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Original Article

Effect of Notch1-Dll4 signaling pathway in mouse model of oxygen-induced retinopathy

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Abstract: Objective: To investigate the role of Notch1-Dll4 signaling pathway in the retinal neovascularization of oxygen-induced retinopathy mouse model. Methods: Thirty C57BL/6J mouse pups in experimental group were exposed to (75 ± 2)% oxygen for 5 d and then returned to room air. Another 30 mice in control group were subjected to room air. The expression of retinal Dll4, vascular endothelial growth factor 1 (VEGFR-1) and VEGFR-2 was measured by immunohistochemical analysis and RT-PCR. Results: In both groups, Dll4, VEGFR-1 and VEGFR-2 proteins were expressed in retina. In 17-d-old (P17) mice, positive rate of VEGFR-1 in experimental group was significantly lower than that in control group (P<0.05). Positive rate of VEGFR-2 did not significantly differ between two groups (all P>0.05). At P12 and P17, positive rates of Dll4 considerably differed between two groups (both P<0.05). In experimental group, positive rates of VEGFR-1 and Dll4 tended to decline over time (both P<0.001), whereas an increasing tendency for VEGFR-2 (P<0.05). In control group, positive rate of VEGFR-1 tended to decrease, whereas VEGFR-2 tended to elevate over time (P<0.001). VEGFR-1 mRNA expression significantly differed between two groups at P17 (P<0.05). VEGFR-2 mRNA did not dramatically differ between two groups (all P>0.05). The expression of Dll4 mRNA significantly differed between two groups at P12 and P17 (both P<0.05). In experimental group, the expression of VEGFR-1 and Dll4 proteins declined (both P<0.05), whereas VEGFR-2 protein was elevated over time (P<0.05). In control group, the expression of VEGFR-2 protein was significantly up-regulated over time (P<0.05), whereas no changes for VEGFR-1 and Dll4 proteins (both P>0.05). Dll4 expression was positively correlated with VEGFR-1 (r=0.905, P<0.001). Conclusion: Dll4 and VEGFR-1 expression were both inhibited during neovascularization, and Dll4 expression positively correlated with Dll4 expression. Notch1-Dll4 signaling pathway was probably involved in retinal neovascularization.

Keywords: Notch1-Dll4 signaling pathway, retinopathy, VEGFR-1, VEGFR-2, VEGF

Introduction

Retinopathy of prematurity (ROP) is a type of proliferative retinal vascular disease, mainly pathologically characterized by retinal neovascularization. The pathogenesis of retinal neovascularization is largely unknown and effective treatment is still lacking. Consequently, the etiology of retinal neovascularization has been focused on retinopathy in preterm infants. Retinal vascular development is affected by a variety of factors. Vascular endothelial growth factor (VEGF) is the most potent proangiogenic factor and plays a pivotal role in retinal neovascularization. Notch signaling pathway is widely prevalent in invertebrate and vertebrate animals. Notch1-Dll4 signaling pathway exerts a biological effect upon regulating retinal vascular growth by negative feedback. However, whether Notch1-Dll4 signaling pathway is suppressed and the exact role in regulating VEGF has been rarely reported in preterm infants with retinopathy. In this study, an oxygen-induced retinopathy mouse model was established to investigate the role of Notch1-Dll4 signaling pathway on VEGF regulation in preterm infants with retinopathy.

Materials and methods

Establishment of mouse model of oxygen-induced retinopathy

Experimental animals: Sixty 7-day old C57BL/6J mouse neonates, both male and female,

