four ROBOs (1, 2, 3 and 4; ROBO4 is also called magic roundabout) have been identified in mammals. In addition to its repulsive role in neuronal guidance [7], SLIT/ROBO signaling also functions as a repellent to regulate the migration of leukocytes [8], vascular smooth muscle cells [9], and muscle precursor cells [10]. These data indicate that this signaling system functions as a fundamentally conserved guidance machinery for somatic cells. The SLIT/ROBO signaling complex is indispensable for organogenesis in some systems especially for the heart [11] and blood vessels [12]. In addition, SLIT2 has been implicated in breast cancer metastasis [13], and ROBO1 has been implicated in heptacellular carcinoma [14]. In colorectal

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carcinoma, ROBO1 and ROBO4 are differentially regulated [15]. Activation of the SLIT/ROBO signaling is linked to tumor angiogenesis; blocking this pathway suppresses tumor angiogenesis and inhibits tumor growth [16]. Furthermore, ROBO4 has been identified as a vascular-specific receptor for SLITs [17]. These data substantiate the significance of the SLIT/ROBO signaling system in vascular development.

The SLIT/ROBO system has also been found in various nonneuronal tissues, including lung [18], kidney [19], and mammary gland and skin [20] as well as the ovary [21]. Very limited data suggest the expression of ROBO4 in the placental arteriole and venule [22] and SLIT3 in the placenta [20]. We have recently shown immunohistochemically that SLIT2 and ROBO1 are located in syncytiotrophoblast and ROBO1 in normal human placental villous capillary endothelium, implicating an autocrine/paracrine role for this system in regulating placental angiogenesis and trophoblast function [23]. However, detailed information on the expression of this system in human placentas and whether PE alters the expression patterns have not been reported. Thus, this study was designed specifically to examine whether other members of the SLIT/ROBO system are expressed in the placenta and if yes, whether they are differentially expressed in normal and PE placentas. Because the SLIT/ROBO molecules are differentially expressed in various tumors, hypothetically regulated by hypoxia [16], and hypoxia is recognized as a critical factor for placental development and angiogenesis [2], we also determined the effects of hypoxia on SLIT/ROBO expression in placental endothelial and trophoblast cells in vitro.

MATERIALS AND METHODS

Placental Tissues

A total of 11 placentas, five from healthy term (normal) and six from PE, were used in this study. Placenta collection was approved by the University of California San Diego Institutional Review Board with written informed consent. Among the PE women, five had severe PE and one had hemolysis, elevated liver enzymes, and low platelets. The clinical characteristics of the patients are summarized in Table 1. All the placentas were obtained immediately after delivery. After being thoroughly washed with cold PBS, the villous tissue beneath the chorionic and basal plates were quickly dissected, sliced into small pieces (100–500 mg), snap-frozen in liquid nitrogen, and stored in a −80°C freezer. Placental tissues were also fixed in 4% (wt/vol) paraformaldehyde overnight at 4°C for immunohistochemical analysis. Normal pregnancy is defined as a pregnancy in which the mother had normal blood pressure of less than 140/90 mm Hg, no proteinuria, and no medical or obstetrical complications. PE is defined according to the guidelines of National Institutes of Health publication No. 00–3029 [24]: onset of hypertension during mid- or late pregnancy with systolic and diastolic blood pressure equal or higher than 140/90 mm Hg on at least two occasions, separated by 6 h, and proteinuria more than 300 mg in a 24-h period after 20 wk of gestation. Preeclampsia is considered severe with one or more of the following symptoms: maternal blood pressure equal or higher than 160/110 mm Hg on two separate readings at least 6 h apart; proteinuria 3+ by dipstick or 5 g/24 h; visual disturbances; pulmonary edema; epigastric or right upper quadrant pain; impaired liver function; thrombocytopenia; or fetal growth restriction.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA were extracted and quantified by their optical densities at 260nm/280nm as described previously [25]. The RNA samples were reverse-transcribed using AMV Reverse Transcriptase (Promega), and the cDNAs were used for determining the steady-state levels of mRNA of *SLIT/ROBO* molecules. The PCR primer pair for each target was designed to span at least two exons of the gene to discriminate the amplification that might arise from chromosomal DNA (Table 2). PCR was performed in a reaction mixture (25 μl) containing $MgCl_2$ (1.5 mM), deoxyribonucleotide triphosphates (dNTP; 0.2 mM), and 1 units of JumpStart Taq DNA Polymerase (Sigma) under the following conditions (RPL19 [ribosome protein L19], 25 cycles; SLIT/ROBO molecules, 30 cycles): denaturation at 94°C for 30 sec; and annealing for 30 sec at 60°C and extension at 72°C for 30 sec. The PCR products were confirmed by sequencing. Total RNA samples (kindly donated by Dr. Paul Lu) extracted from 5-mo-old rat cortex and Postnatal Day 1 (P1) rat brain were used as controls for PCR analysis.

