

## BASIC SCIENCE ARTICLE



# Propranolol ameliorates retinopathy of prematurity in mice by downregulating HIF-1 $\alpha$ via the PI3K/Akt/ERK pathway

Shaomin Su<sup>1,2,6</sup>, Peicen Zou<sup>3,6</sup>, Guangran Yang<sup>4</sup>, Yajuan Wang<sup>5</sup>✉, Lei Liu<sup>1</sup>, Ying Liu<sup>1</sup>, Jinjing Zhang<sup>1</sup> and Yijun Ding<sup>1</sup>

© The Author(s), under exclusive licence to the International Pediatric Research Foundation, Inc 2022

**BACKGROUND:** Retinopathy of prematurity (ROP) is the leading cause of blindness in infants, and elevation of HIF-1 $\alpha$  through the PI3K/Akt and ERK pathways is implicated in ROP pathogenesis. The mechanism of action of propranolol in ROP remains controversial. We investigated the effect of propranolol on ROP and explored its potential mechanisms of action in an oxygen-induced retinopathy (OIR) mouse model.

**METHODS:** OIR mice were first treated with propranolol intraperitoneally, and the retina integrity was measured by FITC-dextran and hematoxylin-eosin staining. The expression of HIF-1 $\alpha$ , VEGF, and key signaling pathway proteins was determined using real-time PCR and western blotting.

**RESULTS:** FITC-dextran staining showed that propranolol treatment reduced damage to retinal morphology in OIR mice. Mice treated with propranolol showed a reduced number of nuclei of vascular endothelial cells penetrating the inner limiting membrane of the retina, confirming the therapeutic effect of propranolol on ROP. Further analysis showed that HIF-1 $\alpha$  and PI3K/Akt/ERK pathway protein levels were significantly elevated in OIR mice. In contrast, propranolol treatment downregulated the expression of these proteins, indicating that the PI3K/Akt/ERK/HIF-1 $\alpha$  axis is associated with propranolol-induced ROP alleviation.

**CONCLUSIONS:** Propranolol has a therapeutic function against ROP, likely through the downregulation of HIF-1 $\alpha$  via the PI3K/Akt/ERK pathway.

*Pediatric Research* (2023) 93:1250–1257; <https://doi.org/10.1038/s41390-022-02211-8>

**IMPACT:**

- Propranolol can reduce the formation of abnormal retinal neovascularization in oxygen-induced retinopathy (OIR) models, and reduce leaking, tortuous, and abnormally expanding retinal blood vessels.
- Propranolol possibly improves OIR by inhibiting the activated ERK and HIF-1 $\alpha$  pathways. Furthermore, propranolol may downregulate HIF-1 $\alpha$  via the PI3K/Akt/ERK pathway to ameliorate retinopathy of prematurity.
- This study elucidated that the therapeutic effect of propranolol in OIR mice does not involve the VEGFR-2 pathway.

**INTRODUCTION**

Retinopathy of prematurity (ROP) is characterized by abnormal retinal vascular development in premature infants and is the leading cause of blindness in infants,<sup>1–3</sup> and a major challenge in protecting children's vision. ROP incidence varies greatly between countries; in developed countries such as the United States, the incidence of ROP is approximately 15.58%,<sup>1</sup> whereas, in developing countries, the incidence of ROP is much higher. For example, in northern India, an incidence between 26.6 and 49% in 20 years has been reported.<sup>4,5</sup> Currently, China does not have any statistics on ROP incidence. However, an analysis of existing studies in some regions shows that the incidence is between 10 and 20%.<sup>6–9</sup> Blencowe et al.<sup>10</sup> estimated that as many as 32,200 infants lost their vision owing to ROP in the year of diagnosis. Globally, Solebo et al.<sup>11</sup> estimated that approximately 14 million children have blindness,

with ROP and cataracts being the most common causes. The overall incidence of ROP has increased due to the improved survival rate of low and very low birth weight babies and premature babies because of advancing neonatal medicine.

ROP occurs due to premature exposure to oxygen outside the womb, causing physiological vascular occlusion, which then promotes the expression of angiogenic factors and, in turn, causes pathological changes, such as retinal vessel hyperplasia. Among the animal models of human ROP, the oxygen-induced retinopathy (OIR) model is currently the most widely used. The roles of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in ROP have been relatively well studied in OIR models. VEGF plays an important role in ROP.<sup>12,13</sup> Hypoxia and other factors can increase the transcription of VEGF and stabilize the transcribed VEGF mRNA, thereby increasing the

<sup>1</sup>Department of Neonatology, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, Beijing, China. <sup>2</sup>Department of Neonatology, Shenzhen Children's Hospital, Shenzhen, China. <sup>3</sup>Department of Neonatology, Capital Institute of Pediatrics, Beijing, China. <sup>4</sup>Department of Endocrinology, Beijing Tongren Hospital, Capital Medical University, Beijing, China. <sup>5</sup>Department of Neonatology, Children's Hospital, Capital Institute of Pediatrics, Beijing, China. <sup>6</sup>These authors contributed equally: Shaomin Su, Peicen Zou. ✉email: cxswyj@vip.sina.com

