Effect of the Notch4/Dll4 signaling pathway in early gestational intrauterine infection on lung development

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Abstract. Intrauterine infection is an important risk factor for bronchopulmonary dysplasia (BPD). BPD is characterized by arrested lung alveolarization and impaired pulmonary vascularization. The Notch4 signaling pathway is a key regulator of vascular remodeling and angiogenesis. Therefore, the presents study investigated the expression of Notch4, delta-like canonical Notch ligand 4 (Dll4) and related factors in an *in vivo* rat model and in rat pulmonary microvascular endothelial cells (PMVECs) *in vitro*, to study the mechanisms by which intrauterine infection affects rat lung development. A rat model of intrauterine infection was established by endocervical inoculation with *Escherichia scoli* on embryonic day 15. The date of birth was counted as postnatal day 0 (P0). Then, the lung tissues were collected from pups at days P3-P14. The expression of Notch4, Dll4 and related factors was measured by reverse transcription-quantitative PCR and western blotting. In addition, the γ-secretase inhibitor DAPT was used to examine the effect of Notch4 signaling on PMVECs. Intrauterine *E. coli* infection impaired normal lung development, as indicated by decreased microvessel density, fewer alveoli, fewer secondary septa, and larger alveoli compared with the control group. Furthermore, Notch4, Dll4 and NF-κB levels were significantly increased in the *E. coli*-infected group at P3 compared with the control group. Similarly, the mRNA expression levels of fetal liver kinase 1 (Flk-1, a VEGF receptor) were significantly increased in the *E. coli*-infected group at P3 and P7. In PMVECs, the inhibition of Notch4 signaling contributed to decreases in lipopolysaccharide (LPS)-induced expression of VEGF and its receptors. Furthermore, the inhibition of Notch4/Dll4 signaling accelerated cell proliferation and decreased the apoptosis rate of LPS-induced PMVECs. LPS-induced NF-κB expression in PMVECs was also attenuated by the Notch4/Dll4 inhibitor. In conclusion, intrauterine *E. coli* infection impaired normal lung development, possibly through Notch4/Dll4 signaling and effects on VEGF and its receptors.

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Key words: Notch4, delta-like canonical Notch ligand 4, intrauterine infection, bronchopulmonary dysplasia, VEGF, angiogenesis

Introduction

Bronchopulmonary dysplasia (BPD) is one of the most common complications in extremely preterm infants and is associated with many factors, including mechanical ventilation, hyperoxia, genetic predisposition, infections and inflammation (1). New BPD manifests as impaired lung alveolarization and aberrant microvasculature (2). Evidence indicates that angiogenesis is crucial for lung development, while alveolarization impairment due to disrupted angiogenesis is critical to the development of BPD (3-5). A great deal of clinical and animal research has shown that there is a relationship between infection/inflammation and BPD (6-8). BPD was increased in preterm infants who had a history of chorioamnionitis (6). In animal models, intra-amniotic lipopolysaccharide (LPS) administration impaired the growth of alveoli and vessels (7,8). Although increasing evidence shows that intrauterine inflammation can impair alveolarization, the exact mechanism remains unclear, and no effective therapies are currently available to prevent BPD.

Notch signaling is an evolutionarily conserved intercellular signaling mechanism that has a major role in regulating cell fate in mammals and other vertebrates. Notch signaling is activated when Notch ligands bind to Notch receptors that are expressed on the surface of the adjacent cell. In mammals, there are five Notch ligands [jagged canonical Notch ligand (Jag) 1, Jag2, delta-like canonical Notch ligand (Dll) 1, Dll3 and Dll4] and four Notch receptors (Notch1-4) (9,10). Among these Notch receptors and ligands, Notch4 and its ligand Dll4 are primarily expressed in the vascular endothelium. Notch4 has been reported to be associated with arteriovenous malformations. Notch4 or Dll4 overexpression in the vasculature results in defective embryonic angiogenesis, while Dll4 or Notch receptor deficiency leads to embryonic lethality with profound vascular defects (11). These results suggest that balanced levels of Notch4/Dll4 signaling are necessary for normal vascular development. However, the role of Notch4/Dll4 signaling during vascular remodeling in BPD has not been well studied.

The current study focused on the expression of Notch4, Dll4 and related factors *in vivo*, in a rat model, and *in vitro*,

in rat pulmonary microvascular endothelial cells (PMVECs), to verify the effect of Notch4/Dll4 signaling in intrauterine infection on lung development.

Materials and methods

Animal model. Twenty pregnant Sprague-Dawley rats were purchased from the Laboratory Animal Center of Zhejiang Academy of Medicine in China and randomized into two groups: The *Escherichia coli*-infected group (n=10) and the control group (n=10). All animals were housed under standard laboratory conditions at 22±2˚C with a constant 12-h light/dark cycle. On embryonic day E15, pregnant rats were anesthetized with 2% sodium pentobarbital (40 mg/kg, intraperitoneal injection). Then, 0.4 ml of either an *E. coli* suspension (2.5-4x10^8 colony-forming units per ml) or saline was injected into the uterine cervix of the rats (12,13). All animal procedures and protocols were reviewed and approved by the Animal Care Committee of Zhejiang University.