Real-Time Quantitative PCR Analysis

The primer pairs for quantitative-PCR (q-PCR) analysis are listed in Table 2. They were designed specifically from different exons of human genes to amply the amplicons around 100 bp. The primer pair for FLT1 amplifies both full-length and soluble FLT1. All the targets were verified by PCR amplification and sequencing. Real-time q-PCR was performed with ABI 7300 Real-Time PCR System (Applied Biosystems) using QuantiTect SYBR Green PCR Kit (Qiagen). Briefly, in a reaction mixture of 25 μl, half volume (12.5 μl) of QuantiTect SYBR Green Master Mix was mixed with equal volume (12.5 μl) of a mixture of primers (final, 0.2 μM) and 40 ng of cDNA synthesized as described above. The reaction was carried out under the following conditions: 94°C, 15 sec; 55°C, 30 sec; and 72°C, 34 sec for 40 cycles after the initial activation at 95°C for 15 min. Serial dilutions (5×) of human umbilical vein endothelial cells (HUVEC) cDNA were used as internal controls. Levels of mRNA signals were determined using the standard curve of cycle thresholds. Amplification of all the targets for each sample was adjusted within the corresponding standard curve for all the targets. The q-PCR was performed in triplicate; data were averaged for mRNA levels of targets amplified for each sample and then normalized to that of RPL19.

Immunoblotting Analysis

Protein extracts were prepared from homogenates of placental villous tissues in a nondenaturing buffer as described previously [25]. Equal amount of protein extracts (20 μg) were subjected to SDS-PAGE and electrically transferred to polyvinylidene fluoride membranes as described [25]. SLIT2 antibody (1:500) was kindly provided by Dr. Jian-guo Geng (University of Minnesota), which has been successfully used in human studies previously [16]. SLIT3 antibody (1:250) was purchased from Santa Cruz Biotechnology; and antibodies of ROBO1 (1:500; ab7279), ROBO4 (1:1000; ab10547), and FLT1/sFLT1 (1:500; ab32152) were from Abcam. These SLIT/ROBO antibodies have previously successfully used in human studies [26–28]. Anti-

TABLE 1. Clinical characteristics of the patients (mean ± SD).*

Characteristic	Control (n = 5)	Preeclampsia (n = 6)	*P* value
Maternal age (yr)	31.6 ± 5.9	27.3 ± 7.2	NS
Primigravida (%)	0	2 (33)	NS
Prepregnancy BMI (kg/m^2)	26.2 ± 5.0	28.9 ± 4.9	NS
Gestational age at delivery (wk)	40.0 ± 1.0	32.8 ± 5.2	<0.05
Blood pressure			
Systolic (mm Hg)	118 ± 3	151 ± 21	<0.01
Diastolic (mm Hg)	67 ± 7	100 ± 13	<0.001
Proteinuria (≥300 mg/24 h)	0	6	NS
Birth weight (g)	3440 ± 458	2118 ± 1271	0.056
Fetal weight percentile (%)	52.0 ± 34.2	30.2 ± 24.8	NS
Fetal growth restriction (%)	0	4 (67)	NS
No. of vaginal deliveries (%)	2 (40)	2 (29)	NS

* BMI, body mass index; NS, not significant.

TABLE 2. Primers used for amplification of *SLIT/ROBO* mRNAs by RT-PCR and q-PCR.