Received: 28 October 2021 Revised: 13 June 2022 Accepted: 28 June 2022

Published online: 19 August 2022

expression of VEGF mRNA and protein. This, in turn, induces angiogenesis and vascular permeability, resulting in ROP. The oxygen concentration regulates HIF-1 $\alpha$ . Under hypoxia, HIF-1 $\alpha$  cannot be hydroxylated, leading to an increase in HIF-1 $\alpha$  concentration. By regulating the expression of various factors in the body, it promotes new blood vessel formation and other processes.<sup>14,15</sup> OIR models have shown that HIF-1 $\alpha$  increases with VEGF and retinal neovascularization. This finding suggests that HIF-1 $\alpha$  is closely related to retinal neovascularization. The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway is involved in angiogenesis and has been reported to play an important role in ROP development.<sup>16–18</sup> In addition, the PI3K/Akt pathway is associated with HIF-1 $\alpha$  expression.<sup>19</sup> However, the role of the PI3K/Akt pathway in ROP remains unclear.

Propranolol, a non-selective  $\beta$ -adrenergic blocker, can lead to the treatment of hemangioma regression, possibly by intervening in the formation of new blood vessels and inducing apoptosis of vascular endothelial cells. Previous studies have shown that propranolol can inhibit pathological retinal neovascularization and blood–retinal barrier rupture by downregulating VEGF expression in OIR models.<sup>20–22</sup> Furthermore, clinical studies conducted by Filippi et al.<sup>23,24</sup> found that oral administration of propranolol reduced the risk of ROP progression and the occurrence of adverse ocular events, such as strabismus. Therefore, the OIR model is considered the most representative model for studying human ROP. However, it has also been reported that propranolol can neither affect VEGF expression nor inhibit pathological neovascularization in OIR models.<sup>10</sup> Therefore, the effectiveness of propranolol in the treatment of ROP remains controversial. In this study, we aimed to confirm the therapeutic value of propranolol in ROP and further explore the underlying mechanisms in an OIR mouse model.

## MATERIALS AND METHODS

### Animals

Forty-eight specific antigen-free C57BL/6J mice aged 7 days, both male and female, were bred together with lactating mice (provided by the Military Experimental Animal Center of the Chinese People's Liberation Army Medical College). All animal experiments were approved by the institutional ethical review board and performed in accordance with the "Regulations on the Administration of Laboratory Animals" set by the National Science and Technology Commission.

### Oxygen-induced retinopathy (OIR) animal model

The OIR animal model was established according to the classical method proposed by Smith.<sup>25</sup> Mice were randomly grouped, with six baby mice and one lactating mouse placed in a sealed oxygen chamber. Then, 100% humidified medical oxygen with a flow rate of 10 L/min was introduced, and was changed to a small flow rate of 0.5 L/min after about 10 min. Thereafter, once the oxygen concentration detector reached the range of 75  $\pm$  2%, it was stabilized for 5 min. The temperature was set at 25  $\pm$  2  $^{\circ}$ C. The chamber conditions were recorded every 8 h. The chamber was illuminated with fluorescent lamps, and the photoperiod was 12 h/12 h. After 5 days, when the mice were at postnatal day (PD) 12, the mice were removed from the chamber and kept in normal air for 5 days (until the baby mice were 17 days old). The vital signs and mobility of the mice were monitored daily.

### Animal grouping and treatment

This study followed a previous method,<sup>26</sup> and the methods of eye drop administration and intraperitoneal injection were compared. Both methods showed that propranolol intervention reduced the occurrence of oxygen-induced retinal neovascularization; however, the eye drop delivery method was superior to intraperitoneal injection. As the expected results were obtained using both methods, a technically mature intraperitoneal injection was adopted in this study. In addition, propranolol at 2, 20, and 50 mg/kg was administered thrice a day in preliminary experiments, and it was found that 20 and 50 mg/kg propranolol caused a high mortality rate, whereas there was little difference in retinal vascular changes among the

three groups. The mice were then divided into the normal control, disease, and propranolol groups. In the normal control group, the mice were raised to PD 17 in a normal environment without intervention. For the propranolol-treated OIR, at PD 12–17, mice were intraperitoneally injected with propranolol (2 mg/kg of body weight) in citrate buffer (Sigma-Aldrich, St. Louis, MO) thrice a day. OIR mice were intraperitoneally injected with citrate buffer at the same volume and frequency as those in the propranolol group.

