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Original Article

Temporal and spatial expression change of GPR124 in mouse retinal development and in oxygen-induced retinopathy model

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Abstract: G protein-coupled receptor 124 (GPR124), a member of the class II GPR family, is up-regulated in endothelial cells during central nervous system and tumor angiogenesis. However, the expression change of GPR124 in normal retinal development and in oxygen-induced retinopathy (OIR) model remains to be elucidated. In this study, neonatal C57BL/6J mice were randomly divided into normal group and OIR group, eyes were enucleated at each time point, then qRT-PCR, immunohistochemistry and immunofluorescence double staining were performed to observe the location and expression change of GPR124 in normal mice retina and in OIR retina. GPR124 was progressively up-regulated in the normal retinal development, peaked at P14, and then gradually decreased. In OIR retina, the expression of GPR124 was weak at P10 and P12, then elevated gradually and peaked at P17. GPR124 expression was located in ganglion cell layer and inner nuclear layer in a time-dependent manner in both groups, and also expressed in the abnormal pre-retinal neovascularization in OIR. Cellular localization indicated that GPR124 was expressed in endothelial cells and in retinal ganglion cells. The spatiotemporal changes of GPR124 expression demonstrated that GPR124 involved in retinal neurovascular development and neovascularization. We conclude that GPR124 might become a new target for the study of retinal development and to investigate the occurrence of pathological retinal neovascularization, and could be used as a potential candidate to prevent the retinal neovascular diseases.

Keywords: GPR124, endothelial cells, retinal ganglion cells, retinal development, retinal neovascularization

Introduction

Vascular endothelium cells, neurons, glial cells interdependently form neurovascular unit in the retina, which are involved in the regulation of retinal angiogenesis. Retinal neovascular (RNV) diseases, including retinopathy of prematurity (ROP) and diabetic retinopathy (DR), are the neurovascular related diseases can lead to severe visual impairment [1]. With the improvement of living standards and the raised survival rate of premature infants, the incidence of the DR and ROP are significantly increased. It has been confirmed that there are many pro-angiogenic factors involved in RNV [2]. Undoubtedly, vascular endothelial growth factor (VEGF) is the most important angiogenesis factor, playing a crucial role in the development of RNV disease and anti-VEGF agents have become the most effective treatment in clinic [3, 4]. However, the application of these agents are limited in some ways for many patients are not respond to it and for their potential side-effects such as inflammation reaction, endophthalmitis, retinal fibrosis, glial toxicity, etc [5, 6]. Moreover, scholars speculated that anti-VEGF therapy could cause retinal vascular regression, further intensify retinal ischemia and induce upregulation of placental growth factor, fibroblast growth factor or other pro-angiogenic factors, which in turn reactivate the neovascularization formation [2]. Therefore, it is urgent to explore the pathogenesis of RNV and find a more effective and economical therapeutic target.

The G protein-coupled receptor 124 (GPR124), also known as tumor endothelial marker 5 (TEM5), is a member of the class II GPR family [7, 8]. Recent studies have found that GPR124

played a significant role in the central nervous system (CNS) angiogenesis [9-11]. Various studies have determined that GPR124 was expressed in endothelial cells and pericytes during mouse embryogenesis, most prominently in the forebrain and neural tube [9]. Both global deletion and endothelial-specific deletion of GPR124 in mice resulted in embryonic lethality for the defective angiogenesis of the forebrain and spinal cord, characterized by the delayed vascular penetration, pathological glomeruloid tufts formation, and hemorrhage [9-11]. In contrast, over-expression of GPR124 in mice resulted in the CNS-specific hyperproliferative vascular malformations [9]. In addition, GPR124 was found abundantly elevated in endothelial cells during tumor angiogenesis [7].

As a part of the CNS, the formation and the characteristics of retina blood vessels are similar with the cerebral vessels, such as the blood barrier function, the interactions among pericytes, neurocytes and glial cells. Previous study has shown that GPR124 was expressed in endothelial cells of adult mouse retina [9]. However, the expression changes of GPR124 in the process of retinal angiogenesis and in the oxygen-induced retinopathy (OIR) model have not been reported. In the present study, we observed the expression and distribution changes of GPR124 in normal mice retina and in OIR retina, and further explored the cellular localization of GPR124. These results may gain an insight into the possible roles of GPR124 in the normal retinal development and in the pathogenesis of RNV, further developing a new direction for the study of RNV.