Gene	Forward	Reverse	Size (bp)
SLIT1	TGGCCTTCCCTGACTTCAGGTGTG	GTTCCTTGTAGCCAGTCTTCACCC	528
SLIT2	CAGATCAAAAGCAAGAAATTCCGTTG	GAACATCTTATGCTGCACATTTTCC	399
q-PCR	CACATTCCCCAGTACACTG	GATTCCTGTGGCTTCCAAC	72
SLIT3	CCTCTGTCAGCATGAGGCCAAGTGC	CGTGGCCCTGGTACAGCTCCAGTG	509
q-PCR	GACCCCAGGCCAACATC	TCCAGTGCCAGGGGGTC	91
ROBO1	CAGCCATGCATCTGGTAGCAGC	CACTATCTGCTCCTTGAAATTCATT	494
q-PCR	GCATATGGAATTAGTGATCCAAGC	CCTGCTTGTGGTCCACC	92
ROBO2	GATCAGATTGTTGCTCAAGGTCG	GTAAATCCCTCCTTTAACCAGC	407
ROBO3	GGGAAGCTGATGATGTCACATAC	TCCTTCTGCCAGAAGATGGCAG	494
ROBO4	GCAGCAGCAGCCTCAGCAGTCG	TCTGCAGGGGCCAGAGACAAGC	304
q-PCR	AGCAGCCTCAGCAGTCG	TCTGGAAGCAGGGGCAC	107
RPL19	ATCGCCAATGCCAACTCCC	GTCTGCCTTCAGCTTGTGG	351
q-PCR	AGACCCCAATGAGACCAATG	GTGTTTTTCCGGCATCGAGC	129
FLT1			
q-PCR	ATACAGGTAGACCTTTCGTAG	TGGAAACTTTTTTAAAGTAACA	131

ACTB antibody (1:1000) was from Ambion. Anti-HIF1A antibody was from BD/Invitrogen. Digital images were captured by using the ChemiImager Imaging System (Alpha Innotech). Where indicated, integrated relative densities of individual bands were digitized by multiplying the absorbance of the surface areas using NIH's ImageJ.

Immunohistochemistry

Immunohistochemical staining was carried out with the SuperPicture kit from Invitrogen following the manufacturer's protocol as described previously [25]. The concentrations of the antibodies tested were: 10 μg/ml anti-SLIT2, 1 μg/ml anti-SLIT3, 1 μg/ml anti-ROBO1, and 1 μg/ml anti-ROBO4. Corresponding concentrations of anti-IgG (anti-immunoglobulin G) served as nonspecific controls.

Cell Culture and Hypoxia Treatment

HUVEC was purchased from ScienCell, cultured in ECM medium (ScienCell), and used at passages 2–4 as previously described [29]. BeWo choriocarcinoma trophoblast cells were purchased from ATCC and cultured in DMEM/F12 medium containing 10% (vol/vol) fetal bovine serum and 1% (vol/vol) antibiotics (Gibco). BeWo cells and HUVEC were cultured until they reached 80% confluence under a humidified atmosphere (all the gasses in vol/vol) of 95% air and 5% CO_2 at 37°C. Cells were washed once with 1× PBS and then cultured in fresh medium under hypoxia (2% O_2, 5% CO_2, and 93% N_2) or normoxia (10% O_2, 5% CO_2, and 85% N_2) for 24 or 48 h. A SevenGo dissolved oxygen meter (Mettler Toledo) was used to monitor the oxygen levels in the cultured medium. Additional experiments using 100 μM deferoxamine mesylate (DFO) for 24 h to induce chemical hypoxia [30] were performed to verify the effects of hypoxia. All the experiments were performed in triplicate, and protein and total RNA samples were prepared for expression analyses.

Statistics

All the results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SigmaStat (Systat Software Inc.). For comparison between multiple groups, analysis was carried out using the Student-Newman-Keuls test of one-way analysis. Paired Student *t*-test was used for comparisons between the two groups. Significance was defined as $P < 0.05$.

RESULTS

Expression of SLIT/ROBO *mRNAs in Healthy Term Placentas*

We first determined if *SLIT/ROBO* mRNAs were expressed in healthy term placenta. RT-PCR was carried out using RNA samples isolated from normal placental villi for determining the expression of SLIT/ROBO molecules. We also studied the expression of SLIT/ROBO in HUVEC as a reference EC model. High mRNA levels of *SLIT2*, *ROBO1*, and *ROBO4* and low levels of *SLIT3* and *ROBO2* were detected in placental villous tissues. High mRNA levels of *SLIT2, SLIT3, ROBO1*, and *ROBO4* and low level of *ROBO2* mRNA were detected in HUVEC. *SLIT1* and *ROBO3* mRNAs were not detected in the placenta and HUVEC. We also used neonatal rat cortex and P1 rat brain as positive controls and found that mRNAs of all the *SLIT* and *ROBO* molecules were detected (Fig. 1).