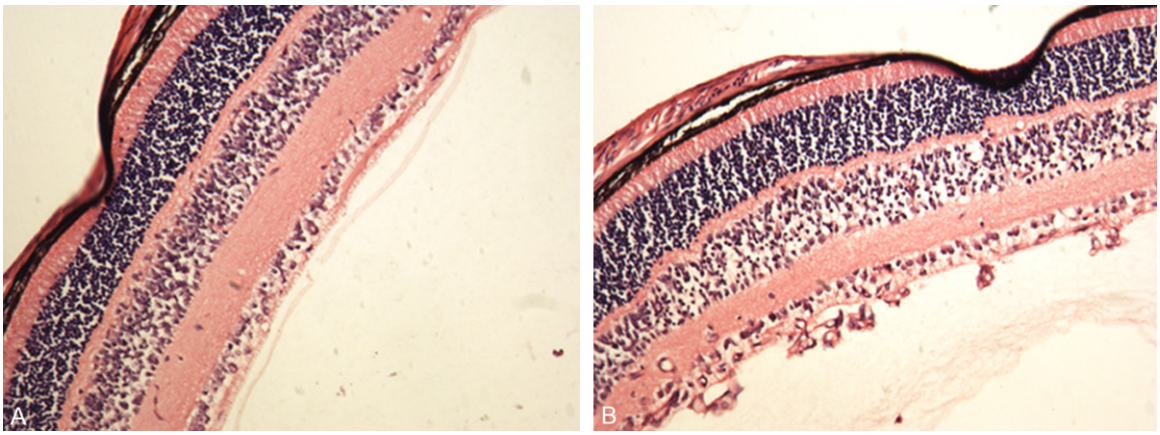

Figure 1. H&E staining of the retina in control group (A) and experimental group (B) (× 200).

weighed approximately 3-4 g, were selected. The mouse pups and mothers were housed in a SPF grade environment in the experimental animal center of Sun Yat-sen University.

Establishment and grouping of mouse model: The mouse neonates (regardless of gender) were randomly assigned into the experimental (n=30) and control groups (n=30). Control mice (n=30) and mothers were exposed to room air in conventional cages. The mouse neonates and mothers in the experimental group were exposed to (75 ± 2)% oxygen at a flow control of 0.5-1 L/min for 5 d and then transferred to room.

Immunohistochemical staining of VEGFR-1, VEGFR-2 and Dll4 expression

The ocular globes were removed from 5 neonates in each group at postnatal days 7, 12 and 17, fixed for 24 h (15 sections for each eye) and then subject to immunohistochemical staining. The expression of VEGFR-1, VEGFR-2 and Dll4 was observed under an optical microscope and the images were photographed. The mean percentage of positive cells was calculated and statistically analyzed.

RT-PCR

Total RNA was extracted from the mouse retinal tissues and subject to RT-PCR detection. The mRNA levels of target genes were measured. TBP was used as internal standard.

Statistical analysis

Measurement data were expressed as mean ± standard deviation (SD). SPSS 16.0 statistical software was utilized for data analysis. Independent *t*-test was used to detect the difference between two groups. Enumeration data were expressed as rate and percentage and analyzed with *chi*-square test. The correlation of VEGFR-1, VEGFR-2 and Dll4 mRNA was analyzed with Pearson correlation analysis. A value of $P<0.05$ was considered as statistically significant.

Results

Quantitative analysis of oxygen-induced retinal neovascularization

After H&E staining, different layers of the retina could be explicitly observed under light microscope. In control mice, almost no endothelial neovascularization, beyond the internal limiting membrane of retina, was noted (**Figure 1A**), and the mean number of neovascular nuclei was 1.10 ± 1.54 for each slice. In the experimental group, a substantial quantity of neovascular endothelial cells that were present beyond the internal limiting membrane were formed in a single or cluster pattern, as illustrated in **Figure 1B**. The mean number of neovascular nuclei was 44.62 ± 19.08 for each slice, significantly higher than that in the control group ($t=14.523$, $P<0.001$).

