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Expression and function of Delta-like ligand 4 in a rat model of retinopathy of prematurity☆

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Abstract

The Delta-like ligand 4/Notch signaling pathway was shown to participate in the process of retinal development and angiogenesis. However, the function of the Delta-like ligand 4/Notch signaling pathway in retinopathy of prematurity requires further study. Retinopathy of prematurity was induced in 5-day-old Sprague-Dawley rats exposed to hyperoxia for 7 days, and then returned to room air. Reverse transcription-PCR and western blot revealed that Delta-like ligand 4 levels decreased at postnatal day 12 and increased at postnatal day 17 in retinopathy of prematurity rats. Flat-mounted adenosine diphosphatase stained retina and hematoxylin-eosin stained retinal tissue slices showed that the clock hour scores and the nuclei counts in retinopathy of prematurity rats were significantly different compared to normal control rats. After retinopathy of prematurity rats were intravitreally injected with Delta-like ligand 4 monoclonal antibody to inhibit the Delta-like ligand 4/Notch signaling pathway, there was a significant increase in the severity of retinal neovascularization (clock hours) in the intravitreally injected eyes. The nuclei count was highly correlated with the clock hour score. These results suggest that Delta-like ligand 4/Notch signaling plays an essential role in the process of physiological and pathological angiogenesis in the retina.

Key Words

neural regeneration; peripheral nerve injury; Delta-like ligand 4; retinopathy of prematurity; retinal neovascularization; vascular endothelial cells; vascular endothelial growth factor; Notch signaling pathway; oxygen-induced retinopathy; optic nerve disease; photographs-containing paper; neuroregeneration

Research Highlights

(1) Delta-like ligand 4 expression was observed in a rat model of retinopathy of prematurity.
(2) Adenosine diphosphatase was observed histochemically in all blood vessels, including new vessels. Tissue morphology was maintained for 1 to 2 weeks. This method was suitable to investigate pathogenesis of neovascularization.
(3) Hematoxylin-eosin staining, and histochemical staining of adenosine diphosphatase could detect the severity of retinal neovascularization.
(4) Intravitreal injection of Delta-like ligand 4 monoclonal antibody inhibited the Delta-like ligand 4/Notch signaling pathway and produced pathological changes in the retina of rat models.
(5) Results indicate that Delta-like ligand 4/Notch signaling plays an essential role in angiogenesis in the retina.

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INTRODUCTION

Retinopathy of prematurity, previously called retrolental fibroplasia, is a vasoproliferative disorder of the developing retina that accounts for blindness in over 70 000 children worldwide[1]. The major risk factors for retinopathy of prematurity are decreased gestational age and decreased birth weight[2]. The natural course of retinopathy of prematurity leads to blindness. Irreversibly impaired vision may also hinder cognitive and psychomotor development of the affected children[3]. The underlying mechanism of retinopathy of prematurity is multifactorial and complex. Retinal neovascularization is a major pathological form of retinopathy of prematurity in which hypoxia is thought to play an important role[4].

Previous studies have demonstrated that the Delta-like ligand 4/Notch signaling pathway and vascular endothelial growth factor are critical mediators of neovascularization under hypoxic conditions[5]. Delta-like ligand 4 is an endothelium specific Notch ligand which functions as a regulating factor during physiological and pathological angiogenesis[6]. Haploinsufficiency of Delta-like ligand 4 results in embryonic lethality due to severe vascular defects in mice[7], a phenotype previously only reported for vascular endothelial growth factor haploinsufficiency in angiogenic pathways[5].

Although much progress has been made, the relationship between Delta-like ligand 4 and retinopathy of prematurity is not clear and further research is needed because the disease remains the leading cause of blindness in premature babies. Oxygen-induced retinopathy in the neonatal rat has been widely used as an animal model for retinopathy of prematurity[8]. This study sought to evaluate the role of Delta-like ligand 4 in the development of retinal neovascularization in a rat model of retinopathy of prematurity.

RESULTS

Quantitative analysis of experimental animals

A total of 120 Sprague-Dawley rats, aged 5 days, were included in this study, and randomly divided into four groups: normal control group (n = 35), oxygen-induced retinopathy group (n = 35), oxygen-induced retinopathy with PBS group (n = 25), oxygen-induced retinopathy with Delta-like ligand 4 monoclonal antibody group (n = 25). Eighty-five 5-day-old rats were exposed to 80% oxygen for 7 days (12-day-old), then returned to room air until 17 days old. Another 35 rats in the normal control group were fed in the normal environment. Similar to previous studies[9-10], not all oxygen-induced retinopathy rats survived. Specifically, 66 of 85 rats (survival rate was 77.65%) completed the study. Throughout the experiment, inflammation, retinal detachment, and vitreous hemorrhage were not noted in any of the rats.

