NOTCH4 signaling controls EFNB2-induced endothelial progenitor cell dysfunction in preeclampsia

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Abstract

Preeclampsia is a serious complication of pregnancy and is closely related to endothelial dysfunction, which can be repaired by endothelial progenitor cells (EPCs). The DLL4/NOTCH–EFNB2 (ephrinB2) cascade may be involved in the pathogenesis of preeclampsia by inhibiting the biological activity of EPCs. In addition, both NOTCH1 and NOTCH4, which are specific receptors for DLL4/NOTCH, play critical roles in the various steps of angiogenesis. However, it has not been determined which receptor (NOTCH1, NOTCH4, or both) is specific for the DLL4/NOTCH–EFNB2 cascade. Accordingly, we performed a series of investigations to evaluate it. EFNB2 expression was examined when NOTCH4 or NOTCH1 was downregulated, with or without DLL4 treatment. Then, the effects of NOTCH4 on EPC function were detected. Additionally, we analyzed NOTCH4 and EFNB2 expression in the EPCs from preeclampsia and normal pregnancies. Results showed that NOTCH4 downregulation led to decreased expression of EFNB2, which maintained the same level in the presence of DLL4/NOTCH activation. By contrast, NOTCH1 silencing resulted in a moderate increase in EFNB2 expression, which further increased in the presence of DLL4/NOTCH activation. The downregulation of NOTCH4 resulted in an increase of EPC biological activity, which was similar to EFNB2 silencing. NOTCH4 expression, consistent with the EFNB2 level, increased notably in preeclampsia EPCs compared with the controls. These findings suggest that NOTCH4, not NOTCH1, is the specific receptor for the DLL4/NOTCH–EFNB2 cascade. Blockade of this cascade may enhance the angiogenic property of EPCs, and act as a potential target to promote angiogenesis in patients with preeclampsia.

Reproduction (2016) **152** 47–55

Introduction

Preeclampsia is a hypertensive complication that affects 5–7% of all pregnancies. It is associated with significantly higher rates of maternal–fetal morbidity and mortality (ACOG 2013). The generally accepted pathogenesis of preeclampsia is a two-stage model (Agata Szpera-Gozdziewicz 2014). The first stage (before the 20th gestational week) is triggered by abnormal embryo implantation and poor placentation that leads to placental hypoxia. The second stage, which represents endothelial dysfunction, occurs after the 20th gestational week and is a result of the release of anti-angiogenic factors due to hypoxia into the placental and maternal circulation. However, this dysfunction cannot be automatically repaired by terminally differentiated endothelial cells. Endothelial progenitor cells (EPCs) are considered to have the capacity for vascular endothelial repair (Agata Szpera-Gozdziewicz 2014).

EPCs are heterogeneous populations of endothelial cell precursors that are thought to play a role in endothelial homeostasis and vascular remodeling (Hur *et al.* 2004, Burger & Touyz 2012). Reduction in

number or malfunction of EPCs may be a risk factor for cardiovascular diseases (Hill *et al.* 2003, Di Mambro *et al.* 2010), and EPCs act as a treatment for animal ischemia models (Oh *et al.* 2007, Ye & Poh 2015). Moreover, recent evidence supports the idea that EPCs may play a part in placental vasculature and the pathophysiology of preeclampsia (Robb *et al.* 2007, Sipos *et al.* 2010). EPCs increase gradually with gestational age in an uncomplicated pregnancy (Sugawara *et al.* 2005). The decrease and functional disorder of EPCs has been shown to reflect damaged endothelial repair ability in preeclampsia (Lin *et al.* 2009, Luppi *et al.* 2010). Therefore, defects in EPCs may be used as a diagnostic tool, while enhancement of EPCs may be used as a therapeutic tool with regard to preeclampsia.