All pregnant rats were allowed to give birth naturally. They were checked daily to confirm whether they gave birth, and the day of birth was considered as postnatal day 0 (P0) for the pups. Most of the rats were delivered at 21-22 days. Furthermore, there was no significant difference between the control group and the *E. coli*-treated group regarding the duration of pregnancy and day of delivery. The period of the human or rat lung development has been divided into five stages: Embryonic stage, pseudoglandular stage, canalicular stage, saccular stage and alveolar stage. BPD is mainly observed at the saccular stage. One pup per litter from each group was sacrificed at P3 (saccular stage), P7 and P14 (alveolar stage). The rats were anesthetized with 2% sodium pentobarbital (40 mg/kg, intraperitoneal injection). These rats were euthanized by exsanguination via excision of the inferior vena cava before the lung tissues were collected. Thus, lung tissue samples from P3 to P14 were harvested in both groups (n=10 per time point). The right upper lobe was fixed for 24 h at room temperature in 10% neutral formaldehyde for histopathological examination. Other specimens were frozen in liquid nitrogen and stored at -80˚C for further analysis. Intrauterine infection was confirmed by the presence of inflammation in histological sections of the uterus and placenta, as described in a previous study (12).

Histology and immunohistochemistry. Lung samples were fixed overnight in 10% formalin at a pressure of 20 cm H_2O. Then, the tissues were dehydrated in graded ethanol and embedded in paraffin. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin (H&E). Radial alveolar counts (RACs) were measured by drawing a perpendicular line from the respiratory bronchiole to either the pleura or the nearest connective tissue septum, and the number of alveoli transected by this line was counted. Three sections per time point were randomly selected for analysis. Five fields from each section were used to perform morphometric analysis and RACs using a light microscope (magnification, x100).

Furthermore, the sections were used for immunohistochemical (IHC) staining. Briefly, tissue sections were subjected to antigen retrieval using citrate buffer (pH=6.0). Hydrogen peroxide (3%) was used for 10 min at room temperature for blocking of endogenous peroxidases. The sections were then incubated with primary antibodies targeting CD34 (1:2,500; cat. no. ab81289; Abcam) overnight at 4˚C, followed by incubation with secondary antibodies (HRP-conjugated goat anti-rabbit IgG; 1:200; cat. no. ab6721; Abcam) for 1 h at room temperature. The IHC reaction color was visualized with 3,3 diaminobenzidine (Sigma-Aldrich; Merck KGaA) as the substrate using a light microscope (magnification, x200). Image-Pro Plus version 6.0 software (Media Cybernetics, Inc.) was used for analysis.

Cell culture. Rat PMVECs were obtained from Qingqi (Shanghai) Biotechnology Development Co., Ltd. and were incubated in serum-free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h. PMVECs were pretreated with 10 μmol/l DAPT (cat. no. ab120633; Abcam) or equal volume of vehicle PBS for 24 h according to previous studies (14,15). Subsequently, the cells were stimulated with or without 10 μg/ml LPS in serum-free DMEM for 48 h, resulting in the following three experimental groups: Control group, PMVECs treated with PBS vehicle control only; LPS group, PMVECs treated with LPS only; and DAPT+LPS group, PMVECs treated with DAPT and LPS (16-18).

MTT assay. Cell proliferation and viability were measured with an MTT assay. Briefly, exponentially growing cells (4x10^4/well) were seeded in a 96-well plate and cultured for 72 h. For each group, the wells were randomly sampled every 24 h, and MTT solution (5 mg/ml, 20 μl/well; Sigma-Aldrich; Merck KGaA) was added to the selected wells. After the wells were incubated for an additional 2 h at 37˚C, 200 μl of DMSO was added to the wells, and the cell viabilities were determined by measuring the optical density values at 490 nm with a microplate reader (ELX-800; BioTek Instruments, Inc.).

Apoptosis assay. The effect of Notch4/Dll4 signaling on PMVEC apoptosis was evaluated by Annexin V staining. PMVECs were collected, washed 3 times with PBS buffer and suspended in binding buffer at a density of 10^6 cells/ml. Then, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide using an Annexin V FITC apoptosis detection kit (JingMei Biotechnology Co., Ltd.), according to the manufacturer's protocol. Apoptotic cells were subsequently analyzed by flow cytometry (LSRFortessa SORP; BD Biosciences) and FlowJo v7.6 software (FlowJo LLC).

RNA preparation and reverse transcription-quantitative PCR. Total RNA was extracted from small pieces of frozen lung tissue or from cells by using a RNeasy Mini kit (Qiagen GmbH). The RNA was then reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Invitrogen; Thermo Fisher Scientific, Inc.), which was used for quantitative PCR on an ABI PRISM 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.) as previously described (12). A SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.) was used for the qPCR analysis using the $2^{-\Delta\Delta Cq}$ method (19). The primers (Table I) were designed using Primer Express 3.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and synthesized by Takara Biotechnology Co., Ltd. GAPDH was used as an endogenous control gene to normalize the amount