Expression of Delta-like ligand 4 in retina of oxygen-induced retinopathy rats

We detected the mRNA and protein expression of Delta-like ligand 4 in the retina using reverse transcription-PCR and western blot assay. Figures 1 and 2 show the mRNA and protein bands of Delta-like ligand 4 in the oxygen-induced retinopathy group and the normal control group. Delta-like ligand 4 mRNA and protein were expressed at low levels in normal developing retinas, and the expression levels at postnatal day 17 were slightly lower than those at postnatal day 12 (P > 0.05). Compared with normal retinas, the level of Delta-like ligand 4 mRNA (P < 0.05) and protein (P < 0.05) decreased dramatically in oxygen-induced retinopathy retinas at postnatal day 12, then the expression levels of Delta-like ligand 4 mRNA (P < 0.01) and protein (P < 0.05) increased at postnatal day 17. The mRNA levels of Delta-like ligand 4 changed to the same extent as the protein levels (Table 1).

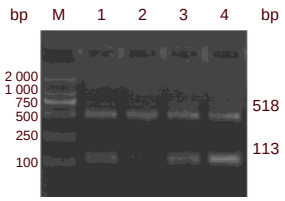

Figure 1 Expression of Delta-like ligand 4 (Dll4) mRNA in the retinas of oxygen-induced retinopathy (OIR) and normal rats by reverse transcription-PCR.

The product size of Dll4 was 113 bp and β-actin was 518 bp. M: Marker 2000; 1: normal postnatal day 12 (P12); 2: OIR P12; 3: normal P17; 4: OIR P17.

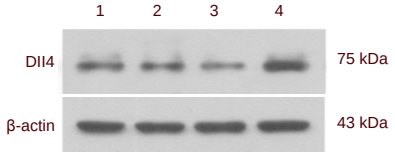

Figure 2 Expression of Delta-like ligand 4 (Dll4) protein in the oxygen-induced retinopathy (OIR) and normal control groups by western blot assay.

The resulting bands of Dll4 and β-actin were visualized by chemiluminescence. 1: Normal postnatal day 12 (P12); 2: normal P17; 3: OIR P12; 4: OIR P17.

Table 1 Expression level of Delta-like ligand 4 (Dll4) mRNA and protein (absorbance ratio to β-actin) in the retina at postnatal day 12 (P12) and P17 of normal and oxygen-induced retinopathy (OIR) rats

Group	Dll4 mRNA	Dll4 protein
Normal control P12	0.81±0.05	1.20±0.06
OIR P12	0.33±0.15[b]	0.44±0.21[b]
Normal control P17	0.67±0.15	1.05±0.07
OIR P17	1.22±0.21[a]	1.66±0.20[b]

Data are expressed as mean ± SD from 10 rats in each group at each time point. Each experiment was performed in triplicate. [a]$P < 0.01$, [b]$P < 0.05$, *vs*. normal control group using the independent-samples *t*-test.

Retinal neovascularization in oxygen-induced retinopathy rats

The degree of hypoxia-induced neovascularization was quantified in serial paraffin cross sections by counting the number of vascular endothelial cell nuclei on the vitreous side of the internal limiting membrane. The oxygen-induced retinopathy retinas had higher median nuclei counts than the room air control retinas ($P < 0.01$; Figure 3, Table 2). Within each group, 10 adenosine diphosphatase stained retinas were technically readable and could be scored. None of the room air control retinas was scored positive for pre-retinal neovascularization. The clock hour scores in the oxygen-induced retinopathy retinas ranged from 1 to 3. There were significant statistical differences in the severity of neovascularization between oxygen-induced retinopathy and normal retinas ($P < 0.01$; Figure 4, Table 2).

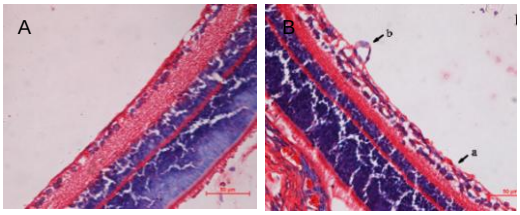

Figure 3 Morphology of postnatal day 17 rat retinas in 6 mm paraffin sections (hematoxylin-eosin staining, light microscope; scale bars: 50 μm).