Materials and methods

Experimental animals and OIR Model

C57BL/6J pregnant mice in this study were obtained from the Animal Laboratory of Zhongshan Ophthalmic Center (Guangzhou, China). The neonatal mice were randomly divided into normal group and OIR group. In normal group, all mice were kept in room air during the whole experiment. In OIR group, the model was induced as described as our previously studies [12-14]. Briefly, the neonatal C57BL/6J mice with their nursing mothers were exposed to 75% O_2 for five consecutive days from postnatal day (P) 7 to P12. At P12, the mice were returned to room air. All procedures with animals in our study were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center.

Quantitative Real-Time PCR (qRT-PCR) Analysis

GPR124 mRNA was detected by qRT-PCR and total RNA was isolated using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and concentration of RNA samples were measured by violet spectrophotometry at 260 and 280 nm. Reverse-Transcriptase reaction was performed using reverse-transcriptase reagent kit with gDNA Eraser (Takara, Tokyo, JAPAN). qRT-PCR was performed using the SYBR Green qRT-PCR Master mix, according to manufacturer's protocol (Biotool, Houston, USA). The sample in each tube was repeated in triplicate. The CT value was normalized against β-actin and the $2^{-\Delta\Delta Ct}$ method was used to calculate GPR124 expression. The primers for mice GPR124 and β-actin were as follows: GPR124 Forward 5'-ACC ACC GTC TAG GTC CAG AT-3', GPR124 Reverse 5'-GGG TTA CCC TAG GGA CCG AT-3'; β-actin Forward 5'-CAT CCG TAA AGA CCT CTA TGC CAA C-3', β-actin Reverse 5'-ATG GAG CCA CCG ATC CAC A-3'.

Immunohistochemistry

Eyes were enucleated and fixed overnight in an admixture fixative solution consisting of formalin, dehydrated alcohol, and glacial acetic acid, then were embedded in paraffin and cut into a thickness of 6 μm. The sections were dewaxed, rehydrated and rinsed in PBS. Microwave-based antigen retrieval was performed in citrate buffer (10 mM; pH 6.0). The sections were incubated with 3% hydrogen peroxide for 25 min to block the endogenous peroxidase. Subsequently, sections were incubated with primary antibody against GPR124 (1:100; Abcam) overnight after blocked the nonspecific binding sites with 3% BSA for 30 min. On the following day, sections were incubated with HRP conjugated goat anti-rabbit IgG (1:200, KPL) for 50 min. Finally, sections were counterstained, dehydrated and mounted after DAB (DAKO) staining. Images were observed with optical microscope (Zeiss).

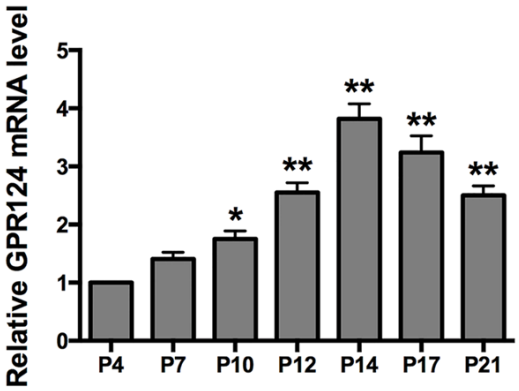

Figure 1. GPR124 mRNA expression in the normal mice retina. Mice retinas from normal group were harvested at P4, P7, P10, P12, P14, P17 and P21 and detected by qRT-PCR. qRT-PCR was performed at least three times and the expression of GPR124 was normalized by β-actin. Significantly different compared with P4. These data were presented as means ± SEM. *P<0.01, **P<0.001.