Immunohistochemical staining of Dll4, VEGFR-1 and VEGFR-2 expression

The cells with positive VEGFR-1, VEGFR-2 and Dll4 expression were all located on the retinal internal limiting membrane, strata ganglionaris and inner nuclear layer (**Figures 2**-**4**). Over aging, the expression of VEGFR-1, VEGFR-2 and Dll4 was altered correspondingly. At P7 and

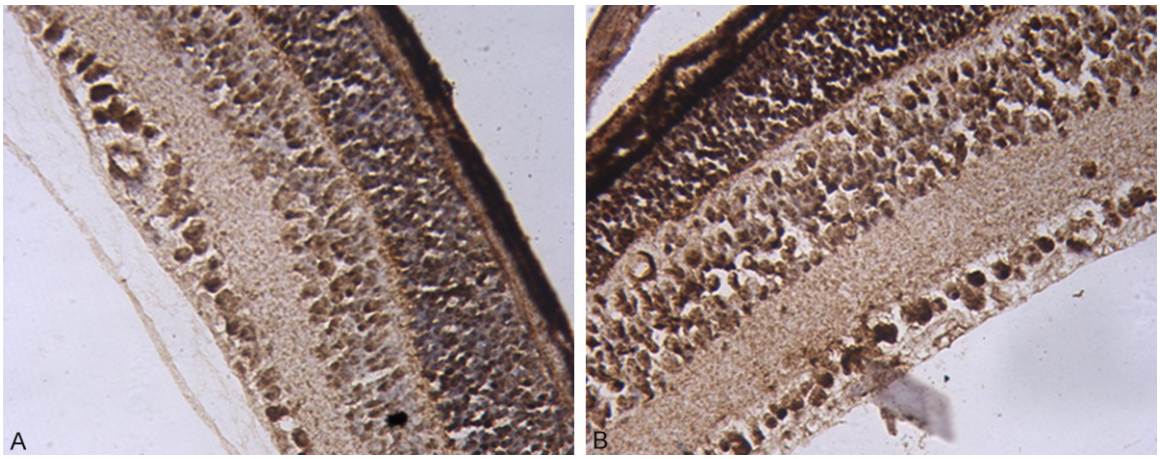

Figure 2. VEGFR-1 expression in the mouse retina of experimental group (A) and experimental group (B) at P17 (× 200).

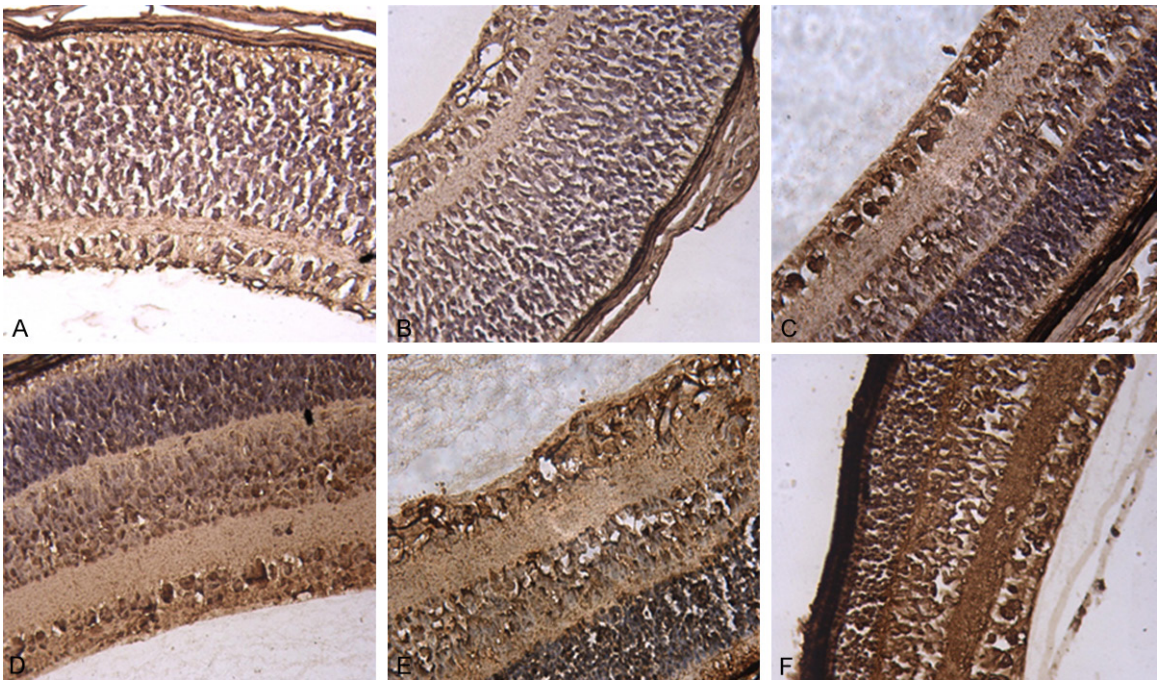

Figure 3. VEGFR-2 expression in the mouse retina of experimental group (A, C, E) and experimental group (B, D, F) at P7, P12 and P17, respectively (× 200).