Our recent studies have provided the novel insight that DLL4/NOTCH–EFNB2 (ephrinB2) cascade is part of the pathological mechanism of preeclampsia in the regulation of EPC activity (Liu *et al.* 2015). The evolutionarily conserved NOTCH signaling pathway plays a crucial role in intercellular communication and deciding the fate of cells during development

(Artavanis-Tsakonas *et al.* 1999). The NOTCH family consists of four NOTCH receptors (NOTCH1, 2, 3, and 4) and five ligands (Jagged 1 and 2 and Delta-like 1, 3, and 4) in mammals (Weinmaster 1997). Notably, DLL4 is a pivotal vascular-specific element of the NOTCH pathway and contributes to embryonic vascular development and arteriogenesis (Duarte *et al.* 2004, Gale *et al.* 2004), by linking endothelial cell bioactivity to the tissue microenvironment via NOTCH ligand receptor signaling (Noguera-Troise & Gale 2006, Ridgway *et al.* 2006). DLL4 was found to only interact with NOTCH1 and NOTCH4, endothelial cell-specific NOTCH receptors, which played a prominent role in angiogenesis and homeostasis during development (Shutter *et al.* 2000, Shawber *et al.* 2003). Strikingly, the activation of DLL4/NOTCH signaling led to repression of EPC proliferation, differentiation, and migration (Liu *et al.* 2015). NOTCH1 was also discovered to be involved in the inhibition of EPC activity (li *et al.* 2010); however, the influence of NOTCH4 on EPCs has not been thoroughly explored. EFNB2 is a membrane-bound ligand for EPH receptor tyrosine kinases, which are involved in numerous developmental processes (Christian Hafner 2004). EFNB2 is specifically expressed in endothelial cells, arterial angioblasts, and perivascular mesenchymal cells as a critical regulator of embryonic vessel development and vascular remodeling (Wang *et al.* 1998). EFNB2 has the ability to regulate the biological functions of differentiated endothelial cells (Steinle *et al.* 2003, Kuijper *et al.* 2007), including their growth, sprouting, migration, and proper assembly with other endothelial cells to form vascular structures (Salvucci & Tosato 2012). As the downstream of DLL4/NOTCH signaling pathway, EFNB2 has been also discovered to play an inhibitory role on EPC function (Liu *et al.* 2015).

This study is based on the hypothesis that the decrease and dysfunction of EPC in preeclampsia could be rescued by appropriately modulating DLL4/NOTCH–EFNB2 cascade. Although the vital role of DLL4/NOTCH–EFNB2 cascade in EPC bioactivity was determined, the specific receptor that interacts with DLL4 was not fully determined. DLL4 binding to different receptors exhibited different biological effects on vascular development. NOTCH1 or NOTCH4, or even both, are the suggested receptors for this cascade in EPCs. This hypothesis was tested by a number of investigations to evaluate the relationship between EFNB2 and DLL4/NOTCH1 or DLL4/NOTCH4.

Materials and methods

Population study

The study groups consisted of 12 normotensive women and 12 preeclampsia women. All of the study participants were from the Department of Obstetrics and Gynecology, Union

Table 1 Clinical characteristics of study population.

Parameters	Normal (n=12)	Preeclampsia (n=12)	P-value
Maternal age (years)	29.65 ± 1.04	31.00 ± 1.55	>0.05
BMI in pregnancy	25.66 ± 0.57	26.07 ± 0.87	>0.05
Gestational weeks at delivery	38.34 ± 0.23	36.54 ± 0.56	<0.05
SBP at delivery	113.42 ± 1.20	158.74 ± 6.71	<0.05
DBP at delivery	75.42 ± 1.34	102.16 ± 2.90	<0.05
Proteinuria	0% (0/12)	100% (12/12)	<0.05
S/D ratio of umbilical artery	2.21 ± 0.09	2.30 ± 0.04	>0.05
Birth weight (g)	3410 ± 82.13	2997 ± 83.61	<0.05
Apgar score	9.05 ± 0.18	8.02 ± 0.33	<0.05

Data is presented as mean ± S.D. or percentage (number/total). BMI, body mass index in pregnancy (kg/m²); SBP, systolic blood pressure; DBP, diastolic blood pressure; S/D ratio, systole/diastole (S/D) ratio; Proteinuria (quantity of 24 h urine protein excretion), higher or equal to 300 mg/24 h is proteinuria positive; lower than 300 mg/24 h is proteinuria negative.

Hospital, Huazhong University of Science and Technology (HUST) and signed the informed consent. Ethical approval was obtained from the hospital's Ethics Committee. All subjects were beyond the 32th gestational week. Preeclampsia was clinically described as hypertension (blood pressure greater or equal to 140/90 mmHg) and proteinuria (greater or equal to 300 mg/24 h) that occurred after the 20th gestational week (ACOG 2013). Members of control group were healthy pregnant women without pregnancy complications or any chronic problems. Exclusion criteria included multiple pregnancies, rupture of membranes, fetal structural, or genetic anomalies (e.g., hepatic disease, diabetes, cardiovascular diseases, blood diseases, or other organic and metabolic disorders, and infectious disease). Clinical characteristics of the two groups are given in Table 1. After cutting the umbilical cord, cord blood (40–60 mL) was immediately collected and processed for cell culture.