(A) Section from retina of normal control group.

(B) Section from retina of the oxygen-induced retinopathy group. The number of vascular endothelial cell nuclei increases in the retinas of the oxygen-induced retinopathy rats (arrow a). Arrow b indicates the nuclei above the internal limiting membrane.

Effect of Delta-like ligand 4 monoclonal antibody on retinopathy of prematurity retina

To determine whether Delta-like ligand 4 impacts on retinal neovascularization, Delta-like ligand 4 monoclonal antibody was intravitreally injected into the right eye of oxygen-induced retinopathy rats to inhibit the Delta-like ligand 4/Notch signaling. Oxygen-induced retinopathy rats, intravitreally injected with PBS, served as controls.

Table 2 Clock hour score and nuclei count of retinal neovascularization of normal control, oxygen-induced retinopathy (OIR), OIR with PBS, and OIR with Delta-like ligand 4 (Dll4) monoclonal antibody rats at postnatal day 17

Group	Clock hour score	Nuclei count
Normal control	0	0.10±0.32
OIR	1.50±0.71[a]	32.30±4.85[a]
OIR + PBS	1.63±0.79	35.10±5.17
OIR + anti-Dll4	5.80±1.23[b]	63.60±11.59[b]

Data are expressed as mean ± SD from 10 rats in each group at each time point. Each experiment was performed in triplicate. [a]$P < 0.01$, *vs*. normal control group; [b]$P < 0.01$, *vs*. OIR + PBS group using the independent-samples *t*-test.

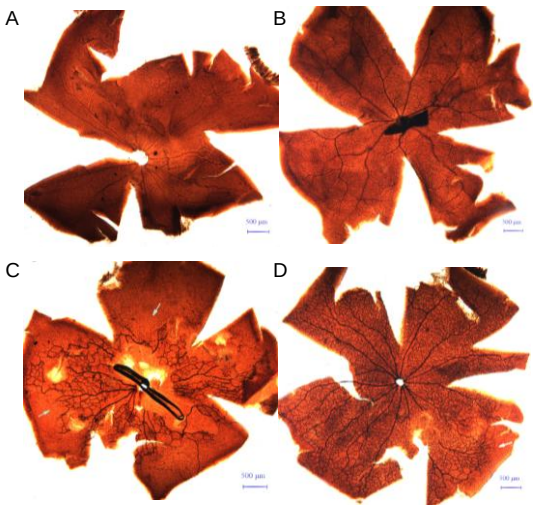

Figure 4 Changes of retinal vascular morphology in oxygen-induced retinopathy and normal control groups (adenosine diphosphatase-stained retina, light microscope; scale bars: 500 μm).

(A) The vessels formed a fine radial branching pattern in the superficial retinal layer in the retina of the normal postnatal day 12 (P12) rat.

(B) The retina of the normal P17 rat had both superficial and deep vascular layers (joined by connecting vessels) that extended from the optic nerve to the periphery. Pre-retinal neovascularization was not observed in the retina of normal rats.

(C) Obliteration of retinal vessels and avascular area (arrows) were observed at P12 in oxygen-induced retinopathy rats.

(D) At P17 in oxygen-induced retinopathy rats, retinal neovascularization (arrow) was detected.

The results of these experiments showed that Delta-like ligand 4 monoclonal antibody caused a 67.16% reduction in Delta-like ligand 4 protein expression (Figure 5). The inhibition of Delta-like ligand 4 protein expression level was significant ($P < 0.05$). Downregulation of the

expression of Delta-like ligand 4 resulted in a significant increase of retinal neovascularization clock hours as measured in flat-mounted adenosine diphosphatase-stained retinas (Figure 6). The number of nuclei structurally adjacent to the vitreous side of the internal limiting membrane also increased (Figure 7). Both the clock hour scores ($P < 0.01$) and the nuclei counts ($P < 0.01$) were significantly different (Table 2). The clock hour scores in the oxygen-induced retinopathy group with Delta-like ligand 4 monoclonal antibody treated retinas ranged from 4 to 8. There were no statistical differences in the nuclei counts and the clock hour scores between the oxygen-induced retinopathy group and oxygen-induced retinopathy with PBS group ($P > 0.05$).