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Primer	Sequence (5'-3')	PCR product length (bp)
Dll4	Forward	AGAGGAGAAGGAGGAGGAATG	130
	Reverse	CCCTTGACTCTTCCCTTGATG	
Notch4	Forward	ATTCCCAGTGCTGGCTTCTC	177
	Reverse	GAGCGTTGTTGCAGCCTTTC	
NF-κB	Forward	AGCTGCTATTGGATTACAC	109
	Reverse	AGATGGCTAGAAAGAACAC	
Flk-1	Forward	TTACTGTCCAGCCTGCTAC	173
	Reverse	CCAAAGAGCGTCCAAGTTC	
Flt-1	Forward	ATAGCGTGGGACAGTAGG	164
	Reverse	CTCGGTGGGCTTATTTGG	
VEGF	Forward	GAGTCTGTGCTCTGGGATTTG	188
	Reverse	TCCTGCTACCTCTTTCCTCTG	
GAPDH	Forward	GTCGGTGTGAACGGATTTG	181
	Reverse	TCCCATTCTCAGCCTTGAC	

Dll4, delta-like canonical Notch ligand 4; Flk-1, fetal liver kinase 1; Flt-1, FMS-like tyrosine kinase 1.

of cDNA. The amplification procedure was performed in two steps: Initial denaturation at 95˚C for 3 min and 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec.

Western blot analysis. Total protein was extracted from frozen lung tissue samples (40 mg) or cells using RIPA buffer (Abcam). Equal amounts of protein determined using the BCA method (50 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting. Then, the membranes were blocked with 5% nonfat dried milk for 2 h at room temperature and incubated with primary antibodies overnight at 4˚C. The following primary antibodies were used: Anti-Dll4 (1:1,000; cat. no. ab183532; Abcam), anti-Notch4 (1:1,000; cat. no. ab184742; Abcam), anti-VEGF (1:1,000; cat. no. ab72807; Abcam), anti-fetal liver kinase 1 (Flk-1; 1:2,000; cat. no. ab221679; Abcam), anti-FMS-like tyrosine kinase 1 (Flt-1; 1:2,000; cat. no. ab184784; Abcam), anti-NF-κB (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.) and anti-GAPDH (1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.). The next day, the membranes were washed with TBST (containing 0.05% Tween 20 in TBS buffer) three times for 10 min, followed by incubation with anti-rabbit (cat. no. ab6721), anti-mouse (cat. no. ab6728) and anti-goat (cat. no. ab6741) HRP-conjugated secondary antibodies (all 1:5,000; all from Abcam) for 2 h at room temperature. Finally, the proteins were detected by chemiluminescence (ECL Plus Detection kit; Beyotime Institute of Biotechnology). Then, the membranes were exposed to X-ray film to visualize the protein bands. Protein bands were scanned and quantified using a computer-based image analysis system (Gel-Pro analyzer software 4.0; Media Cybernetics, Inc.). GAPDH was used as the endogenous control.

Statistical analysis. All data are presented as the mean ± standard deviation (SD). A t-test was used to measure the statistical significance of the difference between the means of two groups (*E. coli*-infected group and control group). The differences among multiple groups of samples (PMVEC treatment groups) were evaluated using analysis of variance with Tukey's post hoc test. Data analyses were performed using the SPSS software package version 18 for Windows (SPSS Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Intrauterine infection impairs lung development. During the present study, no dams died after intrauterine *E. coli* administration. There were few intrauterine fetal deaths and pregnancy losses in the two groups. Ninety-eight live pups from the control group and ninety-four live pups from the *E. coli*-infected group were eligible for this study (Table II). Compared with that of pups in the control group (Fig. 1A), the lung morphology of the pups in the *E. coli*-infected group displayed fewer alveolar numbers, larger alveoli, fewer secondary septa and thickened alveolar septa (Fig. 1B). Furthermore, a higher microvessel density (CD34 expression in lung tissue) was observed in the control group (Fig. 1C) compared with the *E. coli*-infected group (Fig. 1D). There was no marked difference in the histological evidence of alveolar structure destruction or fibrosis between the two groups. The RAC, as an important index of lung development, increased gradually from P3 to P14. Notably, the RAC of the control group at P7 and P14 was significantly higher compared with that of the *E. coli*-infected group (both $P<0.05$; Fig. 1E).

Notch4, Dll4, NF-κB, VEGF, Flt-1 and Flk-1 are aberrantly expressed in the rat lung after intrauterine infection. To investigate whether intrauterine infection altered the expression of Notch4, Dll4, NF-κB, VEGF, Flt-1 (also known as VEGFR1) and Flk-1 (also known as VEGFR2) in the rat lung, PCR and western blot analysis were performed. Compared with those

Table II. Induction of pregnancy losses by intrauterine infection.

Time points	Litters/fetuses	*Escherichia coli*-treated group (n=10)	Control group (n=10)
P3,7,14	Pregnant rats/fetuses	10/94	10/98
	Stillborn litters	1	0
	Stillborn pups	5	3