Isolation and culture of EPCs

The method adopted for EPC isolation from cord blood was as described previously (Yan *et al.* 2013). It has been demonstrated that EPCs can be induced and differentiated from mononuclear cells (MNCs) in peripheral blood (Hur *et al.* 2007). MNCs were isolated from 50 mL of cord blood by the density gradient centrifugation method (400 g, 30 min, 20°C). The collected MNCs were washed twice using a phosphate-buffer solution (PBS) and then re-suspended (1 × 10⁶ cells/mL) in endothelial basal medium-2 (EBM-2; Lonza, Allendale, NJ, USA) containing 10% fetal bovine serum (FBS) and cultured in pre-coated six-well culture dishes (Corning) with fibronectin (Sigma-Aldrich). The cells were incubated in a 5% CO₂ humidified atmosphere at 37°C and the medium was replaced every 3 days. Attached cells formed small EPC clusters after 3 days. Cells from the central core of these clusters gradually became elongated and acquired spindle-shaped. Colony-forming units (CFU) (Hur *et al.* 2007) began to materialize on day 7, when all experiments were carried out. The identification of EPCs on day 7 has been performed in our previous study (Yan *et al.* 2013).

Quantitative RT-PCR

Total RNA was extracted from cultured cells using RNAiso Plus (Takara Bio). This was followed by cDNA synthesis using PrimeScript™ 1st RT Master Mix (Takara, Japan). The primer sequence was as follows: *GAPDH* (an internal control), forward primer: 5'-ACCACAGTCCATGCCATCAC-3', reverse primer: 5'-TCCACCACCCTGTTGCTGTA-3'. *NOTCH4*, forward primer: 5'-CACTAGGCGAGGACAGCATTG-3', reverse primer: 5'-ATTCTGGGGAGGAGTTAGCA-3'. *NOTCH1*, forward primer: 5'-GAGAAGGGAAGTTGAACGAGCAT-3', reverse primer: 5'-TGCCTCCGTTTGCCTCTGG-3'. *EFNB2*, forward primer: 5'-GGTGGTCTCTTGCTGAAGT-3', reverse primer: 5'-CGCTGACCTTCTCGTAGTGA-3'. *HES1*, forward primer: 5'-CTACCCAGCCAGTGTCAA-3', reverse primer: 5'-AACGCAGTACCGGCGAGTG-3'. *HEY1*, forward primer: 5'-CGGCAGGAGGGAAAGGCTA-3', reverse primer: 5'-CGGGTGATGTCCAAAGGCAG-3'. Quantitative RT-PCR (qRT-PCR) was performed on the StepOne™ Real-Time PCR System with SYBR Premix Ex Taq™ (TaKaRa, Japan) according to the manufacturer's protocol. The qRT-PCR reaction consisted of a 95°C denaturation step for 30 s, 40 cycles (95°C for 5 s, 65.5°C for 30 s, 60°C for 45 s) and extension at 72°C for 60 s. The expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

EPCs with different treatment or from controls and preeclampsia patients were lysed in a RIPA lysis buffer. The supernatant protein concentration was determined using a BCA protein assay kit (Beyotime, China), after which 50 µg proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated with primary antibodies against NOTCH4 (1.0 µg/mL; Millipore), NOTCH1 (1.0 µg/mL; Abcam), EFN2 (0.5 µg/mL; Abcam), and GAPDH (0.5 µg/mL; Affinity, Cincinnati, OH, USA) at 4°C overnight. Incubated membranes were developed by appropriate anti-rabbit secondary antibodies (0.5 µg/mL; ProteinTech, Chicago, IL, USA) for 1 h at room temperature and signals were visualized using enhanced chemiluminescence (ECL; Beyotime, China).