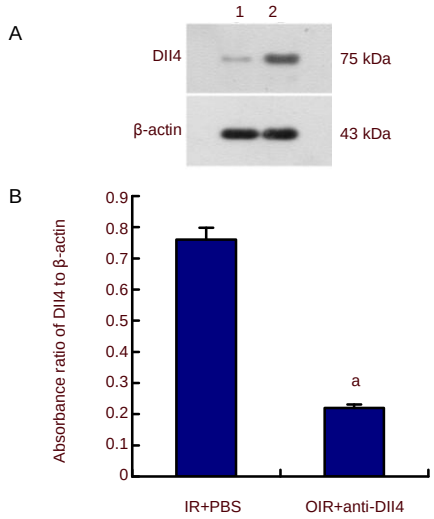

Figure 5 Expression of Delta-like ligand 4 (Dll4) protein in the retinas treated with Dll4 monoclonal antibody or PBS by western blot assay.

(A) Dll4 protein bands by western blot assay. 1: Oxygen-induced retinopathy (OIR) with the Dll4 monoclonal antibody group; 2: OIR with the PBS group.

(B) Quantitative analysis of Dll4 protein expression. Data are expressed as mean ± SD from10 rats in each group at each time point. Each experiment was performed in triplicate. $^aP < 0.05$, *vs*. OIR with PBS group using the independent-samples *t*-test.

DISCUSSION

Retinopathy of prematurity develops in two distinct stages. First, the hyperoxic insult leads to obliteration of immature retinal vessels. Second, upon resumption of the breathing of normal air, there is an adverse compensatory neovascularization response, in which formation of new vessels is excessive and leaky[11]. In growing blood vessels, sprouting of endothelial tip cells is inhibited by Notch signaling, which is activated by binding of the Notch receptor to Delta-like ligand 4[12].

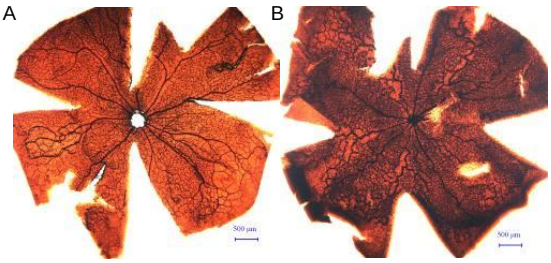

Figure 6 Neovascularization in the retinas of postnatal day 17 oxygen-induced retinopathy (OIR) rats treated with Delta-like ligand 4 (Dll4) monoclonal antibody or PBS (adenosine diphosphatase-stained retina, light microscope; scale bars: 500 μm).

(A) Pre-retinal neovascularization was observed in the OIR with PBS group.

(B) The clock hour scores and the vascular density in the OIR with the Dll4 monoclonal antibody group were higher than those in the OIR with PBS group.

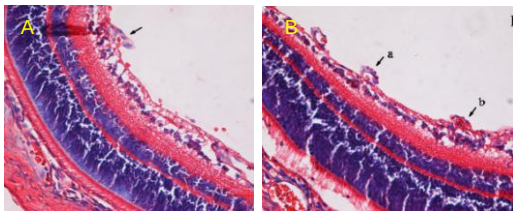

Figure 7 Morphology of postnatal day 17 oxygen-induced retinopathy (OIR) rat retinas in 6 mm paraffin sections (hematoxylin-eosin staining, light microscope; scale bars: 50 μm).

(A) Section from retina of OIR with PBS group. Arrow indicates the nuclei above the internal limiting membrane.

(B) Sections from retinas of OIR with Delta-like ligand 4 monoclonal antibody group. Arrows indicate the nuclei above the internal limiting membrane showing (a) vascular structures or (b) nuclei without lumen.

Several studies have provided insights into the function of Delta-like ligand 4/Notch signaling in angiogenesis and the underlining mechanism of vascular defects resulting from attenuated Delta-like ligand 4/Notch signaling activity. The experimental models used in these studies include *in vitro* three-dimensional culture of primary endothelial cells, developing zebra fish, neonatal mouse retina, mouse embryogenesis models, and murine tumor models[13]. These studies suggest that the Delta-like ligand 4/Notch signaling pathway is likely to be part of the mechanism of angiogenesis[14-17]. The mechanism may include inhibiting endothelial cell proliferation, and suppressing tip cell phenotype, and stalk cells sprouting[18-19]. However, the expression and exact role of Delta-like ligand 4 in the retinopathy of

prematurity retina have not been completely identified.