Immunohistochemical Localization of SLIT/ROBO Proteins in Healthy Term Placentas

We have shown recently that SLIT2 and ROBO1 are localized in the syncytiotrophoblast and ROBO1 in capillary endothelium of normal human placental villi [23]. Since *SLIT3* and *ROBO4* mRNAs are detected in human placentas and HUVEC, we further determined if SLIT3 and ROBO4 are also localized in the villous trophoblast and capillary endothelial cells along with SLIT2 and ROBO1. As shown in Figure 2, SLIT3, ROBO1, and ROBO4 were immunohistochemically localized in the endothelium (arrowheads). Of note, ROBO4 is specifically expressed in the placental capillary endothelium.

FIG. 1. mRNA Expression of SLIT/ROBO molecules in the human healthy term placenta. Total RNA (2 μg) samples were reversed transcribed (RT), and polymerase-chain reaction (PCR) was used for measuring mRNA levels. Total RNA without RT served as the no-RT control (not shown). The ribosome protein L19 (RPL19) served as a loading control. Lanes: 1, 5-mo-old rat cortex; 2, Postnatal Day 1 rat brain; 3, human term placental villi; and 4, HUVEC.

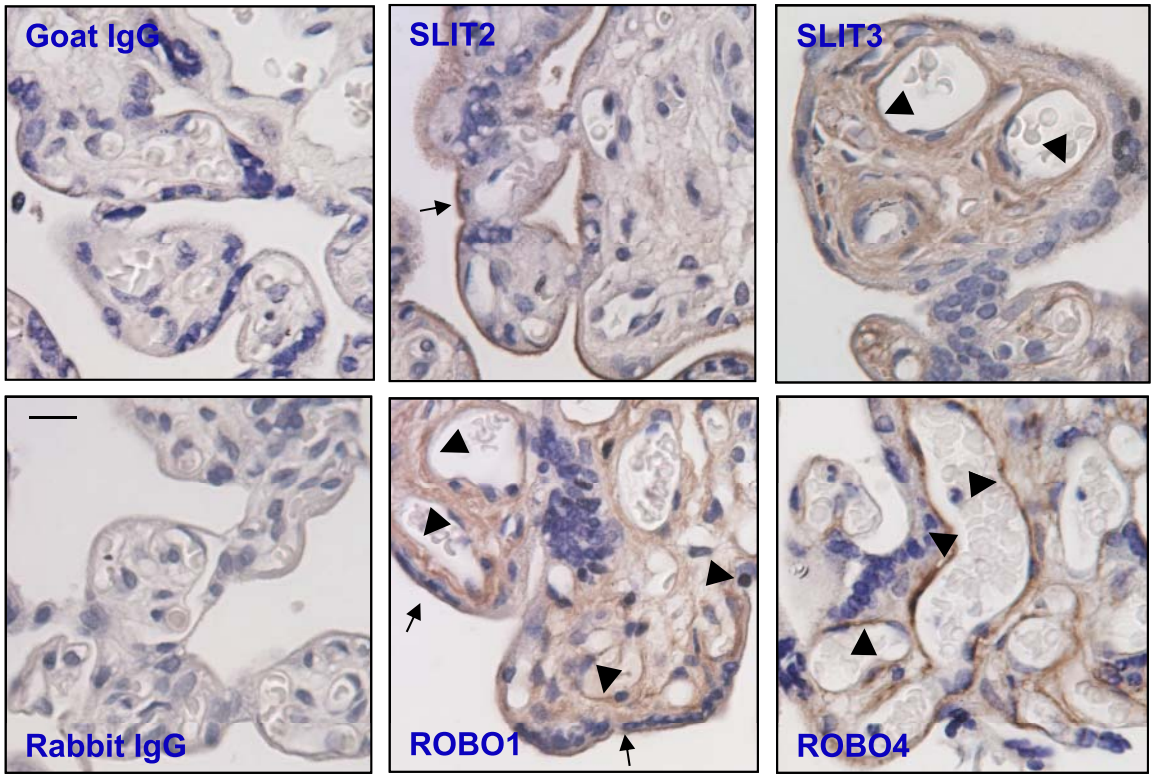

FIG. 2. Immunolocalization of SLIT/ROBO molecules in human placentas. Paraffin embedded sections (6 μm) of human healthy term placentas were processed for immunohistochemical analysis of SLIT2, SLIT3, ROBO1, and ROBO4 with specific anti-SLIT2 (goat, 10 μg/ml), anti-SLIT3, anti-ROBO1, and anti-ROBO4 antibodies (rabbit, 1 μg/ml) using the Zymed/Invitrogen SuperPicture kit. Negative controls were run in parallel with goat (10 μg/ml) or rabbit (1 μg/ml) IgGs. All the panels are in the same magnification. Arrowheads denote capillary endothelial cells, and arrows represent syncytiotrophoblasts. Bar = 30 μm.