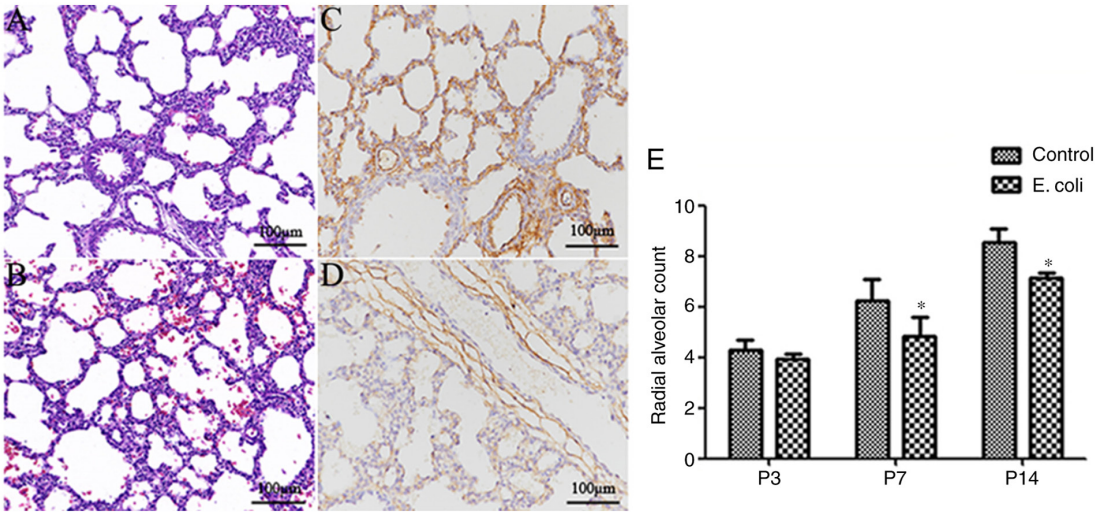

Figure 1. Lung morphology, immunohistochemistry and the radial alveolar counts of both groups. (A) Representative images of H&E-stained lung sections from control pups at P7 and (B) from *E. coli*-infected pups at P7. (C) Representative images of CD34 immunohistochemistry staining (a blood vessel marker) of lung tissues from control pups at P7 and (D) from *E. coli*-infected pups at P7. Magnification, x200. Scale bar, 100 μm. (E) Radial alveolar counts in the control and *E. coli*-infected groups on P3, P7, P14. Data are presented as the mean ± SD (n=10 per group). *P<0.05. P, postnatal day.

of the control group, the mRNA expression levels of Notch4 (Fig. 2A) and Dll4 (Fig. 2B) after intrauterine infection were significantly upregulated at P3 (P<0.05). A similar trend in Notch4 and Dll4 protein expression was observed by western blotting (Fig. 2G). However, there was no significant difference in expression between the two groups at the other time points. There was a significant difference in the Flk-1 mRNA expression levels at P3 and P7 between the two groups (both P<0.05; Fig. 2E), while the mRNA expression levels of Flt-1 were significantly increased at P14 compared with those of the control group (P<0.05; Fig. 2F). Additionally, the results demonstrated that the protein and mRNA expression levels of NF-κB were significantly increased in the *E. coli*-infected group at P3 compared with the control group (P<0.05; Fig. 2C and G). The protein expression levels of Flk-1 in lung tissues from the *E. coli*-infected group were significantly higher compared with those of the control group at P3 (Fig. 2H); however, there was no difference in VEGF or Flt-1 protein expression levels between the two groups at P3 (Fig. 2H).

LPS induces the expression of Notch4, Dll4, VEGF, Flt-1 and Flk-1 in PMVECs. PMVECs were exposed to different concentrations of LPS to determine the optimal concentration for further experiments. The dose of 10 μg/ml LPS was selected because it induced the greatest mRNA upregulation of Notch4 and Dll4 (Fig. 3A). Subsequently, RT-qPCR analysis revealed that the mRNA expression levels of Notch4 and Dll4 in the LPS-treated PMVEC group were significantly increased compared with those of control cells (both P<0.05; Fig. 3B and C). The Notch4 and Dll4 mRNA levels in the LPS group were increased by 3.3 and 4.6-fold, respectively. In addition, the mRNA expression levels of VEGF and its receptors Flt-1 and Flk-1 in PMVECs showed a similar increase as that of Notch4/Dll4 (all P<0.05; Fig. 3D-F). Compared with those of the control group, the mRNA expression levels of VEGF, Flt-1 and Flk-1 in the LPS group were elevated by 6.5, 6.7 and 5.3-fold, respectively. Similar results were also observed for the protein levels by western blot analysis (Fig. 3G). The present findings suggest that LPS treatment can induce the expression of Notch4, Dll4, VEGF, Flt-1 and Flk-1 in PMVECs.

Notch4/Dll4 inhibition reverses the LPS-induced upregulation of VEGF, Flt-1 and Flk-1 in PMVECs. To determine whether the Notch4/Dll4 signaling pathway is involved in the LPS-induced upregulation of angiogenic markers in PMVECs, the present study determined the levels of VEGF, Flt-1 and Flk-1 following inhibition of Notch4/Dll4 signaling. Notch4/Dll4 inhibition by DAPT pretreatment suppressed LPS-induced VEGF, Flt-1 and Flk-1 mRNA expression by 73, 78 and 70%, respectively, compared with that of the LPS group (Fig. 3D-F). Western blotting also confirmed that VEGF, Flt-1 and Flk-1 protein expression in the DAPT+LPS group was significantly decreased compared with that in the LPS group (P<0.05; Fig. 3G). These data demonstrate that the Notch4/Dll4 pathway may regulate LPS-induced VEGF, Flt-1 and Flk-1 expression in PMVECs.