Transfection

We established the silence model in normal EPCs to further study the relationship between EFN2, NOTCH4 and NOTCH1 in EPCs. Cultured EPCs (4×10^5 cells/well) were divided into five groups: (1) nontransfected cells (control group); (2) transfected cells with DNA plasmid of nonspecific sequence as a negative control (shNC group); (3) transfected cells with DNA plasmid against NOTCH4 expression (shNOTCH4 group) (Genechem, Shanghai, China); (4) transfected cells with DNA plasmid against NOTCH1 expression (shNOTCH1 group) (Genechem, Shanghai, China); and (5) negative control for shNOTCH1 group (shNC1 group). Transfection was completed using Neofect (Neofect Biotechnologies, Beijing, China) according to the manufacturer's protocol. The inhibition efficiency of target genes was verified using qRT-PCR and western blot assay.

Reagents

The NOTCH signaling pathway was activated in EPCs using s-DLL4 recombinant protein (0.2 µg/mL; PeproTech, Rocky Hill, NJ, USA). Normal and transfected EPCs were incubated with s-DLL4 for 24 h (Liu et al. 2015). According to the manufacturer's instruction, recombinant human s-DLL4 comprises the extracellular signaling domain of DLL, a member of a structurally-related family of single-pass type I transmembrane proteins that serve as ligands for NOTCH receptors. s-DLL4 functions to specifically activate the NOTCH1 and NOTCH4 receptors.

Cell proliferation assay

The influence of NOTCH4 on cell proliferation was evaluated using the Cell Counting Kit (CCK-8) assay. EPCs (2.5×10^4 cells/well) with different treatment were cultured on 96-well plates in five replicates. After 24 h, 10 µL of a CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well and incubated for 4 h at 37°C. The absorbance of each well was measured at 450 nm using a microplate reader (Model 550; Bio-Rad). All experiments were carried out three times.

Cell differentiation assay

Cell differentiation assay was carried out as described previously (Yan et al. 2013). EPCs (2.5×10^4 cells/well) with different treatment were re-suspended and re-plated on 96-well fibronectin-coated culture dishes. After 24 h incubation at 37°C in a 5% CO₂ incubator, the number of elongated and spindle-shaped cells, representing differentiated EPCs according to the report by Asahara (Asahara et al. 1997), was counted in three random fields at 200× magnification using a phase-contrast microscope. We also normalized differentiated cells to the total number of cells.

Migration assay

The migration ability of EPCs was detected using Transwells (8.0 µm pore size; Corning). Transfected cells were briefly re-suspended in 200 µL of EBM-2 and then added to the upper chamber (5×10^4 cells/chamber). At the same time, the lower chamber was filled with 550 µL of a medium containing 10% FBS and 50 ng/mL of recombinant human VEGF₁₆₅ (PeproTech, Rocky Hill, NJ, USA) as a chemoattractant. After 24 h incubation at 37°C, nonmigrated cells were removed using a cotton-tipped swab while migrated cells were fixed with 4% formaldehyde polymerisatum and stained with crystal violet. The migrated cells were imaged and counted (three randomly selected fields per Transwell at 200× magnification) using an inverted microscope.

Adhesive assay

EPC adhesion was carried out as described previously (Liang et al. 2009). EPCs (2.5×10^4 cells/well) from different groups were re-suspended in 0.25% trypsin and incubated in fibronectin-coated culture plates containing EBM-2 with 10% FBS. After further incubation for 0.5 h, nonadherent cells were

washed off using PBS. Adherent cells were counted in three random fields (at 200× magnification).

In vitro HUVEC tube formation assay

On day 7, EPCs failed to form tube-like structures but promoted human umbilical vein endothelial cell (HUVEC) to form tube-like structures by secreting angiogenic cytokines (Hur *et al.* 2004). Therefore, we conducted HUVEC tube formation assay *in vitro*. First, the medium that had cultured transfected cells was collected. HUVECs (3.5×10^4 cells/well) were then harvested, re-suspended in 100 μ L of a conditioned medium, and re-seeded on 96-well plates coated with growth factor reduced Matrigel (BD, Franklin Lakes, NJ, USA). After 8 h incubation at 37°C, tube-like structures were formed and observed by microscopy (at 100× magnification). To quantitate the progression of angiogenesis, the mean number of branching points with at least three tubules was counted in five randomly selected fields per well (Huang & Zheng 2006).

Statistical analysis

We used GraphPad Prism 5 for data analysis. The analysis of variance (ANOVA) followed by *post hoc* test was applied in the comparison among groups, and the results were expressed as means $\pm$ s.d. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Clinical characteristics of study population

The clinical characteristics of study population were summarized in Table 1. There was no statistical difference in maternal age, BMI, and S/D ratio between preeclampsia patients and controls. Preeclampsia patients presented earlier pregnancy termination than controls ($P < 0.05$). Both neonatal weight and Apgar scores were lower in preeclampsia group ($P < 0.05$).