We also confirmed that SLIT2 and ROBO1 proteins were localized in the syncytiotrophoblast of the placental villi (arrows).

Expression of SLIT/ROBO *mRNAs and Proteins in Healthy Term and Preeclamptic Placentas*

Real-time q-PCR analysis was then carried out to determine the mRNA levels of *SLIT2, SLIT3, ROBO1,* and *ROBO4* in placental tissues of healthy term and preeclamptic pregnancies. As summarized in Figure 3A, compared to normal controls, *SLIT2* and *SLIT3* mRNA levels were elevated by 1.83- and 2.4-fold ($P < 0.05$) in PE placentas, respectively, whereas *ROBO1* and *ROBO4* mRNA levels were significantly increased by 2.08-fold ($P < 0.05$) and 5.70-fold ($P < 0.01$) in PE placentas, respectively. Soluble *FLT1* mRNA was also measured for quality control of the placental samples; its level was elevated by 5.70-fold ($P < 0.01$) in PE placentas compared to healthy term placentas. These results verified that the placental samples are of high quality because *FLT1*/s*FLT1* mRNA levels are well known to be increased in PE placentas [31].

We then determined the protein levels of the SLITs and ROBOs by immunoblotting to further confirm the expression of SLIT/ROBO molecules in the placenta. As illustrated in Figure 3B, a distinct ~250-kDa band was detected in the placental villous tissues with anti-SLIT2 antibody, which corresponds to the size of full-length SLIT2. A strong immunoreactive protein (~250 kDa) was detected with the specific anti-SLIT3 antibody. An ~180-kDa protein was detected with anti-ROBO1, corresponding to full-length ROBO1. We were able to detect a distinct ~150-kDa protein with anti-ROBO4 antibody, which corresponds to the glycosylated full-length ROBO4 in the placenta. When the protein levels of SLIT/ROBO molecules in normal and PE placentas were compared, ROBO1 and ROBO4 protein levels in PE placentas were 2.61- and 2.19-fold greater ($P < 0.01$) than those of normal controls. SlLIT2 and SLIT3 levels in PE placentas were 1.53- and 1.30-fold to those of normal controls, respectively; however, they did not reach statistical significance ($P > 0.05$). As controls, both full-length FLT1 and sFLT1 were also detected in the placental tissues. Soluble FLT1 protein level in PE placentas was 1.99-fold greater ($P < 0.05$) than that of normal controls; full-length FLT1 was also increased in PE placentas by 1.27-fold relative to that of normal controls, but again, this did not reach significance ($P > 0.05$).

Effects of Hypoxia on SLIT/ROBO *Expression in BeWo and HUVEC*

Because hypoxia is critical for placental development and angiogenesis [2], we tested the hypothesis that hypoxia regulates *SLIT*/*ROBO* expression in placental cells by using BeWo and HUVEC as the trophoblast and endothelial cell models. Compared to normoxia (10% O_2), treatment with hypoxia (2% O_2) for 24 h significantly stimulated *SLIT2* mRNA expression ($P < 0.001$) and slightly increased *ROBO1* mRNA expression ($P > 0.05$) in BeWo cells (Fig. 4A). *SLIT3, ROBO2,* and *ROBO4* were expressed at very low levels or were not detectable in BeWo cells (data not shown). Treatment with