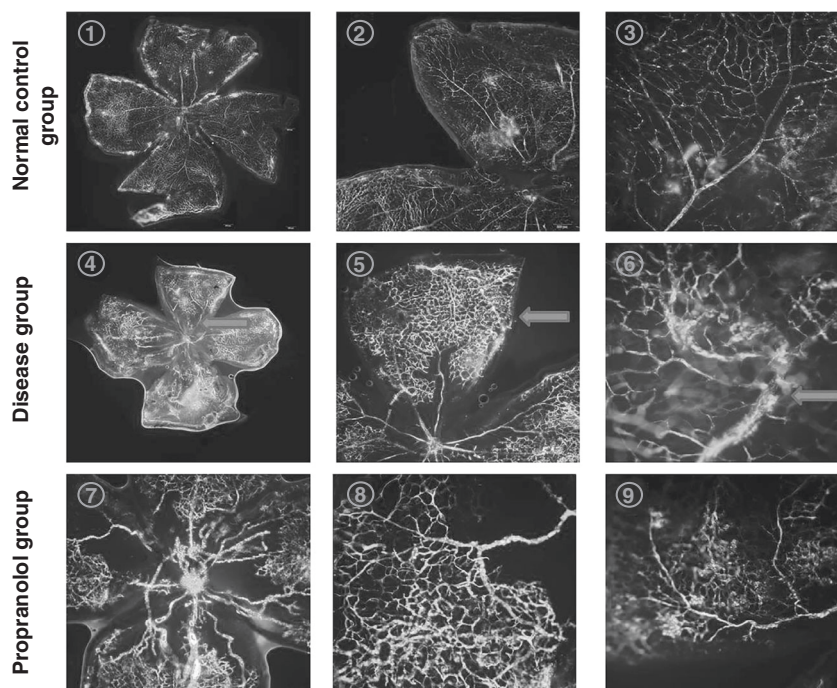
### Histology

(1) Fluorescein-labeled isothiocyanate dextran (FITC-dextran) retinal staining: three mice in each group aged 17 days were anesthetized with a lethal dose of chloral hydrate. The chest cavity was quickly opened, the heart exposed, and 0.3 mL of 50 mg/mL FITC-Dextran (Sigma-Aldrich, St. Louis, MO) was perfused from the apex to the left ventricle. The heart was squeezed gently with forceps to aid perfusion and discoloration of the tongue tip and upper limb indicated that the perfusion was successful. After 5 min, the eyes were removed and placed in phosphate-buffered saline to peel the retina completely. Under the operating microscope, the eyeball was cut circularly along the corneal and scleral limbus, the cornea and lens were removed, and the retina was carefully dissected along the serrated edge to separate it from the choroid. Taking the optic disc as the center, the retina was cut into four equal segments, sealed with glycerin, spread on a glass slide, and placed under a fluorescence microscope (Olympus, Japan) for observation and imaging. The acquired images were stitched together using Adobe Photoshop CS6 to evaluate the OIR model and observe any effect of propranolol on morphology.

(2) Hematoxylin-eosin (HE) staining: the mice were culled by cervical dislocation at the age of 17 days, and their eyes were quickly removed with part of the optic nerve attached. The eyeballs were fixed in 4% paraformaldehyde for 12–24 h, dehydrated, cleared with xylene, and embedded in paraffin. The residual wax at the edge of the eyeball tissue (0.1–0.2 cm) was removed to prevent tissue shrinkage. Serial sectioning was performed parallel to the cornea and optic nerve at a thickness of 3  $\mu$ m and at an interval of 6  $\mu$ m. Ophthalmic tweezers were used to pick up the wax tape and gently spread it on the water surface of 40–45  $^{\circ}$ C, and flatten the slightly wrinkled wax tape naturally. After the slices were fully flattened on the constant temperature water surface, the wax slices were removed to the middle of the glass slide, and the remaining water on the glass slides was discarded; then, the slices were placed in a 60–65  $^{\circ}$ C incubator and baked for 15–30 min to remove and dissolve interstitial paraffin. The sections were then stained with HE (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) and observed under a microscope (Olympus, Japan). Five sections were acquired from each eyeball to avoid optic nerves and peripheral retina sections. The number of vascular endothelial cell nuclei that broke through the inner limiting membrane of the retina was counted; however, endothelial cells that exist in the vitreous cavity, far away from the inner limiting membrane, close to the lens, and no obvious connection with the retina were not counted. Any abnormal neovascularization in the retina and the effects of propranolol were evaluated.

### Western blotting analysis

Western blotting was used to detect the expression of HIF-1 $\alpha$  (NB 100-479), VEGF, PI3K, Akt, phosphorylated (p)-Akt, extracellular signal-regulated kinase (ERK), and phosphorylated (p)-ERK (Cell Signaling Technology, Inc.) in the retinal. Mice were culled at 17 days, and the retina was isolated and shredded by clean tissue scissors. RIPA lysis buffer (containing PMSF final concentration 1 mM) was added to the samples, homogenized, lysed on ice for 20 min, centrifuged for 5 min at 4  $^{\circ}$ C and 12,000 rpm, and the supernatant was obtained. The entire operation was kept on ice. The protein concentration was then quantified with a BCA kit (Beyotime, China), and 50  $\mu$ g of total protein was loaded and subject to sodium dodecyl-sulfate polyacrylamide gel electrophoresis. After electrophoresis, the resolved proteins were transferred onto a polyvinylidene fluoride membrane. The membrane was then blocked with 5% non-fat milk (room temperature (RT), 1 h) and sequentially incubated with primary antibodies (1:1000 dilution, 4  $^{\circ}$ C, overnight) and horseradish-peroxidase-conjugated secondary antibodies (1:5000 dilution, RT, 1 h). Following incubations, the membrane was extensively washed with phosphate-buffered solution, and the immune bands were visualized with Enhanced Chemiluminescence substrate (Beyotime, China).