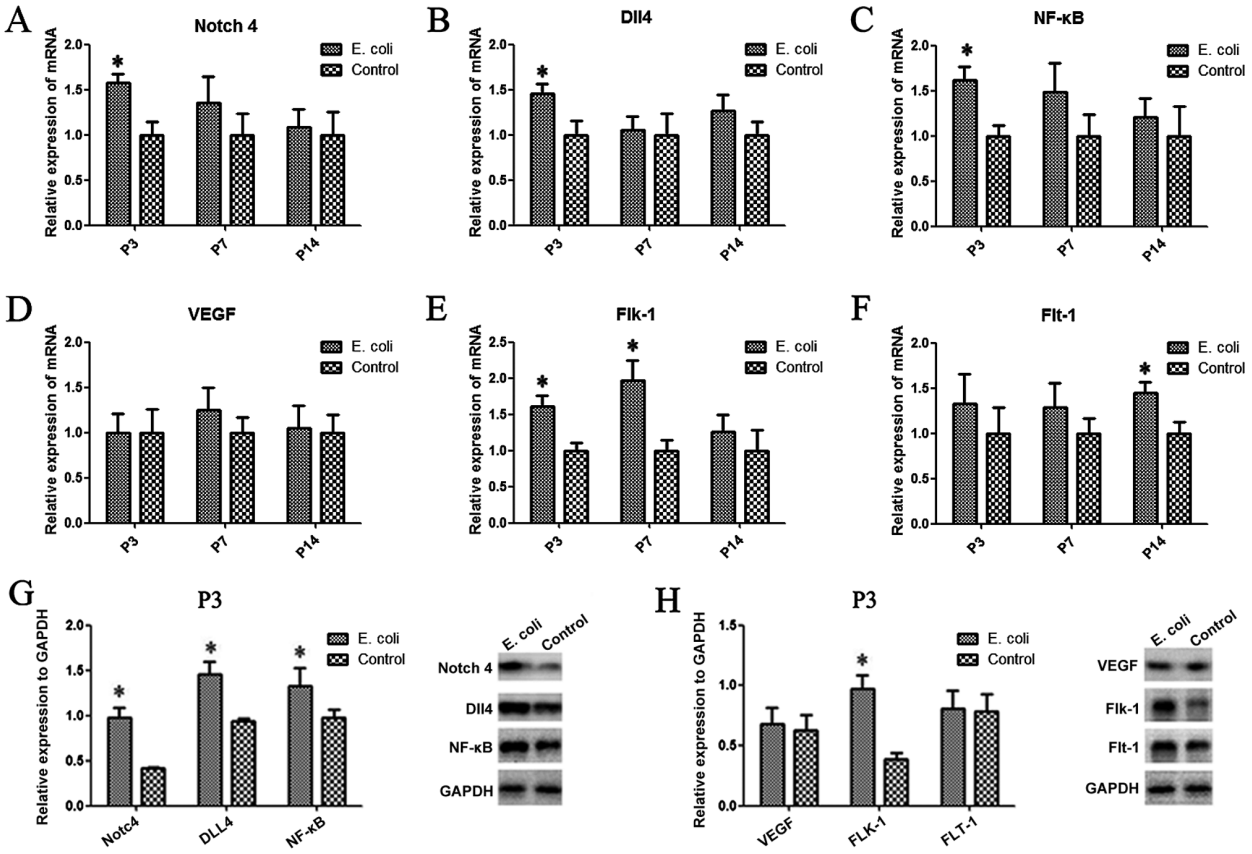

Figure 2. Aberrant expression of Notch4, Dll4, NF-κB, VEGF, Flt-1 and Flk-1 in the rat lung after intrauterine infection. (A) The mRNA expression levels of Notch4, (B) Dll4, (C) NF-κB, (D) VEGF, (E) Flk-1 and (F) Flt-1 were evaluated in the two groups by reverse transcription-quantitative PCR. (G) The protein expression levels of Notch4, Dll4 and NF-κB, and (H) VEGF, Flt-1 and Flk-1 were determined in the two groups at P3 by western blotting. Data are presented as the mean ± SD (n=10 per group). *P<0.05. Dll4, delta-like canonical Notch ligand 4; Flt-1, FMS-like tyrosine kinase 1; Flk-1, fetal liver kinase 1; P, postnatal day.

LPS-induced NF-κB expression is reversed by inhibiting Notch4/Dll4 in PMVECs. The promoters of VEGF, Flt-1 and Flk-1 contain binding sites for the transcription factor NF-κB. To investigate the role of Notch4/Dll4 in the regulation of angiogenic gene expression in PMVECs, the present study determined the expression levels of NF-κB following LPS treatment. The protein expression levels of NF-κB in the LPS group were significantly increased compared with those in the control group (P<0.05; Fig. 4A). Of note, this upregulation was significantly suppressed by DAPT treatment (Fig. 4A). Additionally, LPS induced a 5.9-fold increase in NF-κB mRNA levels compared with those of the control cells (Fig. 4B), while these were decreased by 67% following DAPT treatment (Fig. 4B). These data suggest that the effect of LPS on NF-κB expression in PMVECs may be modulated by Notch4/Dll4 signaling.