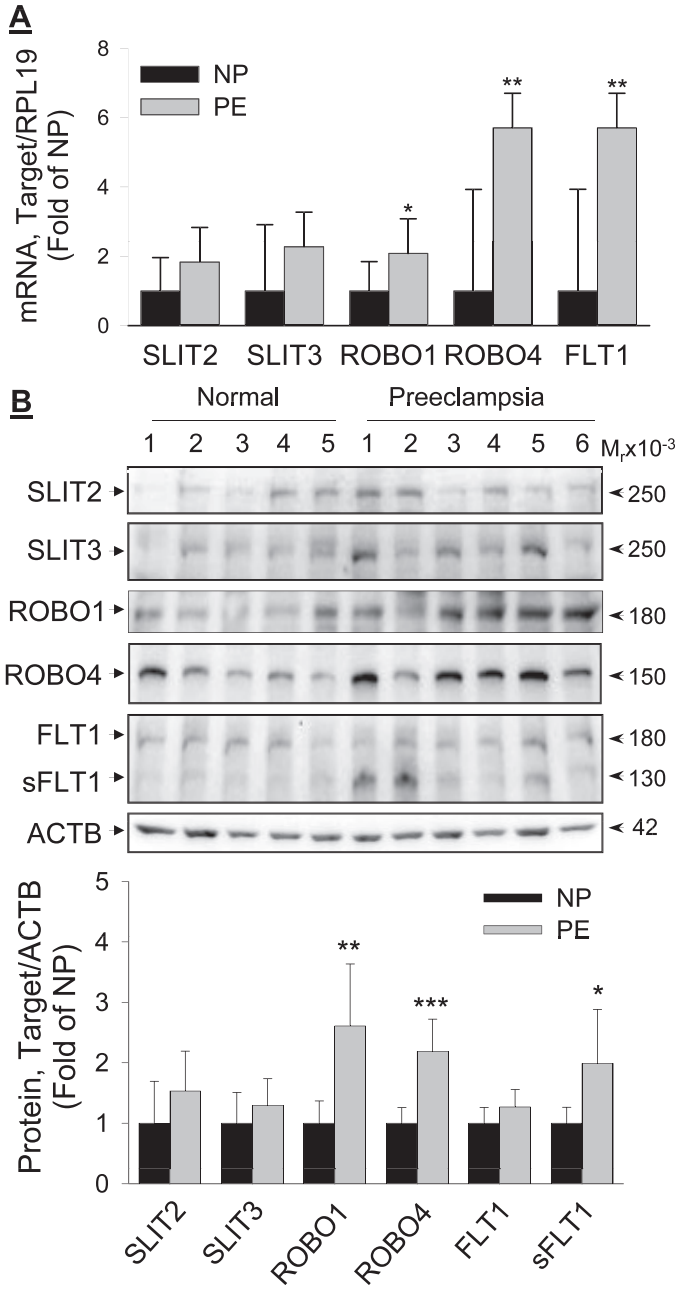

FIG. 3. Messenger RNA and protein expressions of SLIT/ROBO molecules in human placentas from healthy term and PE pregnancies. **A**) Steady-state mRNA levels were measured by real-time q-PCR. Serial dilutions (5×) of HUVEC cDNA were used as internal controls. The mRNA levels were normalized to RPL19 and presented as ratios relative to the controls. The data are expressed as means ± SD, $*P < 0.05$, $**P < 0.01$. **B**) Protein levels were determined by immunoblotting analysis. The bar graph summarizes the data (mean ± SD) from five healthy term (NP) and six preeclamptic (PE) placentas. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

hypoxia significantly stimulated *SLIT3* ($P < 0.05$), *ROBO1* ($P < 0.01$), and *ROBO4* ($P < 0.01$) mRNA expression in HUVEC (Fig. 4B). We further verified these findings with the hypoxia-mimetic DFO (100 μM). Treatment with DFO for 24 h significantly stimulated *SLIT2* mRNA expression in BeWo cells (Fig. 4C) and *SLIT3*, *ROBO1*, and *ROBO4* expression in HUVEC (Fig. 4D). At the protein level, treatment with hypoxia for 48 h significantly stimulated SLIT2 ($P < 0.001$) but not ROBO1 ($P > 0.05$) protein expression in BeWo cells (Fig. 5A). Similarly, hypoxia stimulated SLIT3 ($P < 0.01$), ROBO1

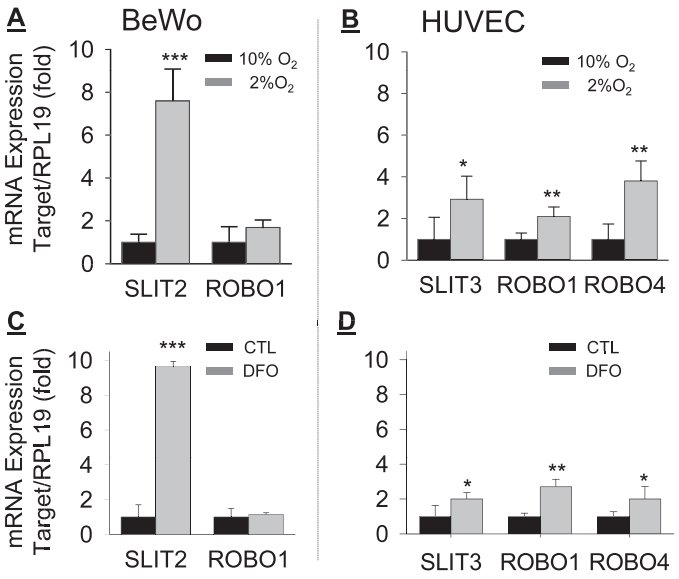

FIG. 4. Hypoxia regulation of *SLIT/ROBO* mRNA expression in BeWo and HUVEC. BeWo (**A** and **C**) and HUVEC (**B** and **D**) were exposed to hypoxia (2% vs. 10% O_2, upper panels) or deferoxamine mesylate (100 μM DFO, lower panels) for 24 h. Total RNA was subjected to q-PCR analysis (**A** and **B**) as indicated in Figure 3A. The bar graph summarizes the immunoblotting data (mean ± SD) relative to normaxia (10% O_2). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. controls.