**Fig. 1 Morphological changes in the mice retinal blood vessels in the oxygen-induced retinopathy (OIR) model and after intraperitoneal injection of propranolol.** Mice at the age of 17 days were anesthetized and perfused with 50 mg/mL FITC-dextran into the left ventricle. Following that, the retina was removed and imaged under a fluorescent microscope. ①–③: normal control group; ④–⑥: disease group; and ⑦–⑨: propranolol group.

### Real-time PCR

Total RNA was extracted from the retina using the RNeasy Mini Kit (Qiagen, Germany) and reverse-transcribed into cDNA using TransScript<sup>®</sup> First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China), according to the manufacturer's instructions. VEGF and  $\beta$ -actin were amplified by PCR using the TransStart<sup>®</sup> Tip Green qPCR SuperMix (TransGen Biotech, China). The relative mRNA level of VEGF was calculated using the  $2^{-\Delta\Delta Ct}$  method with  $\beta$ -actin as the internal control. The following primer pairs were used in the current study:  $\beta$ -actin-F: 5'-GTGACGTTGACATCCGTAAAGA-3',  $\beta$ -actin-R: 5'-GCCGGACTCATCGTACTCC-3'; VEGF-F: 5'-GCACATAGAGAGAATGAGCTTCC-3'; VEGFR: 5'-CTCCGCTGAACAAGGCT-3'; VEGFR-2-F: 5'-TTTGGCAAATACAACCCTCAGA-3'; VEGFR-2-R: 5'-GCTCCAGTATCATTCCAACCA-3'; PI3K-F: 5'-CGAGAGTGTGTCACAGTGTG-3'; PI3K-R: 5'-TGTTGCTTCCACAAACAAG-3'.

### Image processing and statistical analysis

Adobe Photoshop CS6 was used to splice the acquired images. Statistical analysis was performed using SPSS 17.0. One-way analysis of variance followed by Tukey's multiple comparison test was performed to compare the results among the different experimental groups. Statistical significance was set at  $p < 0.05$ . GraphPad Prism 5 was used to plot the graphs.

## RESULTS

### Morphological changes in retinal blood vessels were observed in the OIR mice after propranolol injection

Following FITC-dextran retinal staining, significant differences were observed in the morphology of the retinal blood vessels among the different experimental groups.

(1) Normal control group: major blood vessels branched from the focal fovea and extended around the retina with no obvious deformation. As the distance from the focal fovea gradually increased, the blood vessels became smaller and more evenly distributed around the retina. No obvious absence of blood vessels was observed between the different quadrants. The interspace between blood vessels appeared black, with no sign of fluorescent

markers, indicating that the blood vessel wall remained intact with no leakage (Fig. 1①–③).

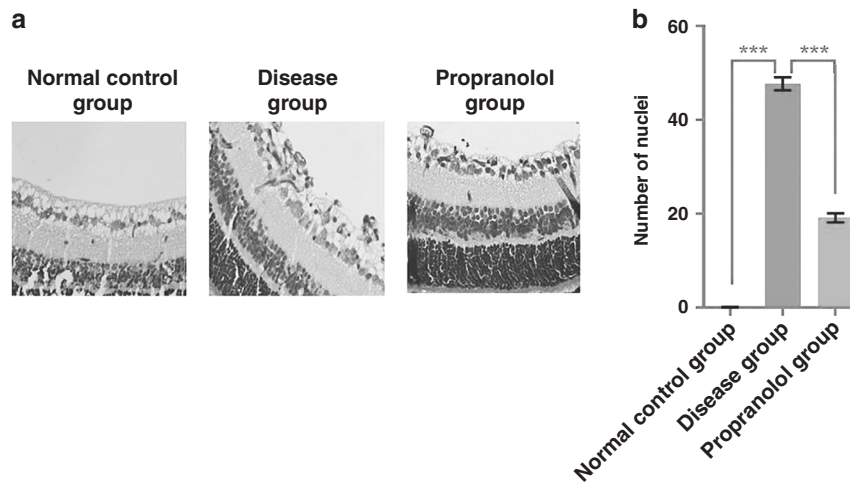
(2) Disease group: major blood vessels branched from the focal fovea, with sublevel vessels becoming smaller further away. However, compared with the normal control group, there were fewer blood vessels around the focal fovea, and the distribution of blood vessels was less even at all levels. Figure 1④ shows the absence of blood vessels in the area near the focal fovea, forming a nonperfusion area. Figure 1⑤–⑥ shows that as the distance from the focal fovea gradually increases, branching blood vessels become more deformed and tortuous. At the edge of the retina, the blood vessels appeared highly disordered and complicated, indicating neovascularization. Leakage of fluorescent markers was also observed on the periphery of the blood vessels, indicating poor integrity of the blood vessel wall. Thus, based on the morphological observations, an OIR model was successfully established.