P12, there were no significant differences in the positive rates of VEGFR-1 between the two groups (P>0.05) (**Table 1**). On P17, the positive rate of VEGFR-1 in the experimental group was 11.20%, significantly lower than that of control group (20.79%, χ^2=3.922, P=0.048). There were no significant changes of the positive rates of VEGFR-2 in the experimental group compared with control group at P7, P12 and P17 (P>0.05). At P7, the positive rate of Dll4 did not significantly change in the experimental group compared with control group (71.25% v.s 70.11%, P>0.05). At P12 and P17, the positive rates of Dll4 in the experimental group were 41.18% and 31.50%, significantly lower than those of control mice (54.86% and 65.73%, respectively) (P=0.034, P<0.001).

Tendency test of positive rate of three parameters at different time points

In the experimental group, the positive rates of VEGFR-1 and Dll4 presented a decreasing ten-

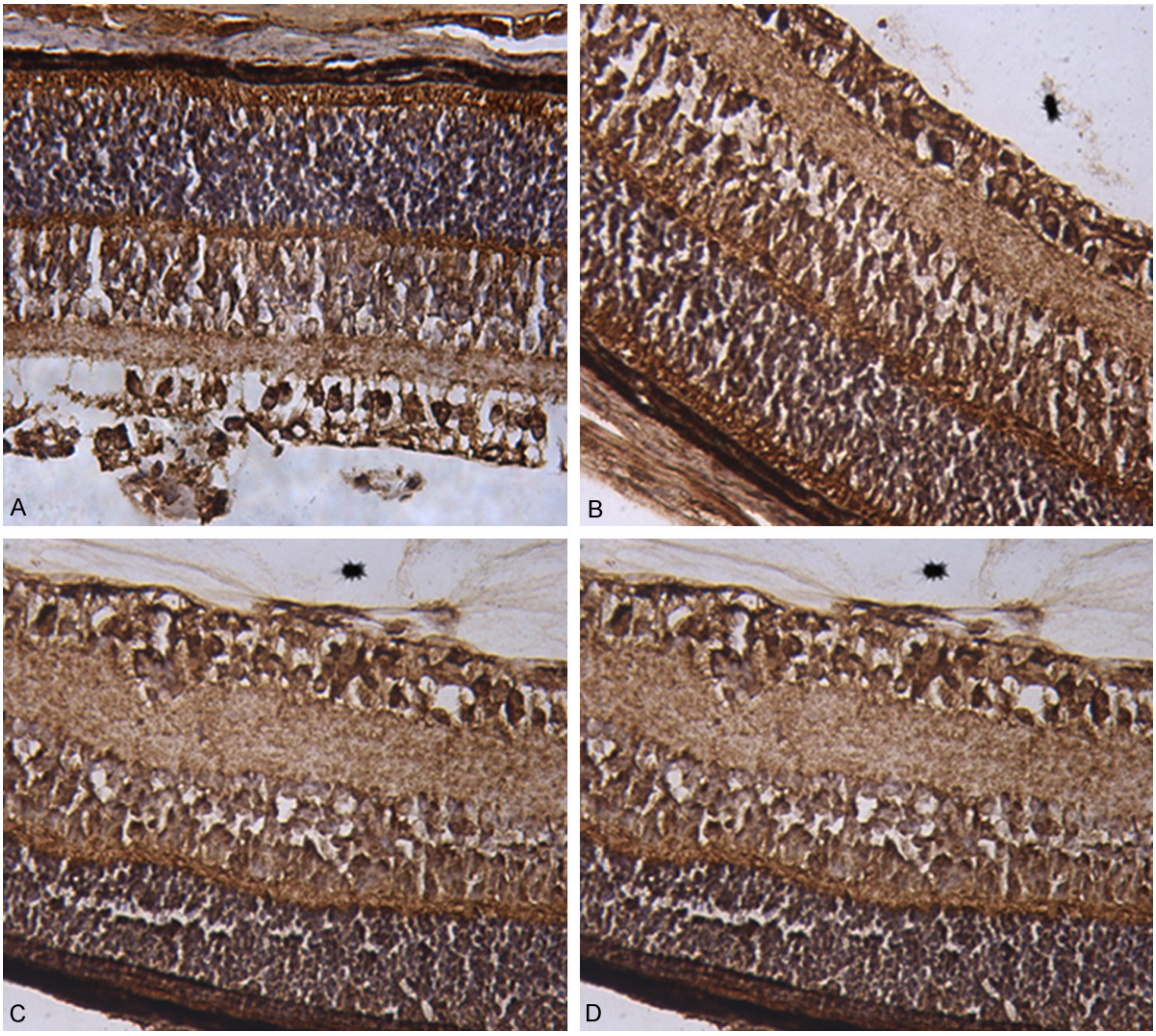

Figure 4. Dll4 expression in the mouse retina of experimental group (A, C) and experimental group (B, D) at P12 and P17, respectively. (× 200).

dency (both P<0.001) (**Table 2**), whereas that of VEGFR-2 tended to elevate (P=0.013). In the control group, the positive rate of VEGFR-1 presented a decreasing tendency at the 3 time points (P<0.017), whereas that of VEGFR-2 tended to increase dramatically (P<0.001). The positive rate of Dll4 did not significantly change at different time points (P=0.464).

VEGFR-1, VEGFR-2 and Dll4 mRNA expression by RT-PCR