This study shows that there are decreased levels of Delta-like ligand 4 expression at postnatal day 12 under hyperoxia conditions and increased levels at postnatal day 17 under hypoxia conditions in the oxygen-induced retinopathy rat retinas. Therefore, we hypothesized that the Notch ligand Delta-like 4 is upregulated by hypoxia and downregulated by hyperoxia. Similarly, the present study indicates that the change in vascular endothelial growth factor expression is similar to that of the Delta-like ligand 4[20-21]. These results are consistent with the studies of vascular endothelial growth factor effects on notch signaling by inducing both receptor and ligand[22-23]. Moreover, we observed that Delta-like ligand 4 was moderately expressed in the normal developing retinas, and the expression level of Delta-like ligand 4 was less as the retina developed. These results suggest that Delta-like ligand 4 plays a more substantial role in the development of neovascularization in normal retinas and oxygen-induced retinopathy retinas.

Various studies have shown Delta-like ligand 4 to be implicated in the pathogenesis of the angiogenesis process *in vitro* and *in vivo*[15, 24-25]. The Delta-like ligand 4/Notch signaling pathway is regulated by hypoxia and this may prevent excessive angiogenesis through the inhibition of angiogenic branching and by triggering vessel maturation[26]. Delta-like ligand 4 expression increases in rat and human during embryonic and developmental phases[27-28] and this is accompanied by increased vascular endothelial growth factor expression[16]. Vascular endothelial growth factor is a prominent factor in retinopathy of prematurity[29]. Elevated concentrations of vascular endothelial growth factor in retinopathy of prematurity retinas have been associated with excessive retinal angiogenesis[29], and induced expression of Delta-like ligand 4[5]. In addition, Delta-like ligand 4 is considered to be a downstream mediator of vascular endothelial growth factor[5]. The combined effects of Delta-like ligand 4 and vascular endothelial growth factor have been shown to be involved in the pathogenesis of tumors and other angiogenesis diseases[15, 30].

Monoclonal antibodies have been widely used in animal and clinical experiments as effective treatments. Our data provide convincing evidence that intravitreal injection with Delta-like ligand 4 monoclonal antibody increases the severity of retinal neovascularization in oxygen-induced retinopathy rats. The nuclei counts were highly correlated with the clock hour scores. Previous studies provide evidence that blockade of Delta-like ligand 4 signaling contributes to the increased level of neovascularization in tumors[31-32]. Delta-like ligand 4 monoclonal antibody inhibition of Delta-like ligand 4/Notch signaling exacerbates the retinal neovascularization in oxygen-induced retinopathy rats. Thus, these results strongly suggest that Delta-like ligand 4 plays an important role in the process of retinal neovascularization in the development of oxygen-induced retinopathy. Delta-like ligand 4 could serve as a potential protection factor for the prevention and treatment of retinal neovascularization disease. However, the specific mechanism of Delta-like ligand 4 remains to be determined. Taken together, the identification of Delta-like ligand 4/Notch signaling during the development of retinal neovascularization provides researchers with a novel pathway to target for treatment of this pathological process in the future.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

The experiments were performed at the Laboratory of Shengjing Hospital, China Medical University, China, between November 2010 and October 2011.

Materials

120 specific pathogen-free 5-day-old Sprague-Dawley rats (Animal Laboratory, Shengjing Hospital, Shenyang, Liaoning Province, China; SCXK (Liao) 2009-0004) were bred. Twelve pups were assigned to a single mother for the duration of the experiment. Mothers and pups received standard laboratory diet and water *ad libitum*. Light was cycled on a 12-hour-on, 12-hour-off schedule, and the room was maintained at approximately 21°C. All experiments were performed in accordance with guidelines set by the Animal Experiment Committee of China Institutes for Biological Sciences. The protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[33].

Methods

Establishment of oxygen-induced retinopathy rat model

Experimental retinopathy of prematurity was induced by high oxygen environment[34]. Sprague-Dawley rats were

exposed to 80% oxygen (mixture of medical grade 100% O_2 and room air measured with an oxygen meter (HZAPO, model CY-12C; APO Instrument Equipment Co., Ltd., Hangzhou, Zhejiang Province, China) for 7 days, then returned to room air for 5 days. The control rats were fed in the normal environment.