(3) Propranolol intraperitoneal injection group: as shown in Fig. 1⑦–⑨, the blood vessels were still absent near the fovea. However, the morphology of the blood vessels appeared less deformed and complex compared to those of the disease group. There was also less blood vessel proliferation and exudation of the fluorescent markers. In previous studies,<sup>26</sup> the mean ratios of the retinal avascular and neovascularized areas to the total retinal area were analyzed between the OIR and the propranolol-treated OIR group ( $38.0 \pm 7.7\%$ ,  $5.4 \pm 0.7\%$  vs  $25.9 \pm 5.0\%$ ,  $2.1 \pm 2.7\%$ ), and the difference was statistically significant ( $p < 0.01$ ).

### Propranolol reduced the number of nuclei in vascular endothelial cells penetrating the inner limiting membrane of the retina

The internal limiting membrane is a homogeneous membrane composed of the Müller cell basement membrane, a small number of glial cells, and vitreous fibers, and the smooth inner surface is in contact with the vitreous cortex. Almost no nuclei penetrated the internal limiting membrane in the normal mouse





**Fig. 2** The number of cell nuclei penetrating the retinal inner limiting membrane after intraperitoneal injection of propranolol. Mice were culled at the age of 17 days, and eyeballs were fixed and embedded in paraffin. The paraffin tissue was then serially sectioned, stained with hematoxylin-eosin (HE), and observed under a microscope. Five sections per eyeball were acquired. **a** Representative HE staining data are shown. **b** The nuclei penetrating the inner limiting membrane were counted ( $n = 5$ ). \*\*\* $p < 0.001$ .

retina, but the number of nuclei penetrating the internal limiting membrane was increased in the OIR mice retina. The difference in the number of nuclei penetrating the inner limiting membrane of the retina was statistically significant ( $p < 0.001$ ) between the different experimental groups. The normal control group (Fig. 2a) showed a clear inner limiting membrane, with an average number of nuclei penetrating the membrane of  $0.13 \pm 0.34$  per section. In the negative control group, however, numerous nuclei penetrated the inner limiting membrane (Fig. 2b). Small circular blood vessels and red blood cells in the lumen were observed in some paraffin sections. The average number of nuclei that broke through the inner limiting membrane was  $47.73 \pm 7.37$  per section, significantly higher than that in the normal control. The difference between the two groups was statistically significant ( $p < 0.001$ ). In OIR mice injected with propranolol, the inner limiting membrane cell nucleus appeared broken, but the number of nuclei penetrating the membrane was significantly less than that in the disease group, with an average of  $19.20 \pm 5.13$  per section.

#### Propranolol ameliorates ROP possibly through the downregulation of HIF-1 $\alpha$ via the PI3K/Akt/ERK pathway

The expression of PI3K, Akt, p-Akt, ERK, p-ERK, and HIF-1 $\alpha$  was determined using western blotting. Our data showed that ROP mice (negative control group) exhibited significantly elevated HIF-1 $\alpha$  expression (Fig. 3). Moreover, expression of key factors of the PI3K/Akt/ERK pathway (PI3K, p-Akt, and p-ERK), which are reported to regulate HIF-1 $\alpha$  expression, were also increased in ROP mice compared to those in normal control mice (Fig. 3). In mice treated with propranolol, the levels of HIF-1 $\alpha$ , PI3K, p-Akt, and p-ERK were lower than those in the ROP mice. These data indicate that propranolol ameliorates ROP by downregulating HIF-1 $\alpha$  via the PI3K/Akt/ERK pathway.