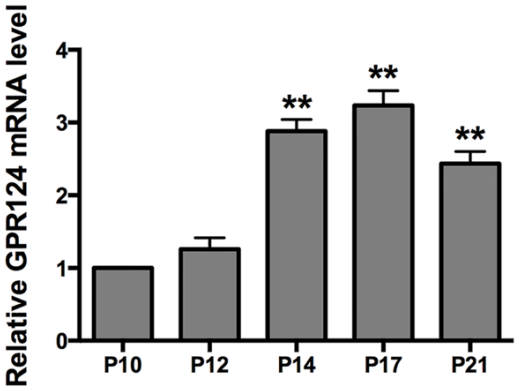

Figure 2. GPR124 mRNA expression in the OIR mice retina. Mice retinas from OIR group were harvested at P10, P12, P14, P17 and P21 and detected by qRT-PCR. qRT-PCR was performed at least three times and the expression of GPR124 was normalized by β-actin. Significantly different compared with P10. These data were presented as means ± SEM. **P<0.001.

Double labeling immunofluorescence staining

Mice in two groups were euthanized at P17, and eyes were enucleated and fixed in 4% paraformaldehyde, then were equilibrated in 30% sucrose and embedded in OCT. Frozen sections were made with a thickness of 6 μm. The sections were permeabilized with 1% Triton X-100 for 20 min, and blocked with 10% normal goat serum for 2 h. Then, sections were incubated overnight at 4°C with rabbit polyclonal primary antibodies for GPR124 (1:200; Novus Biologicals), and co-stained with mouse monoclonal primary antibodies for CD31 (1:200; Santa Cruz) or Brn3a (1:200; Santa Cruz). On the following day, sections were washed in PBS for 30 min, and incubated for 2 h with secondary antibody conjugated anti-rabbit IgG (Alexa Fluor 488, green) and anti-mouse IgG (Alexa Fluor 555, red). The nucleus was labeled with DAPI for 10 min. Images were observed under confocal microscopy (Zeiss 510; Carl Zeiss).

Statistical analysis

Each experiment consisted of at least three replicates per condition and the values were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Bonferroni post hoc test. In all cases, *P* values less than 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS 20.0 software version (SPSS Inc, Chicago, IL).

Results

Changes of GPR124 mRNA expression in the normal mice retina and in the OIR mice retina

qRT-PCR analysis was performed to study the expression pattern of GPR124 mRNA level in different time points in normal retinal development and in OIR model. As shown in **Figure 1**, GPR124 mRNA level in normal mice retina increased from birth, peaked at P14, and then gradually decreased at P17 and P21. Besides, qRT-PCR analysis for GPR124 mRNA in OIR mice retina was performed. As shown in **Figure 2**, the GPR124 mRNA level was elevated gradually from P10 to P17 in OIR mice retina. The most robust increase was found at P17 and declined subsequently. These results demonstrated that the expression of GPR124 mRNA was in a time-dependent manner in both normal and OIR mice retina.

Expression and localization of GPR124 protein in the normal mice retina and in the OIR mice retina

To determine the spatial changes of GPR124 in the normal and OIR mice retina, Immunohistochemistry was performed. Similarly to other rodent, the C57BL/6J mice retina has not been fully developed at birth. Immunohistochemistry