NOTCH4 and NOTCH1 silence in EPCs

Transfection efficiency was verified by qRT-PCR and western blot assay. The mRNA level of *NOTCH4* decreased substantially in the shNOTCH4 group compared with the shNC group (Fig. 1A). Similarly, the expression of NOTCH4 protein was significantly lower in the shNOTCH4 group (Fig. 1B and E). There was a decrease in NOTCH1 mRNA (Fig. 1C) and protein level (Fig. 1D and F) in the shNOTCH1 group compared with the controls.

Activation of NOTCH signaling by s-DLL4

To testify that s-DLL4 recombinant protein can activate NOTCH signaling pathway in EPCs, the mRNA levels of target genes, such as *HES1* and *HEY1* (Williams *et al.* 2006), were monitored by qRT-PCR. Results showed that

Figure 1 Transfection influenced the expression of NOTCH4 and NOTCH1 in EPCs. (A) The mRNA expression of *NOTCH4* in EPCs from shNOTCH4 groups decreased notably compared with shNC groups (0.11 ± 0.02 vs 1.01 ± 0.03 , $P < 0.001$). There was no significant difference of *NOTCH4* mRNA between control and negative control ($P = 0.751$, $P > 0.05$). (B) There was a distinct obvious drop of NOTCH4 protein level in shNOTCH4 groups compared with controls (0.47 ± 0.07 vs 1.06 ± 0.07 , $P < 0.01$). NOTCH4 protein level did not have a significant change between control and negative control ($P > 0.05$). (C and D) There was a drop in NOTCH1 mRNA (0.56 ± 0.14 vs 0.98 ± 0.05 , $P < 0.05$) and protein (0.54 ± 0.01 vs 1.01 ± 0.02 , $P < 0.001$) level in shNOTCH1 group compared with controls. NOTCH1 mRNA ($P > 0.05$) and protein ($P > 0.05$) expression did not have a significant change between control and negative control. (E and F) NOTCH4 and NOTCH1 protein expression in different groups was shown by western blotting. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; shNOTCH4 group (NOTCH4 silence); shNOTCH1 group (Notch1 silence); shNC group (negative control to shNOTCH4 group); shNC1 group (negative control to shNOTCH1 group).

the expression of *HES1* and *HEY1* was higher in DLL4 group than control (Fig. 2A and B).

Effects of NOTCH4 silence on EFNB2 expression

The downregulation of NOTCH4 in EPCs significantly inhibited EFNB2 mRNA (Fig. 2C) and protein expression (Fig. 2D and G) compared with the shNC group. Moreover, EFNB2 mRNA (Fig. 2C) and protein expression (Fig. 2D and G) was also downregulated in NOTCH4-silenced EPCs cultured with s-DLL4 compared with the shNC group. There was no significant difference in EFNB2 expression between the shNOTCH4 group and shNOTCH4+DLL4 group (Fig. 2C, D and G). These indicated that EFNB2 expression may be dominated by NOTCH4 signaling.

Figure 2 Transfection and reagent influenced EFNB2 expression in EPCs. (A and B) The mRNA levels of *HES1* (4.94 ± 0.80 vs 0.99 ± 0.03 , $P < 0.01$) and *HEY1* (6.60 ± 1.54 vs 1.01 ± 0.07 , $P < 0.05$) in EPCs cultured with sDLL4 were higher than in controls. (C and D) The mRNA expression (0.32 ± 0.08 vs 1.02 ± 0.02 , $P < 0.001$) and protein level (0.49 ± 0.06 vs 1.07 ± 0.04 , $P < 0.001$) of EFNB2 in EPCs from shNOTCH4 group significantly dropped compared with shNC group. EFNB2 mRNA (0.37 ± 0.12 vs 1.02 ± 0.20 , $P < 0.001$) and protein expression (0.45 ± 0.17 vs 1.07 ± 0.04 , $P < 0.05$) was also obviously downregulated in transfected EPCs cultured with DLL4, compared with shNC groups. There was no significant difference in EFNB2 expression between shNOTCH4 group and shNOTCH4+DLL4 groups ($P > 0.05$). (E and F) EFNB2 mRNA (2.17 ± 0.28 vs 1.12 ± 0.08 , $P < 0.01$) and protein expression (1.42 ± 0.07 vs 1.09 ± 0.07 , $P < 0.05$) slightly increased in shNOTCH1 group, compared with the shNC1 group. EFNB2 mRNA (6.90 ± 1.05 vs 1.12 ± 0.08 , $P < 0.001$) and protein expression (1.95 ± 0.13 vs 1.09 ± 0.07 , $P < 0.01$) were significantly up-regulated in shNOTCH1+DLL4 group compared to control. Moreover, there was more EFNB2 mRNA (6.90 ± 1.05 vs 2.17 ± 0.28 , $P < 0.01$) and protein (1.95 ± 0.13 vs 1.42 ± 0.07 , $P < 0.05$) expression of shNOTCH1+DLL4 group than shNOTCH1 group. (G and H) EFNB2 protein expression in different groups was shown by western blotting. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; shNOTCH4 group (NOTCH4 silence); shNC group (negative control to shNOTCH4 group); shNOTCH1 group (NOTCH1 silence); shNC1 group (negative control to shNOTCH1 group); shNOTCH1+DLL4 group (NOTCH1-silence EPCs cultured with DLL4); shNOTCH4+DLL4 group (NOTCH4-silence EPCs cultured with DLL4).