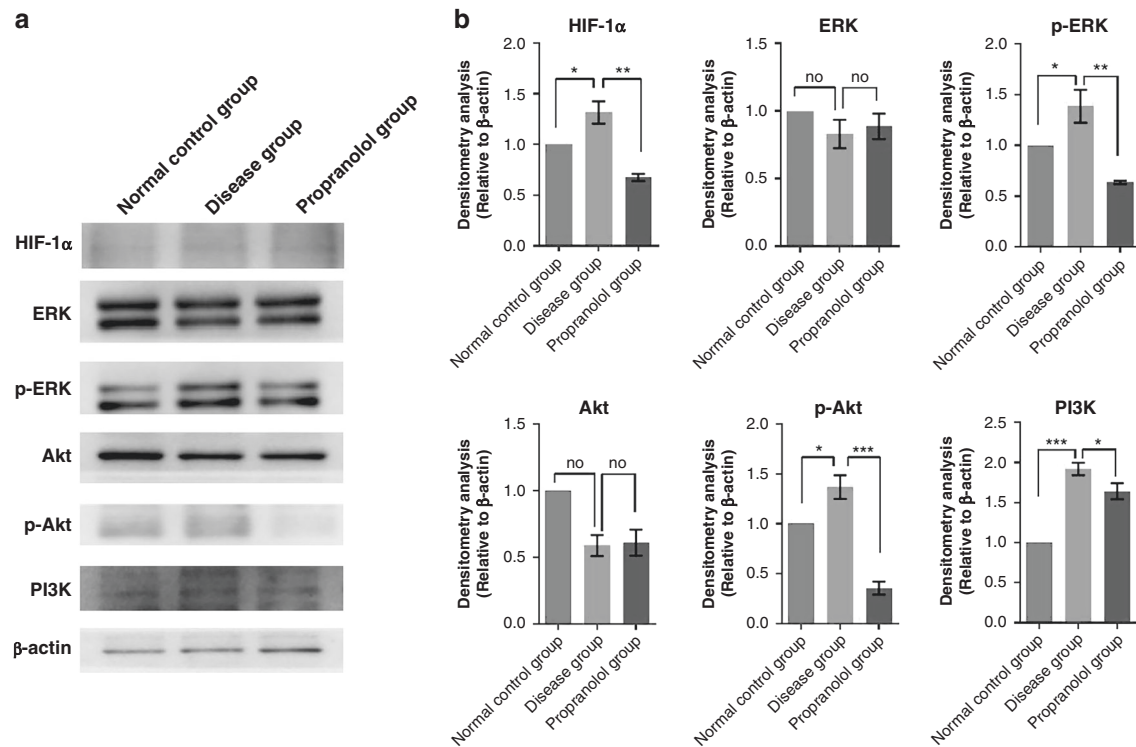
We also examined the involvement of VEGF in propranolol-induced ROP alleviation. As shown in Fig. 4, VEGF mRNA and protein expression were not significantly different between the normal control, disease, and propranolol groups. In addition, the expression of the VEGF receptor VEGFR-2 was also measured, and our data showed that VEGFR-2 was significantly increased in the disease group compared to the normal control group, indicating that VEGFR-2 might be associated with ROP pathogenesis. However, propranolol treatment did not alter VEGFR-2 expression, implying that the therapeutic effect of propranolol does not involve the VEGFR-2 pathway.

#### DISCUSSION

ROP is a retinal vascular proliferative disease of the premature developing retina that remains a major cause of significant visual morbidity, including blindness and visual handicaps, in extremely preterm infants globally. With improved perinatal care and survival of moderately preterm infants, more mature preterm infants are developing severe ROP. ROP occurrence is affected by several independent factors, including birth weight and gestational age, and is negatively correlated with the birth weight and gestational age of preterm infants; that is, the lower the birth weight and the younger the gestational age, the higher the incidence of ROP.<sup>27</sup> Other related risk factors include high oxygen consumption, infection, anemia, and maternal health during pregnancy and the perinatal period.<sup>28</sup>

Although most mild ROP cases do not require treatment and can resolve spontaneously, the prevention and treatment of severe ROP remain a huge challenge. Presently, the main treatment methods for severe ROP include cryotherapy, laser photocoagulation, and intravitreal injection of antiangiogenic drugs. However, these methods can cause adverse ocular effects such as astigmatism and reduced vision. Furthermore, they are expensive and require technologies that are difficult to implement in poorer areas where ROP incidence is usually high. Therefore, there is a need to develop novel and simpler treatments involving drugs.

Propranolol is a non-selective  $\beta$ -adrenergic blocker that is widely used to treat arrhythmia, hypertension, and other diseases.<sup>25,29–31</sup> In recent years, propranolol has been found to inhibit angiogenesis and has been used to treat hemangioma.<sup>32–34</sup> Studies have found that propranolol can also reduce the formation of abnormal retinal neovascularization in OIR models and reduce leakage, tortuosity, and abnormally expanding retinal blood vessels.<sup>21,35,36</sup> Filippi et al.<sup>23,24</sup> conducted propranolol-related clinical studies on some children with ROP and found that propranolol can reduce ROP progression and the occurrence of adverse ocular events, such as strabismus. In this study, the retinas of OIR mice subjected to intraperitoneal injection of propranolol showed no vascular deformation, and the peripheral area showed less proliferation of blood vessels and less exudation than the disease group. We also found that propranolol reduced the number of cells penetrating the inner limiting membrane in the retina of OIR mice. Therefore, intraperitoneal injection of propranolol is considered useful for reducing neovascularization in OIR and improving the structural integrity of the blood vessel wall to a certain extent.