At P7 and P12, the levels of VEGFR-1 mRNA did not significantly differ between the two groups (P>0.05) (**Table 3**), whereas the VEGFR-1 expression in the experimental group was 0.208 ± 0.048 at P17, which was significantly lower than that in the control group (0.380 ± 0.127) (P=0.022). However, the expression of VEGFR-2 mRNA did not significantly differ between the two groups at each time point (all P>0.05). At P7, the level of Dll4 mRNA was not significantly changed in the experimental group compared with control group (0.278 ± 0.128 v.s 0.290 ± 0.207, P>0.05). At P12 and P17, the Dll4 mRNA expression in the experimental group was 0.058+/0.020 and 0.060 ± 0.020, significantly lower than those in the control group (0.100 ± 0.022 and 0.168 ± 0.089, respectively) (both P<0.05).

Tendency test of VEGFR-1, VEGFR-2 and Dll4 mRNA expression at different time points between two groups

In the experimental group, the expression of VEGFR-1 (*F*=27.129, P<0.05) and Dll4 proteins (*F*=24.538, P<0.05) significantly declined over

Table 1. The positive rates of VEGFR1, VEGFR2 and Dll4 at different time points

Time points	VEGFR1		*P* value	VEGFR2		*P* value	Dll4		*P* value
	Experimental group (%)	Control group (%)		Experimental group (%)	Control group (%)		Experimental group (%)	Control group (%)	
p7	36.12	35.23	0.883	19.43	20.61	0.858	71.25	70.11	0.877
p12	20.09	24.68	0.397	33.07	35.28	0.765	41.18	54.86	0.034
p17	11.20	20.79	0.048	35.88	46.17	0.149	31.50	65.73	<0.001

After immunohistochemical staining, the positive rates of VEGFR1, VEGFR2 and Dll4 were analyzed. The differences between the two groups were analyzed with chi-square test.