Effects of NOTCH1 silence on EFNB2 expression

EFNB2 mRNA (Fig. 2E) and protein level (Fig. 2F and H) increased slightly in shNOTCH1 group, compared with the shNC1 group. EFNB2 mRNA (Fig. 2E) and protein expression levels (Fig. 2F and H) were significantly upregulated in NOTCH1-silence EPCs cultured with s-DLL4. Moreover, there was more EFNB2 mRNA (Fig. 2E) and protein (Fig. 2F and H) expression in the shNOTCH1+DLL4 group compared with the shNOTCH1 group. These indicated that DLL4/NOTCH1 signaling pathway was not the direct upstream regulator of EFNB2.

Effects of NOTCH4 on proliferation and differentiation of EPCs

Compared with controls, there was a significant increase in cell proliferation following the downregulation of NOTCH4 (Fig. 3A). The number of differentiated cells in the shNOTCH4 group increased noticeably compared with the control group (Fig. 3B and D). Furthermore, the percentage of differentiated cells among all cells was higher in the shNOTCH4 group compared with the shNC group (Fig. 3C and D).

Effects of NOTCH4 on migration and adhesion of EPCs

Transwell assay showed that more EPCs from the shNOTCH4 group crossed the membrane compared with the shNC group (Fig. 4A and B), and adhesive assay showed that there were more adherent EPCs in the shNOTCH4 group compared with the shNC group (Fig. 4C and D).

NOTCH4 mediated HUVEC tube formation in vitro

We cultured HUVECs with different conditioned media in order to investigate the effect of NOTCH4 on angiogenesis. HUVECs cultured with supernatant collected from EPCs in the shNOTCH4 group formed more tube-like structures than those cells cultured with supernatant from the shNC group (Fig. 4E and F). All of the above suggested that NOTCH4 downregulated EPC function.

NOTCH4 and EFNB2 expression in preeclampsia EPCs and control EPCs

NOTCH4 and EFNB2 expression was tested in EPCs using qRT-PCR and western blot assay. Compared with the controls, the mRNA level of *NOTCH4* increased notably in preeclampsia EPCs (Fig. 5A). Western blot assay showed the same trend for protein level with mRNA expression (Fig. 5B and G). The mRNA and protein levels of EFNB2 in preeclampsia EPCs were higher compared with healthy controls (Fig. 5C, D and H). As shown in

Figure 3 NOTCH4 negatively regulated EPC proliferation and differentiation. (A) Downregulation of NOTCH4 promoted EPC proliferation in shNOTCH4 group (1.03 ± 0.07 vs 0.71 ± 0.04 , $P < 0.01$). (B and C) Histogram showed the average number of spindle-shaped cells increased in shNOTCH4 group (63.22 ± 2.30 vs 36.33 ± 1.05 , $P < 0.001$). The percentage of differentiated cells increased in shNOTCH4 group (20.78 ± 1.16 vs 10.83 ± 0.58 , $P < 0.001$) compared with shNC group. (D) The number of spindle-shaped cells under a phase-contrast microscope represented EPCs differentiation ability. ** $P < 0.01$, *** $P < 0.001$; shNOTCH4 group (NOTCH4 silence); shNC group (negative control to shNOTCH4 group).