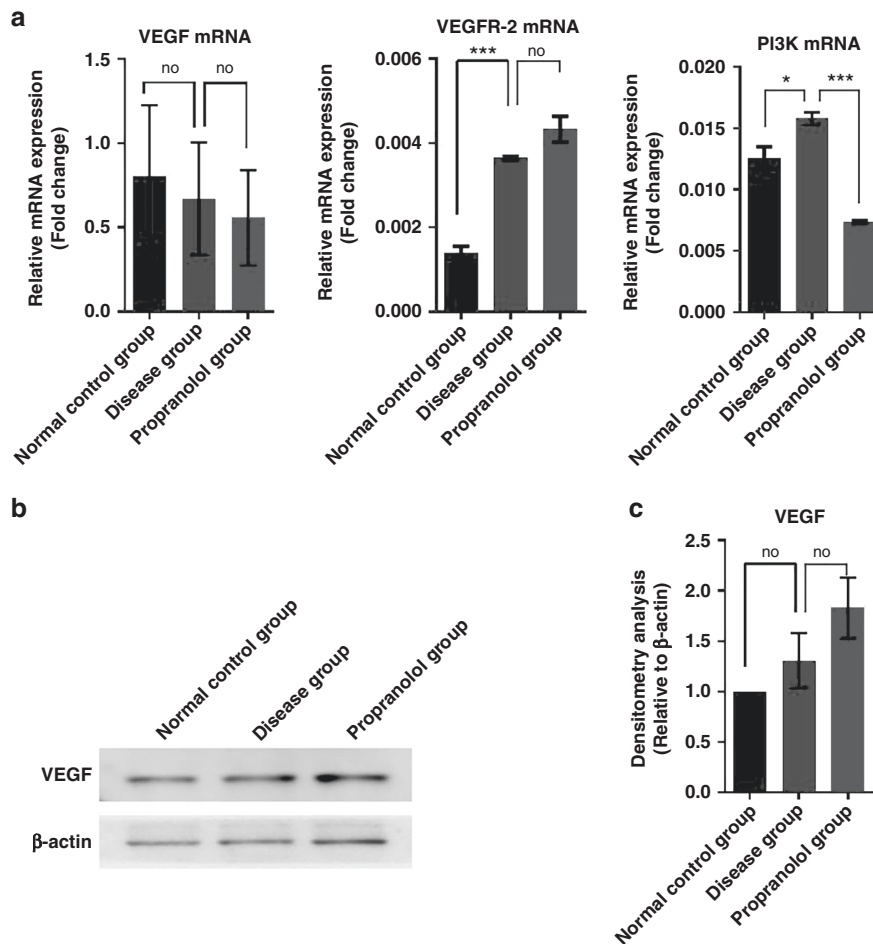
**Fig. 3** Propranolol possibly ameliorates retinopathy of prematurity (ROP) through the downregulation of HIF-1 $\alpha$  via the PI3K/Akt/ERK pathway. Mice were culled at the age of 17 days, and the expression of HIF-1 $\alpha$ , PI3K, Akt, p-Akt, ERK, and p-ERK was assessed by western blotting. **a** One representative result is shown. **b** The relative protein expression was semi-quantified by densitometry analysis. Data shown are mean  $\pm$  SD of three independent experiments. no, not statistically significant; \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001.

VEGF can induce the formation of new blood vessels, increase vascular permeability, and plays an important role in various pathological processes such as the development of tumors, ROP, and diabetic retinopathy.<sup>12,37–39</sup> This study showed that VEGF and VEGFR mRNA expression did not change significantly after intraperitoneal injection of propranolol, suggesting that abnormal angiogenesis in the retina of OIR mice may not be regulated by increasing VEGF expression. VEGFR includes multiple receptors (VEGFR), among which VEGFR-2 plays an important regulatory role in inducing neovascularization and increasing vascular permeability.<sup>40–42</sup> McLeod et al.<sup>43</sup> found that the expression of VEGFR-2 and its mRNA increased significantly in the OIR model, and VEGFR-2 antagonists reduced abnormal retinal neovascularization formation and the intravitreal neovascularization area. Our study found that the VEGFR-2 mRNA expression increased in the OIR and propranolol-treated OIR models and that in the experimental group did not differ significantly from that in the disease group. This suggests that the mechanism by which propranolol improves OIR may not be through regulating the VEGF expression and its receptors.

HIF-1 protein includes  $\alpha$  and  $\beta$  subunits. HIF-1 $\alpha$  is regulated by oxygen concentration. Propranolol can inhibit the expression of HIF-1 $\alpha$  in the OIR model. Under normal oxygen tension, prolyl 4-hydroxylase hydroxylates 402/564 proline residues of HIF-1 prior to its degradation by ubiquitin protease. Under hypoxic conditions, hydroxylase activity is inhibited, allowing the accumulation of HIF-1 $\alpha$  protein. In this study, the expression of HIF-1 $\alpha$  was increased in the OIR model, consistent with the results of Liu et al.,<sup>44</sup> suggesting that the HIF-1 $\alpha$ -related pathways are activated during OIR formation, which in turn promotes abnormal blood vessel formation and increased vascular permeability. In this study, propranolol was injected into the intraperitoneal cavities of OIR mice. After administration, HIF-1 $\alpha$  expression decreased significantly, suggesting its role in inhibiting HIF-1 $\alpha$  expression. Previous

studies have also treated OIR mice with HIF inhibitors, which reduced the retina's abnormal proliferation of blood vessels. This further proves the importance of HIF in the formation of the OIR.<sup>15</sup>