Notch4/Dll4 inhibition increases cell proliferation and decreases the apoptosis rate of LPS-stimulated PMVECs. Compared with that of the control group, LPS suppressed PMVEC proliferation (Fig. 4C). In specific, as the culture time increased, the numbers of viable cells were gradually decreased in the LPS group compared with the control group. Pretreatment with DAPT attenuated this suppression compared with the LPS alone group. The cell viability in the DAPT+LPS group was higher compared with that in the LPS group at each time point, and the differences between the two groups were significant (P<0.05; Fig. 4C).

Next, flow cytometry analysis revealed that the inhibition of Notch4/Dll4 signaling abrogated the LPS-induced apoptosis in PMVECs. The average apoptotic rate of PMVECs in the LPS group (exposed to LPS for 48 h; 49.5% apoptotic cells) was significantly higher compared with that of cells in the control group (4.2% apoptotic cells; P<0.05). In the DAPT+LPS group, the average rate of apoptosis was 20%, which was significantly lower compared with that of the LPS group (P<0.05; Fig. 4D). These results indicate a key role of Notch4/Dll4 signaling in PMVEC proliferation and apoptosis.

Discussion

The present study revealed that intrauterine *E. coli* infection impaired alveolarization and vascular development in the rat lung, which was marked by decreased microvessel density, fewer alveoli, fewer secondary septa, and larger alveoli compared with the control group. Notch4 and Dll4 expression was significantly increased in the *E. coli*-infected group at P3 compared with the control group. Furthermore, the inhibition of Notch4 signaling in PMVECs contributed to decreases in LPS-induced VEGF, Flt-1, Flk-1 and NF-κB expression. The inhibition of Notch4/Dll4 signaling accelerated cell proliferation and decreased the apoptosis rate of LPS-stimulated PMVECs.

Numerous studies have demonstrated the relationship between intrauterine infection/inflammation and BPD. It has