Table 2. The trend of VEGFR1, VEGFR2 or Dll4 positive rates

Group	Factors	Positive rates (%)			χ^2 value	*P* value
		p7	p12	p17		
Experimental group	VEGFR-1	36.12	20.09	11.20	19.631	<0.001
	VEGFR-2	19.43	33.07	35.88	6.196	0.013
	Dll4	71.25	41.18	31.50	33.629	<0.001
Control group	VEGFR-1	35.23	24.68	20.79	5.734	0.017
	VEGFR-2	20.61	35.28	46.17	14.016	<0.001
	Dll4	70.11	54.86	65.73	0.536	0.464

time, whereas that of VEGFR-2 protein was dramatically elevated over aging (F=20.795, P<0.05) (**Table 4**). In the control group, the expression of VEGFR-2 protein was significantly up-regulated over time (F=18.010, P<0.001), whereas the levels of VEGFR-1 or Dll4 proteins did not change over aging (F=2.242, P=0.145; F=2.156, P=0.168). The expression of Dll4 was positively correlated with VEGFR-1 expression (r=0.905, P<0.001) (**Table 5**). No apparent correlation was documented between Dll4 and VEGFR-2 (r=-0.181, P=0.338), or between VEGFR-1 and VEGFR-2 (r=-0.265, P=0.157).

Discussion

Currently, the pathogenesis of ROP is not fully understood yet. Low body weight, premature birth and oxygen inhalation are recognized as the high risk factors of ROP [1-4]. Smith et al. [5] exposed 7-day old mice to 75% oxygen plus 25% nitrogen for 5 days and subsequently returned to room air, and successfully established mouse model of ROP. According to the modified methods by Smith et al., we exposed the mice to (75 ± 2)% oxygen for 5 days and then transferred to the room air. The mouse model was successfully established with high reproducibility and simple procedures. Referring to the animal studies by Connor KM [6], the time points of sampling detection were chosen at postnatal days 7, 12 and 17.

Analysis of VEGFR-1 and VEGFR-2 expression in mouse retina

VEGF plays a pivotal role in regulating the normal development of retinal blood vessels. As a potent proangiogenic factor, the expression of VEGF is positively associated with the quantity of retinal endothelial cells and blood vessels. Both in vivo and in vitro studies have demonstrated that the expression of VEGF mRNA is significantly altered during the neovascularization process of ROP, suggesting that VEGF probably plays a decisive role in the retinal neovascularization and is closely correlated with the pathogenesis of ROP [7-10]. Sonmez K et al. [11] detected the retinal VEGF levels in 22 patients with stage IV ROP and found that the expression of VEGF in the experimental group was significantly up-regulated than that in the control group, suggesting that VEGF plays a vital role in regulating the pathogenesis of ROP, which was corroborated by previous findings [8, 12].

Pieh et al. [13] collected the blood samples from 63 preterm infants of 23-32 weeks gestational age at postnatal 5 days and 15 weeks and found that the plasma levels of VEGFR-1 were almost equivalent between two groups and the VEGFR-2 level in ROP group was significantly elevated. In this study, the positive rate of VEGFR-1 did not differ between two groups at P7 or P12, whereas the positive rate of VEGFR-1 in the experimental group was significantly lower than that in the control group. At P7 and P12, the expression of VEGFR-1 protein did not significantly differ between two groups. However, the level of VEGFR-1 in the experimental group was significantly lower than that in the control group at P17. In the experimental group, tendency test revealed that the expres-

Table 3. The expression of VEGFR1, VEGFR2 and Dll4 mRNA at different time points

Group	Time points	VEGFR1		VEGFR2		Dll4	
		Mean ± S.D	*P* value	Mean ± S.D	*P* value	Mean ± S.D	*P* value
Experimental	p7	0.836 ± 0.315	0.872	0.102 ± 0.030	1.000	0.278 ± 0.128	0.915
Control		0.780 ± 0.682		0.102 ± 0.128		0.290 ± 0.207	
Experimental	p12	0.286 ± 0.086	0.610	0.560 ± 0.208	0.244	0.058 ± 0.022	0.032
Control		0.252 ± 0.115		0.758 ± 0.284		0.100 ± 0.029	
Experimental	p17	0.208 ± 0.048	0.022	0.586 ± 0.165	0.094	0.060 ± 0.020	0.029
Control		0.380 ± 0.127		1.060 ± 0.534		0.168 ± 0.089	

The data are expressed as mean ± S.D (n=5).