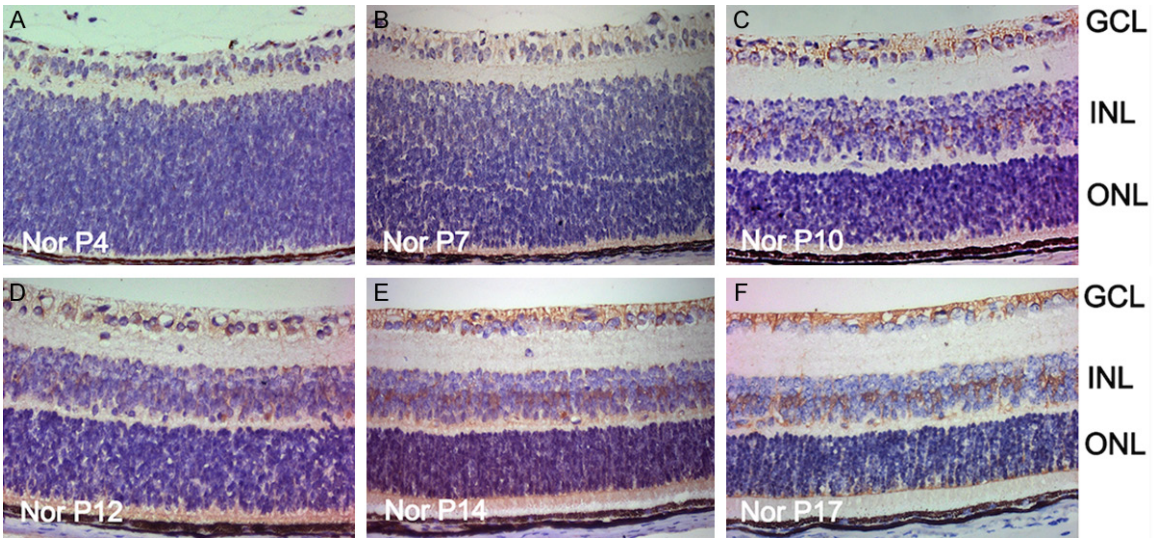

Figure 3. Immunohistochemical detection of GPR124 protein in the normal mice retina. Mice retinal paraffin sections at P4 (A), P7 (B), P10 (C), P12 (D), P14 (E) and P17 (F) from normal group were immunohistochemical staining with GPR124. Original magnification, ×400. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

showed that the retina was composed of three layers in normal retina at P4 (**Figure 3A**): the RPE cell layer, the neuroblast layer and the ganglion cell layer (GCL). At P7 (**Figure 3B**), the neuroblast layer differentiated into inner nuclear layer (INL) and outer nuclear layer (ONL), but the boundary was not clear and the cells were irregular arranged, and the photoreceptor layer still not differentiated into inner-segment/outer-segment (IS/OS). As time goes by, the boundary of INL and ONL became increasingly clear, and the short IS/OS could be observed. At P14 (**Figure 3E**), the mice retinal structure has been improved, manifested as the distinguished ten layers structure of the retina, and the completely separated INL and ONL as the result of the appearance of outer plexiform layer (OPL). At P17 (**Figure 3F**), the retinal structure was more mature as the adult mice retina.

GPR124 immunolabeling signal was observed weakly in the GCL at P4 and P7, and was appeared in the INL at P7, but the expression was still weak. As the retina development, a dramatic increase in the expression of GPR124 was occurred in the GCL and INL from P10 to P17, peaked at P14, and then stabled at P17. In OIR mice retina, GPR124 was weaker at P10 (**Figure 4A**) and P12 (**Figure 4B**) compared with the corresponding time point of normal retina. On P14, vascular expansion (**Figure 4C**, blue arrow) could be seen at the GCL, and the GPR124 expression level was elevated in GCL and INL compared with the OIR mice retina at P12. On P17 in the OIR retina, the neovascularization and the vascular clusters grew within retina and then grew toward the retinal surface or into the vitreous (**Figure 4D**, green arrows), besides, there was an abundance of chromatin condensation, pyknotic nuclei (**Figure 4D**, yellow arrows), and vacuoles (**Figure 4D**, red arrows). In addition to the localization of GPR124 in the GCL and INL in OIR mice at P17, we found that GPR124 was expressed in the new vessels either.

Immunofluorescence localization of GPR124 in the normal mice retina and in the OIR mice retina at P17

To further address the cell types expressing GPR124, double labeling immunofluorescence staining was performed on the P17 retinas from normal mice (**Figure 5**) and OIR mice (**Figure 6**) with retinal ganglion cells (RGCs) specific marker (Brn3a) and with vascular endothelial cells specific marker (CD31). GPR124 was expressed in the endothelial cells as previous researches reported [7, 9, 15], and also expressed in the new vessels of retina from OIR mice (**Figure 6A-D**). In addition, the results revealed that GPR124 expression was co-localized with Brn3a, indicating that the location of GPR124 is in the RGCs either (**Figures 5A-D**, **6E-H**).