Fig. 3E and F, *NOTCH4* mRNA levels were moderately positively correlated with *EFNB2* mRNA ($R = 0.79$, $P < 0.05$), as well as protein level ($R = 0.64$, $P < 0.05$). These results once again indicated that NOTCH4 may act as an upstream of *EFNB2*.

Discussion

EPCs contribute to vascular remodeling and endothelial homeostasis, by their proliferation, migration to the place where vessel formatting, differentiation into mature endothelial cells and secretion of paracrine factors (Sipos *et al.* 2010, Burger & Touyz 2012). EPCs dysfunction may be involved in the pathogenesis of preeclampsia (Szpera-Gozdziewicz & Breborowicz 2014). If the molecular mechanisms that regulate the functions of EPCs can be elucidated, it may be able to discover feasible treatment to promote angiogenesis in patients with preeclampsia. In our recent studies, a number of investigations related to the DLL4/NOTCH-*EFNB2* signaling pathway were carried out in order to evaluate its role on EPCs in the pathophysiology of preeclampsia (Liu *et al.* 2015). Results showed that both DLL4/NOTCH signaling pathway and *EFNB2* inhibited the biological functions of EPCs. Additionally, the activation of the DLL4/NOTCH signaling pathway resulted in the upregulation of *EFNB2* expression. However, it remained unclear if NOTCH4 or NOTCH1 was the specific receptor for DLL4/NOTCH-*EFNB2*

Figure 4 The influence of NOTCH4 on EPC migration, adhesion, and HUVEC-tube formation *in vitro*. (A) The migration ability of EPCs was increased after inhibition of NOTCH4 compared to control (63.22 ± 2.30 vs 36.33 ± 1.05 , $P < 0.001$). (C) NOTCH4 promoted EPCs adhesion compared with shNC group (96.89 ± 2.19 vs 62.78 ± 2.56 , $P < 0.001$). (E) HUVECs cultured in the supernatant collected from shNOTCH4 group formed more branching points than shNC group (34.60 ± 2.68 vs 17.80 ± 2.35 , $P < 0.01$). (B, D and F) Representative images displayed EPC migration, adhesion, and HUVEC tube formation *in vitro*. ** $P < 0.01$; *** $P < 0.001$; shNOTCH4 group (Notch4 silence); shNC group (negative control to shNOTCH4 group).

cascade in preeclampsia. To explore this further, we have carried out a series of investigations from molecular and functional perspectives.

The first part of our research showed that the downregulation of NOTCH4 resulted in a decrease in the *EFNB2* level of EPCs, which indicated that *EFNB2* expression was dominated by NOTCH4 signaling. To further confirm DLL4/NOTCH4-*EFNB2* cascade, we used s-DLL4 to activate the DLL4/NOTCH signaling pathway in NOTCH4-downregulated EPCs and *EFNB2* expression remained low. This phenomenon further clarified that DLL4/NOTCH activation did not influence *EFNB2* expression when NOTCH4 was silenced. At this point, NOTCH4 is likely the upstream regulator of *EFNB2*. However, *EFNB2* increased slightly in NOTCH1-downregulated EPCs. DLL4 contacts with only two receptors, either NOTCH1 or NOTCH4, in a relatively balanced state (Shutter *et al.* 2000, Shawber *et al.* 2003). DLL4/NOTCH4, which enhanced *EFNB2* expression, was the dominant pathway in the case of the downregulation of NOTCH1. *EFNB2* expression increased when NOTCH1 downregulated EPCs were

Figure 5 NOTCH4 and EFNB2 expression in preeclampsia EPCs and control EPCs. (A and B) The mRNA (12.49 ± 4.63 vs 2.49 ± 0.46 , $P < 0.05$) and protein (3.15 ± 0.49 vs 1.08 ± 0.10 , $P < 0.05$) levels of NOTCH4 were higher in preeclampsia EPCs than in controls. (C and D) The mRNA (13.43 ± 3.18 vs 1.33 ± 0.30 , $P < 0.01$) and protein (2.47 ± 0.24 vs 1.06 ± 0.12 , $P < 0.01$) levels of EFNB2 were higher in preeclampsia EPCs than in healthy controls. (E and F) NOTCH4 mRNA levels were moderately positively correlated with EFNB2 mRNA ($R = 0.79$, $P < 0.05$), as well as protein level ($R = 0.64$, $P < 0.05$). (G and H) Representative western blotting showed NOTCH4 and EFNB2 protein expression in different groups. * $P < 0.05$; ** $P < 0.01$.