Table 4. The mRNA expression trend in the two groups

Factors	Time points	Experimental group		Control group	
		F value	*P* value	F value	*P* value
VEGFR-1	p7 p12 p17	27.129	<0.001	2.424	0.145
VEGFR-2	p7 p12 p17	24.538	<0.001	18.010	0.001
Dll4	p7 p12 p17	20.795	0.001	2.156	0.168

Table 5. The correlation of the three mRNA expression

Factors	r	P
Dll4 Vs VEGFR-1	0.905	<0.001
Dll4 Vs VEGFR-2	-0.181	0.338
VEGFR-1 Vs VEGFR-2	-0.265	0.157

sion of VEGFR-1 protein was decreased over time and reached the lowest level at P17, which was consistent with Dll4 expression and opposite to VEGF expression. In the control group, the expression of VEGFR-1 did not significantly alter over different time points. The findings in this study were consistent with previous investigations, suggesting that VEGFR-1 plays an essential role in maintaining the function and survival of blood vessels during early developmental stage. Nevertheless, the expression of VEGFR-1 was down-regulated in the ROP group, indicating that the expression of VEGFR-1 was suppressed along with the increasing bind between VEGFR-2 receptors and VEGF. The tendency of VEGFR-1 and Dll4 expression at protein and mRNA levels was almost similar, hinting that inhibition of Notch1-Dll4 signaling pathway down-regulates the expression of VEGFR-1.

Byrne et al. [14] demonstrated that VEGFR-2 is a functional receptor mediating VEGF signal in vascular endothelial cells. It is more active compared with VEGFR-1. The binding of VEGFR-2 with VEGF-E ligand induces the production of prostacyclin and nitric oxide, accelerates the migration and sprouting of vascular endothelial cells in vitro and form neovascularization. In this study, we found that the positive rate of VEGFR-2 tended to increase over different times points in both the experimental and control groups. Moreover, the expression of VEGFR-2 at protein and mRNA levels was up-regulated over time in two groups. The expression of VEGFR-2 protein was increased over time and peaked at P17, indicating that VEGFR-2 is a receptor of vascular endothelial cells and the expression of VEGFR-2 is up-regulated over the increasing level of VEGF. However, no statistical significance was documented in the positive rate of VEGFR-2 at each time point between two groups. Meantime, the expression levels of VEGFR-2 at both protein and mRNA levels did not significantly differ at different time points between two groups. The discrepancy probably results from limited number of animals and relatively small sample size.

Expression and significance of Notch1-Dll4 in mouse retina

Hellstrom M et al. [14] found that the quantity of neovascular endothelial tip cells was significantly enhanced after Dll4 blockage or Notch1 gene knockdown, accompanied by vascular sprouting. When the Notch1-Dll4 signaling pathway was activated, the number of neovascular endothelial tip cells was dramatically

decreased and the vascular spouting was reduced, suggesting that Notch1-Dll4 signaling pathway could inhibit the generation of endothelial tip cells and suppress the neovascularization. In this study, Dll4 was widely expressed in the internal limiting membrane, strata ganglionare and inner nuclear layer of the mouse retina. In addition, the expression of Dll4 was altered over time. At P12 and 17, the positive rate of Dll4 in the experimental group was significantly lower than that in the control mice. The positive rate of Dll4 tended to decline at different time points in the experimental group and reached the lowest level at P17, suggesting that the suppression of Dll4 is strengthened along with ROP retinal neovascularization and maintained until the peak of neovascularization (P17), which is opposite to the expression of VEGF. In the control group, the positive rate of Dll4 did not significantly change at different time points. At P12 and P17, the expression of Dll4 mRNA in the experimental group was significantly lower than that in the control mice. The expression of Dll4 protein in the experimental group tended to decline over time, whereas no changing tendency was observed in Dll4 expression over time in the control mice. The expression of Dll4 in the ROP mice was significantly lower than that in the control group, suggesting that Dll4 expression is inhibited during the neovascularization and Dll4 is unable to provide negative feedback to the over-expression of VEGF, thereby leading to excessive neovascularization. This outcome has not been reported in previous studies, which is the highlight of this present investigation. These events indicate that Notch1-Dll4 signaling pathway is suppressed during neovascularization in ROP mice. In addition, over-expression of VEGF during the progression of ROP probably results from the inactivated Notch1-Dll4 signaling pathway. In this study, the up-regulated expression of VEGF did not increase the expression of Dll4. Whether it is correlated with the hypoxia-induced changes of regulatory factors in upstream Dll4 signaling pathway remains to be verified.