($P < 0.001$), and ROBO4 ($P < 0.01$) protein expression in HUVEC (Fig. 5B). As controls, treatment with hypoxia significantly increased HIF1A protein levels in both BeWo and HUVEC (Fig. 5).

DISCUSSION

We have shown in this study for the first time that, except for SLIT1 and ROBO3, all other members of the SLIT/ROBO

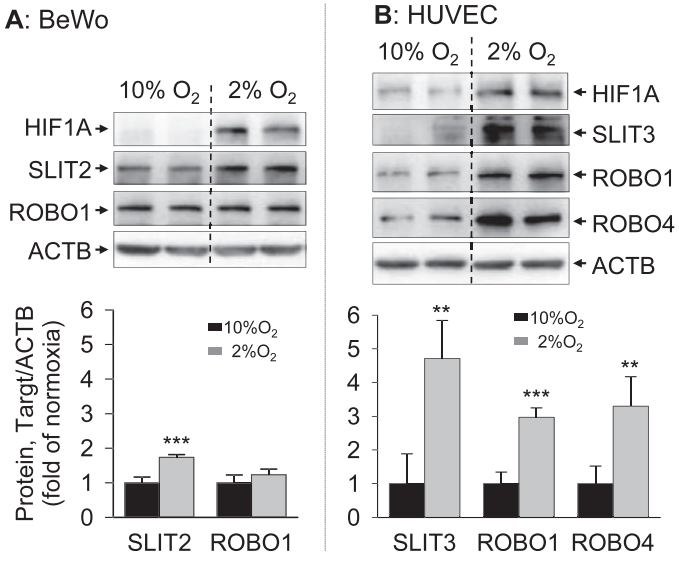

FIG. 5. Hypoxia regulation of SLIT/ROBO protein expression in BeWo and HUVEC. BeWo (**A**) and HUVEC (**B**) were exposed to hypoxia (2% O_2) or normoxia (10% O_2) for 48 h. Total protein extracts were immunoblotted to determine the levels of SLIT/ROBO proteins. HIF1A protein was determined as a positive control of hypoxia treatment. The bar graph summarizes the immunoblotting data (mean ± SD) relative to normoxia (10% O_2). $**P < 0.01$ and $***P < 0.001$ vs. controls.

system are expressed in healthy term human placentas. Specifically, villous syncytiotrophoblasts express high levels of SLIT2 and ROBO1, whereas trophoblastic endothelial cells and HUVEC highly express SLIT2, SLIT3, ROBO1, ROBO2, and ROBO4. Of note, ROBO4 has been identified as an endothelium-specific receptor for SLITs [22]. The coexpression of multiple ligands (SLIT2 and SLIT3) and receptors (ROBO1, ROBO2, and ROBO4) in healthy term placentas further strengthened our recent assessment that this signaling system may function as an autocrine/paracrine regulatory system for regulating placental development and/or maintenance of normal placental functions [23].