treated with s-DLL4, which also may be caused by more DLL4/NOTCH4 activation. These results confirmed that EFNB2 is not a direct target of DLL4/NOTCH1 signaling in EPCs. From genetic and molecular perspectives, these results implied that EFNB2 occurred downstream of NOTCH4 signaling in EPCs. The second part of our research was to test whether NOTCH4 inhibit EPC function, similar to EFNB2. To further explore it, we downregulated NOTCH4 and then detected changes in EPC activity. The results showed that NOTCH4 silence in EPCs stimulated migration, proliferation, and differentiation. In turn, this demonstrated that NOTCH4 acts as an inhibitor of the functions of EPCs, similar to EFNB2 (Liu *et al.* 2015). Additionally, we found that the supernatant, which was collected from NOTCH4-silenced EPCs, promoted the formation of HUVEC tube-like structures, which was also consistent with silenced EFNB2. Functional analyses confirmed that

EFNB2 may be a direct target of NOTCH4 signaling. Finally, we conducted additional investigations to verify the relationship between preeclampsia and NOTCH4–EFNB2. Results demonstrated that NOTCH4 expression, consistent with EFNB2 level, increased notably in preeclampsia EPCs compared with the normal group. Molecular analyses once again proved that NOTCH4 was a possible upstream pathway of EFNB2.

Previous research has demonstrated that the DLL4/NOTCH4 signaling pathway may be positioned to function as an inhibitor of angiogenesis (Leong *et al.* 2002, Williams *et al.* 2006, Lobov *et al.* 2007, Steven Suchting 2007, Li *et al.* 2010). These evidences were consistent with our results, which showed that the inhibition of DLL4/NOTCH4–EFNB2 promoted the functions of EPCs. All of these can be explained as that inhibited NOTCH4 led to repression of $\beta 1$ -integrin, which stimulated VEGF-induced angiogenesis (Leong *et al.* 2002). Moreover, knockdown of NOTCH4 resulted in decreased DLL4/NOTCH4 signaling activation, which upregulated endothelial cell expression of Neuropilin-1 (Williams *et al.* 2006). Neuropilin-1 functioned as a co-receptor for the VEGF-A family, thus strengthening VEGFR2 activity and angiogenesis (Soker *et al.* 1998). In addition, NOTCH4 controlled the expression of EFNB2 in EPCs. EFNB2 suppressed angiogenesis by inhibiting VEGF-induced endothelium proliferation, sprouting and migration (Kim *et al.* 2002), and restraining VEGF2 and VEGF3 secretion (Wang *et al.* 2010).

EPHRINB2 was shown to suppress the function of venous endothelial cell (Kim *et al.* 2002) and EPCs (Liu *et al.* 2015). However, other evidence has demonstrated that EFNB2 promotes the growth, survival, migration, assembly, and angiogenesis of human umbilical vein endothelial cells (Salvucci & Tosato 2012), apparently conflicting with our observations on EPCs. These contrasting results may be due to the different styles of endothelial cells, and the complexity of ‘forward’ and ‘reverse’ signaling in EPH receptor/EPHRIN family (Zhang & Hughes 2006). Furthermore, EPCs comprise a phenotypically heterogeneous cell population and individual sub-populations of EPCs may function differently during angiogenesis and vascular remodeling (Hur *et al.* 2004). Besides, EFNB2 may influence endothelium proliferation, sprouting, and migration through phosphatidylinositol-3-kinase (PI3K) and SRC and mitogen-activated protein kinase (MAPK) pathways (Maekawa *et al.* 2003, Steinle *et al.* 2003). Further research is required to discover more fundamental mechanism of DLL4/NOTCH4–EFNB2 cascade that affects the functions of EPCs.

In conclusion, we demonstrated that NOTCH4, not NOTCH1, was the specific receptor for DLL4/NOTCH–EFNB2 cascade. Blockade of the DLL4/NOTCH4–EFNB2 cascade to enhance angiogenic property of EPCs might act as a therapeutic tool for improving angiogenesis in preeclampsia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Natural Science Foundation of China (No. 81170584 and 81370733 to Li Zou).

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Received 12 March 2016

First decision 5 April 2016

Revised manuscript received 6 April 2016

Accepted 8 April 2016