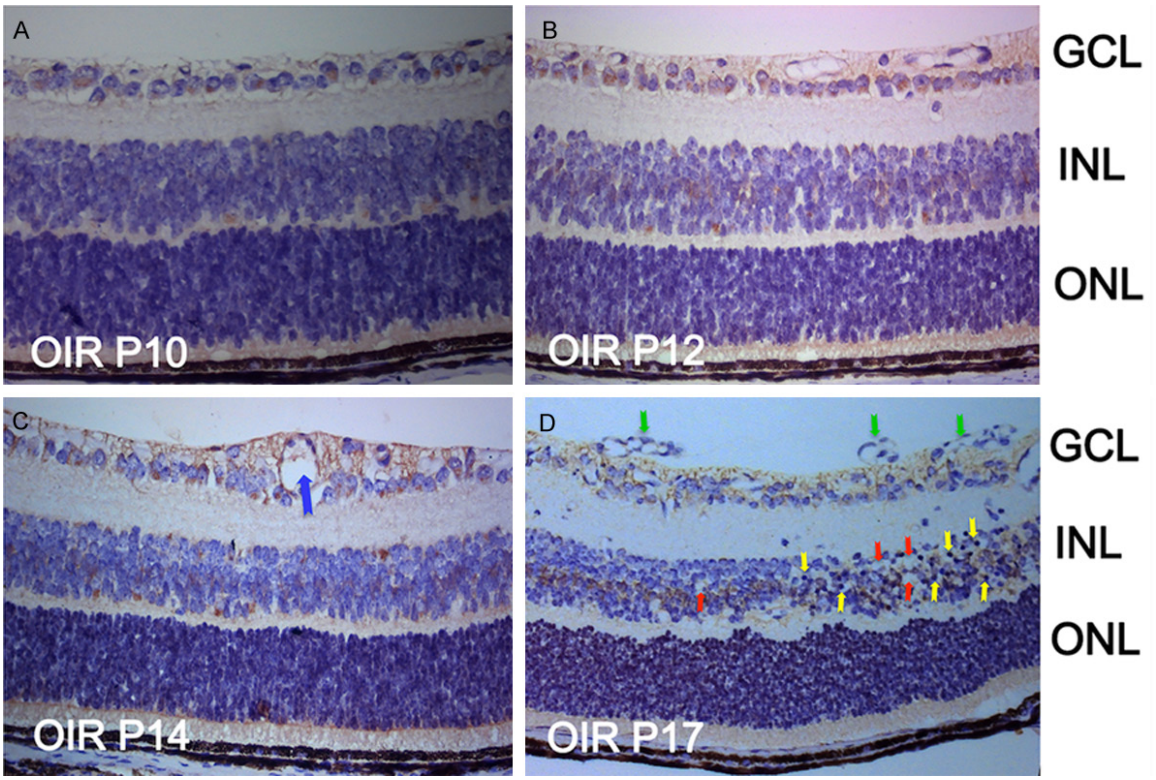

Figure 4. Immunohistochemical detection of GPR124 protein in the OIR mice retina. Mice retinal paraffin sections at P10 (A), P12 (B), P14 (C) and P17 (D) from OIR group were immunohistochemical staining with GPR124. Representative images of vascular expansion (C, blue arrow), chromatin condensation and pyknotic nuclei (D, yellow arrows), vacuoles (D, red arrows) and new vessels (D, green arrows) are shown. Original magnification, ×400. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