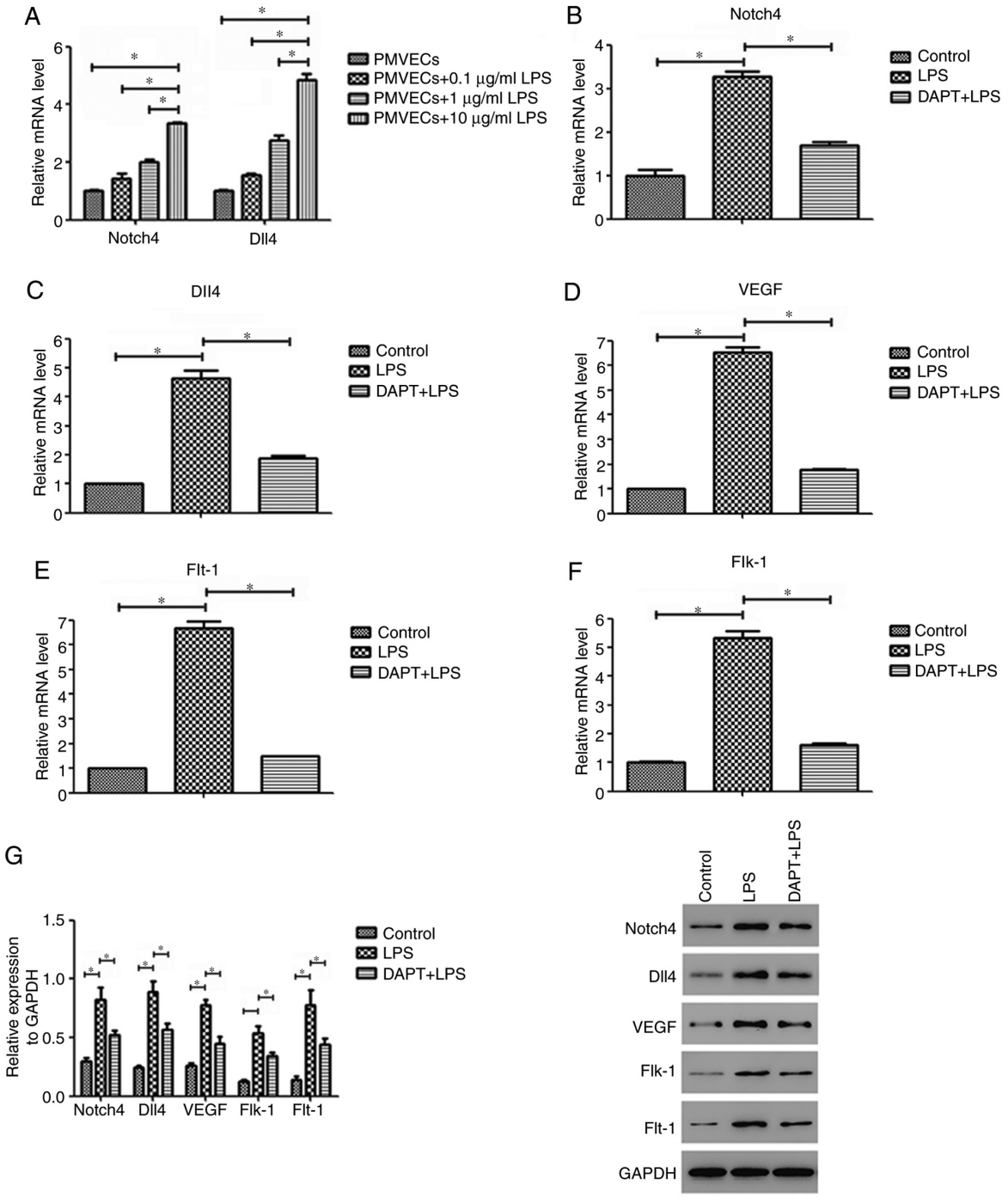

Figure 3. Inhibition of Notch4/Dll4 signaling decreases the expression of VEGF and its receptors in LPS-induced PMVECs. (A) The mRNA expression levels of Notch4 and Dll4 were determined by reverse transcription-quantitative PCR in PMVECs that were exposed to different concentrations of LPS. (B) Notch4, (C) Dll4, (D) VEGF, (E) Flt-1 and (F) Flk-1 mRNA levels were determined in PMVECs in the control, LPS and DAPT+LPS groups. (G) Western blot analysis of protein expression levels in the PMVEC treatment groups. Data are presented as the mean ± SD (n=6 per group). *P<0.05 with comparisons shown by lines. Dll4, delta-like canonical Notch ligand 4; PMVECs, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; Flt-1, FMS-like tyrosine kinase 1; Flk-1, fetal liver kinase 1.

become increasingly evident that intrauterine infection/inflammation impairs pulmonary vascular development in premature infants (6,20). The pulmonary vasculature serves an essential role in normal lung development. Dysregulated angiogenesis during fetal growth may impair alveolarization and contribute to the development of BPD. In the present study, intrauterine infection in a rat model resulted in the arrest of pulmonary alveolarization similar to that in human BPD, and impairments in normal lung vascular development were marked by reduced microvessel density in the lung.

However, the molecular mechanisms of vascular development in BPD remain unclear. Notch receptors and their ligands have important roles in vascular development. The combined loss of Notch4 and Notch1 in mice results in defects in vascular remodeling (21). Among the Notch family members, Notch4 and Dll4 are primarily expressed in vascular endothelial cells.