Intravitreal injections

At postnatal day 12, oxygen-induced retinopathy rats received intravitreal injections as previously described[35]. Rats were anesthetized with an intraperitoneal injection of 3 mL/kg 10% (v/v) chloral hydrate (Department of Pharmacy, Shengjing Hospital, Shenyang, Liaoning Province, China). A topical anesthetic (0.5% tetracaine hydrochloride; Santen, Osaka, Japan) was administered and the pupil was dilated with 1% tropicamide before a 30-gauge needle was inserted just posterior to the limbus to avoid lens damage[36]. Delta-like ligand 4 monoclonal antibody [2.5 μL, (0.5 μg)] (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was injected into the mid-vitreous cavity in the right eye of the oxygen-induced retinopathy Delta-like ligand 4 monoclonal antibody treated group, while the oxygen-induced retinopathy with PBS group (left eye) was injected with 2.5 μL PBS. Then, 0.5% topical TobraDex™ ointment (Alcon Laboratories, Inc., Fort Worth, TX, USA) was applied to prevent infection.

Preparation of retina

The rats from the normal control and oxygen-induced retinopathy groups were sacrificed on postnatal day 12 and postnatal day 17, and the eyes were enucleated to isolate samples. One eye from each rat was stored at –80°C for subsequent western blot analysis and reverse transcription-PCR, and the other was fixed in 4% paraformaldehyde for immunohistochemistry. The rats from the oxygen-induced retinopathy with Delta-like ligand 4 monoclonal antibody treated group and oxygen-induced retinopathy with PBS group were sacrificed on postnatal day 17 and the eyes were enucleated. Delta-like ligand 4 protein levels in the retinas were determined, and the retinal vascular morphology was assessed.

RNA extraction and reverse transcription-PCR

RNA was extracted from the retinas using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For all the RNA preparations, ratios of $A_{260\ nm}$ to $A_{280\ nm}$ were 1.9 to 2.0. The total RNA of retina was reverse-transcribed with a TIANScript RT Kit (Tiangen Biotech, Beijing, China). The primers of Delta-like ligand 4 and β-actin were designed and synthesized by Shanghai Sangon Biotech, China. The primer sequences used are listed in Table 3.

Table 3 The primer sequences of Delta-like ligand 4 and β-actin

Gene	Primer sequence	Product size (bp)
Dll4	Forward: 5'-TGC GGA TAA CCA ACG ACG-3' Reverse: 5'-CCC ACA AAG CCA TAA GGA C-3'	113
β-actin	Forward: 5'-GAG AGG GAA ATC GTG CGT GA-3' Reverse: 5'-GCC TAG AAG CAT TTG CGG TG-3'	518

To verify the mRNA expression of Delta-like ligand 4 and β-actin, reverse transcription-PCR was performed in a volume of 20 μL. After pre-denaturation at 95°C for 5 minutes, 30 cycles were performed at 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. Four microliters out of the 20 μL PCR reaction product was loaded onto a 2% agarose gel, and then visualized under ultraviolet light. Absorbance of bands was quantified using a GIS-2020 image processing system (Technew Tech. Co., Ltd., Shanghai, China) and normalized to the absorbance of the β-actin band.

Western blot analysis

Freshly dissected retinas were pooled, dissociated, and incubated as previously described[37]. Supernatant fractions were collected after homogenization. The prepared lysis buffer was added to the samples, which were centrifuged. The pellet was discarded and the supernatant frozen at –80°C. Before each run, the protein concentrations were determined with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). An equal amount of protein for each sample was heated at 100°C for 10 minutes with an equivalent volume of double strength sample buffer and samples were loaded onto 10% polyacrylamide gels. The proteins were electro-transferred, blocked, and treated with primary rabbit polyclonal antibodies specific for Delta-like ligand 4 of human and rat (1:900; Thermo Fisher Scientific Inc., Rockford, IL, USA). The blots were incubated at 4°C overnight and then exposed to diluted horseradish peroxidase-labeled secondary anti-rabbit IgG (1:10 000; Boster Bioengineering Co., Ltd., Wuhan, Hubei Province, China) at 37°C for 45 minutes. To check for equal loading, membranes were reprobed with an antibody against β-actin (1:15 000; KangChen Bio-tech Inc., Shanghai, China). Exposed Biomax film of bands represented expression of Delta-like ligand 4 protein. Absorbance of bands was quantified using an automated imaging system (ChemiImager 5500 V2.03; Alpha

Innotech, Miami, FL, USA) and normalized to the β-actin band.