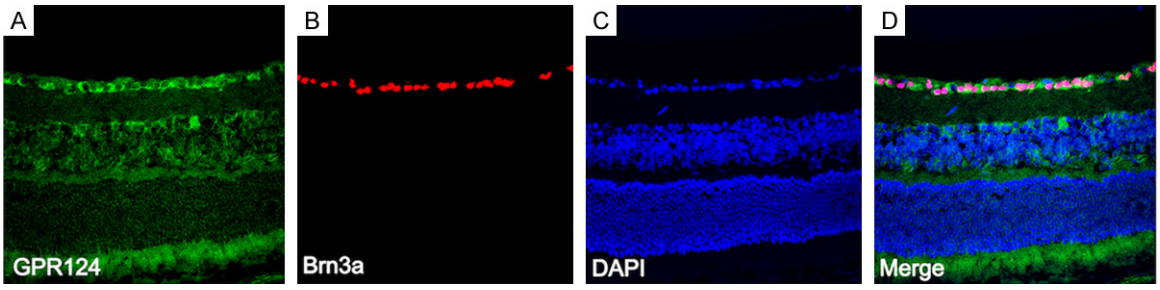

Figure 5. Immunofluorescence localization of GPR124 in the normal mice retina at P17. Mice retinal frozen sections at P17 from normal group were immunostained with GPR124 (A, green), Brn3a (B, red), DAPI (C, blue), and Merge (D, yellow). Original magnification, ×400.

Discussion

GPR124 was originally reported as a gene overexpressed in tumor vessels of human colorectal cancer [7], and was classified as orphan receptor for which the ligand has not been discovered yet [9]. GPR124 is a seven-pass transmembrane receptor, which NH2-terminal extracellular region contains several conserved subdomains and motifs: four leucine rich repeats (LRRs) domains, one LRR of the COOH-terminal domain, an immunoglobulin (Ig) domain, a hormone-receptor (HormR) domain containing an RGD (Arg-Gly-Asp) motif and a membrane proximal GPCR proteolysis site domain [7, 15]. Research has found that the RGD motif of GPR124 was exposed by proteolytic process, and then by directly interacted with integrin $\alpha_v\beta_3$ to mediate endothelial cells adhesion and survival [15]. Previous studies have reported

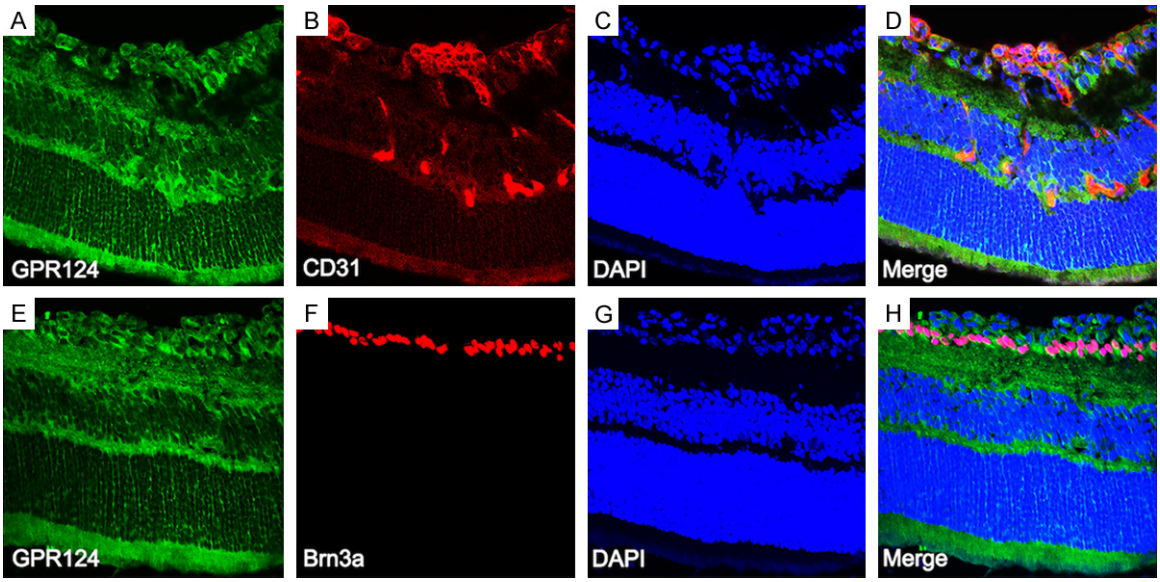

Figure 6. Immunofluorescence localization of GPR124 in the OIR mice retina at P17. Mice retinal frozen sections at P17 from OIR group were immunostained with GPR124 (A, E; green), CD31 (B, red), Brn3a (F, red), DAPI (C, G; blue), and Merge (D, H; yellow). Original magnification, ×400.

that GPR124 displayed elevated expression in endothelial cells during tumor and physiologic angiogenesis [7, 9, 15]. Besides expressed in CNS angiogenesis, GPR124 was also widely expressed in non-CNS embryonic organs or tissues throughout the mouse embryogenesis, including the heart, liver, kidney, epithelium of lung and esophagus and in mesenchyme. In adult mouse, however, the GPR124 was specifically expressed in brain endothelium and angiogenic pericytes and in pericytes of non-neural organs, including the kidney, pancreas, and corpus luteum [9].