Correlation analysis of Dll4 and VEGFR-1 expression in mouse retina

Jakobsson et al. [15] revealed that Notch1-Dll4 signaling pathway and VEGFR act as a potent combination during the neovascularization. Dll4 is capable of affecting the process of neovascularization through regulating VEGFR expression and eventually regulating the reconstruction and function of neovascularization. Dll4 can down-regulate the expression of VEGF by regulating the VEGF receptor family. Previous studies demonstrated that the activation of Notch1-Dll4 signaling pathway could up-regulate the expression of VEGFR-1 in neovascular endothelial cells [16] and competitively bind with VEGF against VEGFR-2, thereby decreasing the bind of VEGF with VEGFR-2, weakening the VEGF signaling pathway and suppressing the neovascularization. In this study, the expression of Dll4 was positively associated with VEGFR-1 expression, which is consistent with previous findings, implicating that VEGFR-1 expression is correlated with the activation of Notch1-Dll4 signaling pathway in vascular endothelial cells. VEGFR-1 serves as one of the factors in the downstream of Notch1-Dll4 signaling pathway. In oxygen-induced retinopathy mouse models, Dll4 can inhibit VEGF expression via up-regulating VEGFR-1 expression during neovascularization and eventually suppress the neovascularization.

Correlation analysis of Dll4 and VEGFR-2 expression in mouse retina

Taylor et al. [17] found that the expression of VEGFR-2 was down-regulated if the Notch signaling pathway was activated. VEGFR-2 expression was up-regulated in vascular endothelial cells with Dll4 gene knockdown and similar findings were observed in endothelial cells with Dll4 blockage [18]. In this experiment, no correlation was documented between Dll4 and VEGFR-2, or between VEGFR-1 and VEGFR-2, which is inconsistent with previous studies. The underlying reasons may be as follows. First, the limited quantity of experimental animals affects the statistical results. Second, the possibility of the expression of alternative VEGF receptors can not be excluded during neovascularization. VEGFR-2 is probably not the key receptor of VEGF expression during retinal neovascularization. Third, HRT1, a factor of the downstream of Notch signaling pathway, is one of promoters of VEGFR-2. Besides the regulatory effect from Notch signaling pathway, whether it is up-regulated by alternative factors and subsequently down-regulates the expression of VEGFR-2 mRNA remains to be further elucidated. Leong

KG et al. [19] found that Notch4 and HRT1 can down-regulate the expression of VEGFR-2 mRNA. HRT1 is a downstream factor of Notch signaling pathway. Consequently, the expression of VEGFR-2 is inhibited via HRT1 after activation of Notch signaling pathway.

Taken together, the expression of Dll4 is suppressed during neovascularization in oxygen-induced retinopathy mouse models, which probably serves as one of the causes of overexpression of VEGF and a substantial quantity of neovascularization. The expression of VEGFR-1 is correlated with the activation of Notch1-Dll4 signaling pathway in vascular endothelial cells. In oxygen-induced retinopathy mouse models, Dll4 is capable of suppressing VEGF expression probably via up-regulating the expression of VEGFR-1 and eventually inhibiting the neovascularization. Experimental outcomes indicate that VEGFR-2 is not a key receptor of VEGF expression. These outcomes may deepen the understanding of ROP pathogenesis and provide reliable foundation for the prevention and treatment of ROP.

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Disclosure of conflict of interest

None.

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