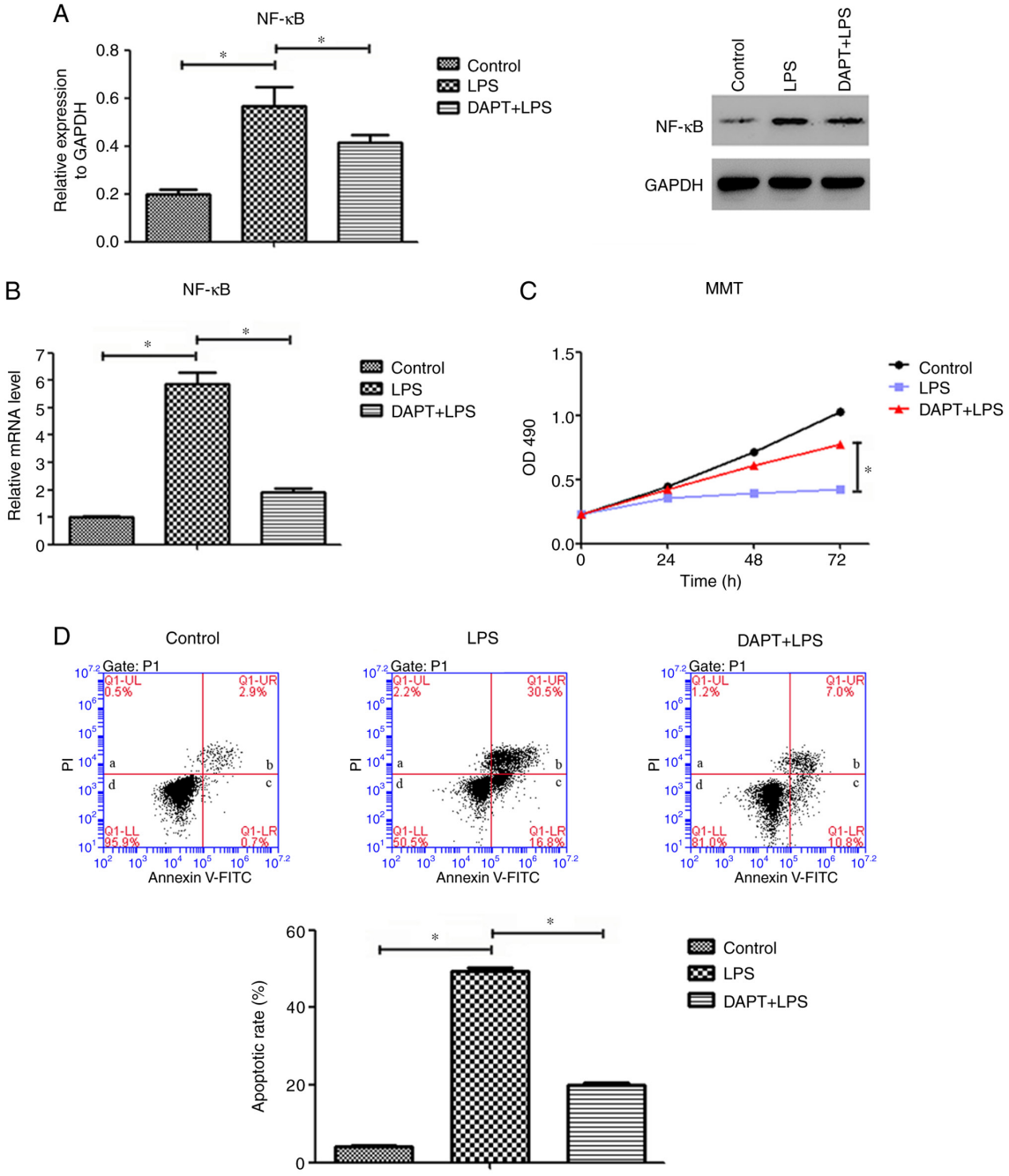

Figure 4. Inhibition of Notch4/Dll4 signaling decreased the expression of NF-κB, inhibited cell apoptosis and promoted cell proliferation in PMVECs. (A) Protein and (B) mRNA expression levels of NF-κB in PMVECs in the control, LPS and DAPT+LPS groups. (C) Cell viability in the control, LPS and DAPT+LPS groups was measured using an MTT assay. (D) Representative plots and quantitative flow cytometric analysis of the apoptotic rates of PMVECs in the control, LPS and DAPT+LPS groups. In the plots, quadrant a shows the proportion of dead/necrotic cells (Annexin V-FITC$^-$/PI$^+$), quadrant b shows the late apoptotic cells (Annexin V-FITC$^+$/PI$^+$), quadrant c shows the early apoptotic cells (Annexin V-FITC$^+$/PI$^-$) and quadrant d shows the live cells (Annexin V-FITC$^-$/PI$^-$). The sum of quadrants b and c was calculated as the apoptotic rate. Data are presented as the mean ± SD (n=6 per group). *P<0.05 with comparisons shown by lines. Dll4, delta-like canonical Notch ligand 4; PMVECs, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; PI, propidium iodide.

The overexpression of activated Notch4 results in vascular patterning defects (22), while Dll4 haploinsufficiency induces major defects in vascular development, resulting in embryonic lethality (23,24). Qiao *et al* (25) also showed that inhibiting Notch signaling with a γ-secretase inhibitor protected against angiotensin II-induced pulmonary vascular remodeling. These findings emphasize the critical role of Notch4/Dll4 in vascular development. Notably, the present results demonstrated that Notch4 and Dll4 expression was significantly increased in the *E. coli*-infected group at P3. *In vitro*, Notch4/Dll4 mRNA expression levels in LPS-stimulated PMVECs were also significantly higher compared with those in the controls. These data reveal that Notch4/Dll4 signaling may be critically involved in intrauterine infection-induced impairments in lung development.

VEGF and VEGFR have key roles in stimulating angiogenesis and vasculogenesis (26,27). The appropriate expression of VEGF not only contributes to the formation of capillaries but

also controls alveolar and bronchial growth (28). Changes in VEGF and its receptors result in angiogenesis disorders, which contribute to detrimental remodeling of small arteries (7). In the current study, there was a significant difference in the Flk-1 mRNA levels at P3 and P7 between the two groups, while the mRNA expression levels of Flt-1 were significantly increased at P14 compared with those of the control group. The present data demonstrated that intrauterine infection disturbed VEGF signaling. Furthermore, inhibiting Notch4/Dll4 signaling decreased apoptosis, promoted cell proliferation, and decreased the expression of VEGF and its receptors Flk-1 and Flt-1 in LPS-induced PMVECs. These results indicate that Notch4/Dll4 and VEGF signaling may be associated with intrauterine infection-induced BPD.

Currently, the role of Notch4/Dll4 signaling in the process of BPD is not well understood. To explore the molecular mechanisms underlying Notch4-mediated regulation of lung development, the present study evaluated the expression of NF-κB. As a pivotal regulator of inflammation, the NF-κB pathway has been shown to promote lung injury in many models (29). In addition, it was observed that the activation of NF-κB promoted alveolarization and pulmonary angiogenesis (29). Alvira (30) suggested that NF-κB might play an essential, unique and beneficial role in lung development. In the present study, LPS induced a large increase in NF-κB expression in PMVECs compared with that of the control cells. Furthermore, the inhibition of Notch4 signaling by a γ-secretase inhibitor significantly decreased NF-κB expression in PMVECs compared with that of the LPS group. Taken together, these results demonstrated that Notch4/Dll4 signaling may have an important role in the development of BPD induced by intrauterine infection, possibly through the NF-κB pathway and downstream target genes, such as VEGF, Flk-1 and Flt-1. However, the present study has certain limitations, such as the lack of Notch4 knockout or overexpression experiments, which would further clarify the effect of Notch4/Dll4 on lung development.

In conclusion, the current study demonstrated that early gestational intrauterine infection arrested alveolarization and vascular development in the lung and disrupted Notch4/Dll4 signaling. Furthermore, Notch4/Dll4 signaling may have an important role in lung development by regulating the expression of VEGF, Flk-1 and Flt-1. Thus, targeting the Notch4/Dll4 pathway might provide a novel therapeutic strategy for the treatment of BPD in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CZ and LC conceived and designed the study, collected and analyzed the data and wrote the manuscript. CZ, YS and JP performed experiments. YS performed the data analysis and interpretation. TY designed the study, analyzed the data and supervised the entire project. CZ and YS confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures and protocols were reviewed and approved by the Animal Care Committee of Zhejiang University (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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