As the extended part of the brain, the development of retina is highly correlated with the CNS. In consideration of the pivotal role of GPR124 in CNS and in tumor angiogenesis, we have reasons to speculate that GPR124 plays a role in retinal angiogenesis and in retinal neovascularization. Our data revealed that GPR124 was progressively up-regulated in the normal retinal development, peaked at P14, and then gradually decreased. As we know, the development of mouse retinal vascular is approximately mature at P14. Thus, the high consistency of time axis between GPR124 expression and retinal angiogenesis indicates that GPR124 participates in the retinal vascular development. In normal tissues, GPR124 was upregulated in endothelial cells during capillary morphogenesis; however, it was not upregulated in mature blood vessels [16]. To further explore the reason, researchers found that GPR124 could mediate contact inhibition of proliferation in endothelial cells during angiogenesis but not to maintain it in mature vessels [17].

OIR is a widely used mouse model for the research on molecular mechanisms of RNV. In the hyperoxic stage of OIR, high oxygen arrest the development of retinal vascular, leading to the existing blood vessels degenerate and the vaso-obliterated zones formation in central retina. In the hypoxic stage of OIR, the ischemic retina triggers a compensatory release of pro-angiogenic factors, leading to abnormal retinal neovascularization. In our present study, the expression of GPR124 was weak at the hyperoxic stage, then elevated gradually and peaked at P17. These results revealed that the expression of GPR124 may be influenced by the oxygen stress and may participate in the formation of retinal neovascularization.

In the next experiments, we investigated the localization and spatial expression change of GPR124. Immunohistochemistry results showed that GPR124 expression was located in GCL and INL in both normal and OIR mice retina, and also expressed in the abnormal preretinal neovascularization in OIR. To further

address the cell types, double labeling immunofluorescence staining was performed. Our findings confirmed that GPR124 was expressed in endothelial cells, consistent with previous report [9]. In addition, the co-localization analysis of GPR124 and Brn3a indicated that GPR124 was expressed in RGCs either. The similar result was found in GPR91, a seven-transmembrane GPR expressed in RGCs, was a mediator of vessel growth in both normal retinal development and RNV and was reported to modulate the expression of VEGF in OIR model [18, 19]. However, whether GPR124 and GPR-91 might have homologous structure and function should be further demonstrated. Besides the vital role in CNS-specific vascularization, GPR124 controlled the establishment of the blood-brain barrier [9, 11] and regulated VEGF/VEGFR signaling in tumor angiogenic processes including endothelial cells interaction, permeability, migration, invasion, and tube formation [20]. GPR form a large protein family that plays an important role in many physiological and pathological processes. It has been revealed that numerous diseases are GPR-related and the GPR have become an excellent therapeutic target in about 50% drugs because of their natural ligands can be mimicked for agonistic or antagonistic purposes [21, 22]. Thus, GPR124 is an attractive target for preventing RNV.

In conclusion, our study demonstrated for the first time that GPR124 was expressed in the normal retinal development and in the OIR mice retina in a time-dependent manner, and located in the endothelial cells of retinal blood vessels and in RGCs. The spatiotemporal changes of GPR124 expression demonstrated that GPR124 might play a role in regulating retinal neurovascular development and retinal neovascularization. Therefore, GPR124 might be used as an anti-angiogenic factor and served as a potential novel target to control the RNV. Further study will be performed on GPR124 gene over-expression or silencing mice to show its role on retinal angiogenesis and neovascularization by construct GPR124 expression vector or GPR124-siRNA vector.

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Disclosure of conflict of interest

None.

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