Counting clock hours of neovascularization

The cornea, lens, and vitreous body were surgically removed from each eye, and each retina was dissected. All dissected retinas were placed in neutral buffered formalin overnight and processed for magnesium-activated adenosine diphosphatase staining as described by Barnett *et al* [38]. Adenosine diphosphatase-stained retinas were temporarily flat mounted on light microscope slides in PBS with a coverslip and photographed (Olympus, Yokohama, Japan). The presence and severity of neovascularization were quantified in a manner that Zhang *et al* [9] have described previously. Briefly, each retina was divided by visual estimation into 12 equal parts (clock hours), and each clock hour was scored for the presence or absence of abnormal neovascularization. In this way, each retina was given a score between 0 and 12 clock hours by the examiner. We also included 10 control retinas from the room air-exposed pups to reduce the bias toward false positives.

Counting cell nuclei

The eyeballs were enucleated from the postnatal day 17 rats and the posterior eyecups were embedded in paraffin for sectioning. Each entire peripheral retina was serially sectioned at 6 μm and stained with hematoxylin-eosin. Nuclei above the internal limiting membrane were then counted in a masked manner using light microscopy (Olympus). The total number of nuclei counted per retina was defined as the nuclei count for further analysis. Our method of counting cell nuclei was the standard quantification method described by Smith *et al* [39].

Statistical analysis

All data from quantitative assays were expressed as mean ± SD. Statistical analysis was performed by the independent-samples *t*-test using SPSS for Windows, Version 15.0 software (SPSS, Chicago, IL, USA). A *P* value of < 0.05 was considered statistically significant.

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Author contributions: Shaoyang Shi designed the experiments, wrote the paper, provided the reagents, materials, and the analysis tools. You Li and Xun Li participated in experimental animal breeding and disposal, and provided data support. Hongwei Yang and Cunwen Pei were responsible for the study analysis and statistical analysis. Xiaolong Chen participated in study concept and design, article authorization, and study instruction. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The experiment was approved by the Animal Ethics Committee of China Medical University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

REFERENCES

[1] Gilbert C. Retinopathy of prematurity: a global perspective of the epidemics, population of babies at risk and implications for control. Early Hum Dev. 2008;84(2):77-82.

[2] Yang CY, Lien R, Yang PH, et al. Analysis of incidence and risk factors of retinopathy of prematurity among very-low-birth-weight infants in North Taiwan. Pediatr Neonatol. 2011;52(6):321-326.

[3] Fortes Filho JB, Eckert GU, Tartarella MB, et al. Prevention of retinopathy of prematurity. Arq Bras Oftalmol. 2011;74(3):217-221.

[4] Rivera JC, Sapieha P, Joyal JS, et al. Understanding retinopathy of prematurity: update on pathogenesis. Neonatology. 2011;100(4):343-353.

[5] Lobov IB, Renard RA, Papadopoulos N, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci U S A. 2007;104(9):3219-3224.

[6] He H, Li B, Zhang H, et al. Effect of DLL4 siRNA on proliferation, migration and tube formation of choroid-retinal endothelial cells under hypoxic conditions. Chin Med J (Engl). 2011;124(1):118-126.

[7] Gale NW, Dominguez MG, Noguera I, et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A. 2004;101(45):15949-15954.

[8] Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. Prog Retin Eye Res. 2010;29(6):500-519.

[9] Zhang S, Leske DA, Holmes JM. Neovascularization grading methods in a rat model of retinopathy of prematurity. Invest Ophthalmol Vis Sci. 2000;41(3): 887-891.

[10] Yan L, Han M, Zhao KX. The anti-thyroid drug methimazole aggravated neovascularization in the oxygen-induced retinopathy rat model of ROP. Zhonghua Yan Ke Za Zhi. 2010;46(7):585-589.