We have further shown that several SLIT/ROBO mRNAs and proteins are increased in PE placentas compared to normal controls. These observations raise an important question as to how SLIT/ROBO is upregulated in the placentas. Physiological O_2 concentration at the maternal-interface especially within the intervillous space is relative low (~3%–8%), possibly due to extremely high metabolism in the maternal-fetal unit [32]. Thus, hypoxia is a key physiological factor for regulating placental gene expression, angiogenesis, and development during placentation. Poor placentation due to insufficient trophoblast invasion into the endometrial spiral artery is a leading cause of PE, which applies to the majority of all PE cases. Deranged vasculature in PE placenta leads to reduced blood flow and even lower O_2 supply to the placenta [2]. In turn, this causes placental ischemia and persistent hypoxia, resulting in trophoblast debris [33] and inducing the secretion of placental factors, such as sFLT1 [3, 34] and inflammatory cytokines [35, 36], to be circulated. Together, these changes lead to the clinically observed maternal manifestations of PE [2, 37]. Thus, it is reasonable to infer that hypoxia is a highly suspicious key factor for regulating placental SLIT/ROBO expression. We therefore have investigated the effects of hypoxia on placental *SLIT*/*ROBO* expression in vitro by using BeWo and HUVEC as the models. Our data clearly demonstrated that both low oxygen (2% O_2, hypoxia) and a chemical hypoxia inducer, 100 μM DFO, stimulated mRNA expression of the *SLIT* and *ROBO* family of molecules that are mainly localized in the placental trophoblast and endothelial cells in vivo. In addition, hypoxia treatment also resulted in increased expression of their proteins. Upregulation of the expression of *SLIT* and *ROBO* genes in these cells seems to transpire through transcription-dependent mechanisms because: 1) all the regulatory regions of *SLIT*/*ROBO* genes possess one or more putative hypoxia-response-element (-ACGTG-) [38], and 2) hypoxia increased hypoxia-inducible factor 1 α (HIF1A) protein in both BeWo cells and HUVEC and DFO is a pharmacological agent that stabilizes HIF1A. Consistent with our findings, it has shown previously that hypoxia induces ROBO4 expression in human dermal microvascular EC [22]. The functional sequelae of the changes in their expression by hypoxia are elusive. It is possible that they might contribute to placental angiogenesis because: 1) placenta is an extremely active organ with angiogenic activity [39], and 2) hypoxia upregulates *SLIT*/*ROBO* in various tumors with increased angiogenic activity [16]. However, this hypothesis needs further investigation.

Previous studies have shown that levels of sFLT1 are elevated in the placenta and maternal blood in PE. These data are consistent with previous studies showing increased sFLT1 production in PE placentas [4, 31, 40]. We have confirmed elevated sFLT1 expression in PE placenta, and full-length FLT1 is not increased concomitantly in PE compared to normal controls (Fig. 3). Increased sFLT1 functions as an endogenous inhibitor that antagonizes the angiogenic activities of vascular endothelial growth factor (VEGF) and placenta-derived growth factor, thereby contributing to the pathogenesis of PE [4]. Obviously, our data showing differential expression of SLIT and ROBO molecules in healthy term and PE placentas suggest that increased SLIT/ROBO expression is likely a consequence of PE. However, our data showing upregulation of placental SLIT/ROBO molecules by hypoxia have raised a key question as to whether SLIT/ROBO signaling contributes to the pathogenesis of PE. Although nothing is known at the moment, it is possible that, like sFLT1, increased expression of SLIT2 and ROBO4 might play an inhibitory role in angiogenesis in the placenta. ROBO4 is recognized as an antiangiogenic factor that inhibits primary EC migration [41]. SLIT2 and ROBO4 inhibit VEGF or FGF-induced HUVEC migration possibly via the RAS-RAF-MAP2K-ERK signaling pathway [42] and VEGF-induced angiogenic responses by blocking SRC family kinase activation [17]. Therefore, further investigations are needed to determine if the expression of this system is developmentally regulated in the placenta and whether the SLIT/ROBO system complicates the signaling network of other growth factors controlling angiogenesis, thereby contributing to the placental pathophysiology of PE.

We also have shown by immunohistochemistry that syncytiotrophoblasts express SLIT2 and ROBO1; in vitro, hypoxia upregulates SLIT2 and ROBO1 proteins in BeWo cells. The significance of trophoblast expression of SLIT2 and ROBO1 has to be determined. However, these data suggest that the SLIT/ROBO signaling also plays a role in regulating trophoblast functions. This system may also play a role in the placenta because SLIT molecules are secreted ligands. To this end, SLIT2 secreted by the trophoblast could regulate placental endothelial functions via binding to the endothelial ROBO1, ROBO2, and ROBO4, whereas SLIT2 and SLIT3 secreted by the endothelial could act on the trophoblasts via ROBO1. From this perspective, the SLIT/ROBO signaling system potentially regulates cell-cell communications between capillary endothelial and trophoblastic cells. It would be intriguing to address if altered placental SLIT secretion contributes to the systemic endothelial dysfunction in PE.

In summary, we have shown that the SLIT/ROBO signaling system is differentially expressed in the placental endothelial and trophoblast cells and their expression is under the influence of PE and hypoxia. Although the functional significance of these specific expression patterns needs to be determined, it is hypothesized that this system has an important role in regulating normal and abnormal placental development and function via an influence on both trophoblastic and endothelial cells.

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