[11] Raghuveer TS, Bloom BT. A paradigm shift in the prevention of retinopathy of prematurity. Neonatology. 2011;100(2):116-129.
[12] Benedito R, Roca C, Sörensen I, et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell. 2009;137(6):1124-1135.
[13] Yan M, Plowman GD. Delta-like 4/Notch signaling and its therapeutic implications. Clin Cancer Res. 2007;13(24): 7243-7246.
[14] Al Haj Zen A, Oikawa A, Bazan-Peregrino M, et al. Inhibition of delta-like-4–mediated signaling impairs reparative angiogenesis after ischemia. Circ Res. 2010; 107(2):283-293.
[15] Dong X, Wang YS, Dou GR, et al. Influence of Dll4 via HIF-1α-VEGF signaling on the angiogenesis of choroidal neovascularization under hypoxic conditions. PLoS One. 2011;6(4):e18481.
[16] Djokovic D, Trindade A, Gigante J, et al. Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis. BMC Cancer. 2010;10:641.
[17] Lv W, Chen L, Zhou DH, et al. Influence of specific blocking of the delta-like ligand 4/notch signal transduction pathway on the biological behavior of human umbilical vein endothelial cells. Cancer Biother Radiopharm. 2010;25(4):449-454.
[18] Holderfield MT, Hughes CC. Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. Circ Res. 2008; 102(6):637-652.
[19] del Toro R, Prahst C, Mathivet T, et al. Identification and functional analysis of endothelial tip cell–enriched genes. Blood. 2010;116(19):4025-4033.
[20] Chen Y, Li XX, Dong JQ. Study on oxygen-induced retinopathy in a mouse model of retinopathy of prematurity. Yanke Yanjiu. 2008;26(2):113-116.
[21] Budd SJ, Hartnett ME. Increased angiogenic factors associated with peripheral avascular retina and intravitreous neovascularization: a model of retinopathy of prematurity. Arch Ophthalmol. 2010;128(5):589-595.
[22] Jakobsson L, Bentley K, Gerhardt H. VEGFRs and Notch: a dynamic collaboration in vascular patterning. Biochem Soc Trans. 2009;37(Pt 6):1233-1236.
[23] Gerhardt H. VEGF and endothelial guidance in angiogenic sprouting. Organogenesis. 2008;4(4):241-246.
[24] Li JL, Harris AL. Crosstalk of VEGF and Notch pathways in tumour angiogenesis: therapeutic implications. Front Biosci. 2009;14:3094-3110.
[25] Stenzel D, Franco CA, Estrach S, et al. Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. EMBO Rep. 2011;12(11): 1135-1143.
[26] Suchting S, Freitas C, le Noble F, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci U S A. 2007;104(9):3225-3230.
[27] Bao ZZ, Cepko CL. The expression and function of Notch pathway genes in the developing rat eye. J Neurosci. 1997;17(4):1425-1434.
[28] Herr F, Schreiner I, Baal N, et al. Expression patterns of Notch receptors, and their ligands Jagged and Delta in human placenta. Placenta. 2011;32(8):554-563.
[29] Stewart MW. The expanding role of vascular endothelial growth factor inhibitors in ophthalmology. Mayo Clin Proc. 2012;87(1):77-88.
[30] Oon CE, Harris AL. New pathways and mechanisms regulating and responding to Delta-like ligand 4-Notch signalling in tumour angiogenesis. Biochem Soc Trans. 2011;39(6):1612-1618.
[31] Oishi H, Sunamura M, Egawa S, et al. Blockade of delta-like ligand 4 signaling inhibits both growth and angiogenesis of pancreatic cancer. Pancreas. 2010;39(6): 897-903.
[32] Ridgway J, Zhang G, Wu Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature. 2006;444(7122):1083-1087.
[33] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[34] Nakamura H, Yamazaki M, Ohyama T, et al. Role of angiotensin II type 1 receptor on retinal vascular leakage in a rat oxygen-induced retinopathy model. Ophthalmic Res. 2009;41(4):210-215.
[35] Levkovitch-Verbin H, Sadan O, Vander S, et al. Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci. 2010;51(12): 6394-6400.
[36] Yang H, Huang Y, Chen X, et al. The role of CTGF in the diabetic rat retina and its relationship with VEGF and TGF-β(2), elucidated by treatment with CTGFsiRNA. Acta Ophthalmol. 2010;88(6):652-659.
[37] Silva VA, Polesskaya A, Sousa TA, et al. Expression and cellular localization of microRNA-29b and RAX, an activator of the RNA-dependent protein kinase (PKR), in the retina of streptozotocin-induced diabetic rats. Mol Vis. 2011;17:2228-2240.
[38] Barnett JM, McCollum GW, Fowler JA, et al. Pharmacologic and genetic manipulation of MMP-2 and -9 affects retinal neovascularization in rodent models of OIR. Invest Ophthalmol Vis Sci. 2007;48(2):907-915.
[39] Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci. 1994;35(1):101-111.

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