PI3K, a second messenger in the cell, is necessary for Akt (also known as protein kinase B, PKB) to be transported to the cell membrane and activated. It can phosphorylate Akt and regulate cell growth, proliferation, survival, and other functions. Activating the PI3K/Akt signaling pathway can inhibit apoptosis that is caused by various factors, accelerate cell cycle progression, and promote cell survival, proliferation, and anti-apoptosis.<sup>45–47</sup> Concurrently, the PI3K/Akt signaling pathway is also involved in the formation of blood vessels and plays a crucial role in the formation and development of ROP.<sup>16–18</sup> PI3K/Akt is an upstream regulator of HIF-1 $\alpha$ .<sup>48</sup> Activation of this signaling pathway has been confirmed to activate the translation initiation factor, thereby participating in the HIF-1 $\alpha$  translation process.<sup>19</sup> In this study, the activation of PI3K and p-Akt in the disease group was evident, indicating that this pathway was activated. After the intraperitoneal injection of propranolol, the expression of PI3K and p-Akt in the mouse retina decreased, and the difference was statistically significant, suggesting that PI3K/Akt inhibition after intraperitoneal injection of propranolol may be important for disease improvement. HIF-1 $\alpha$  is a downstream factor of PI3K/Akt, and its expression may be inhibited when the PI3K/Akt pathway is inhibited in the OIR model, thereby reducing abnormal blood vessel formation in the OIR model and improving retinopathy. VEGF is present both upstream and downstream of AKT. When AKT phosphorylation is restricted, VEGF secretion increases to promote the expression of AKT, and the two develop autocrine forms to jointly maintain cell functions.<sup>49</sup> There are limited studies on the role of the PI3K/Akt pathway in the propranolol-induced improvement of OIR retinopathy. This work has enhanced the current understanding of the role of the PI3K/Akt pathway in retinopathy, while the specific molecular mechanism by which



**Fig. 4** The VEGF pathway is not involved in propranolol-induced retinopathy of prematurity (ROP) alleviation. Mice were culled at the age of 17 days, and **a** the mRNA levels of VEGF, VEGFR-2, and PI3K were analyzed by RT-PCR, and **b**, **c** the protein level of VEGF was analyzed by western blotting. **a**, **c** Data shown are mean  $\pm$  SD of three independent experiments. **b** One representative result is shown. no, not statistically significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

propranolol regulates HIF-1 $\alpha$  expression via the PI3K/AKT signaling pathway remains to be further investigated.

ERK is an important member of the mitogen-activated protein kinase family and an important regulator of growth and development. Some researchers have used ERK blockers in OIR models and found that ERK blockers can reduce the formation of abnormal neovascularization in the retina of OIR mice.<sup>50</sup> In our study, ERK expression in the OIR mouse retina did not increase significantly, but the expression of its active form, p-ERK, increased, which was statistically different from that in the normal control group. After intraperitoneal injection of propranolol, there was no significant change in the ERK expression; however, the expression of p-ERK in the activated form decreased significantly. This difference was statistically significant, suggesting that propranolol has a regulatory effect on the activated MEK/ERK pathway, thereby inhibiting the overexpression of p-ERK. ERK is an upstream regulator of HIF-1 $\alpha$ . Therefore, an increase in its expression can promote HIF-1 $\alpha$ , which promotes the formation of new blood vessels. In this study, propranolol was found to reduce the expression of ERK and HIF-1 $\alpha$  in OIR, suggesting that its mechanism of improving OIR may be inhibition of the activated ERK and HIF-1 $\alpha$  pathways.

In this study, C57BL/6J mice were used to establish the OIR model, which has become one of the most widely used models for studying ROP.<sup>22,25</sup> The number of nuclei breaking through the inner limiting membrane in the immunofluorescence-stained and paraffin

sections confirmed that the OIR model was successfully established. Intraperitoneal injection of propranolol reduced fluorescein leakage and the number of nuclei that broke through the inner limiting membrane, suggesting that propranolol had a therapeutic effect on OIR. Western blotting was used to detect the expression of the relevant factors in the mouse retina. The results showed that the mRNA expression of VEGF and VEGFR did not change significantly before and after intraperitoneal injection of propranolol, indicating that the effect of propranolol may not be associated with the VEGF pathway. Intraperitoneal injection of propranolol may inhibit the overexpression of HIF-1 $\alpha$ , PI3K, p-Akt, and p-ERK in the OIR model, suggesting that propranolol may inhibit the activation of the PI3K/Akt- and ERK-related pathways to regulate the expression of HIF-1 $\alpha$ . This further reduced the development of neovascularization in the OIR model and improved the integrity of the blood vessel wall. The duration of propranolol intervention was from PD 12 to PD 17 when the retinal vasculature develops in a relatively hypoxic period, and neovascular growth is at its peak.<sup>51</sup> However, it is difficult to determine whether the timing of treatment always coincides with the development of neovascularization in clinical settings. Therefore, studies can alter the intervention time in the future, such as starting the intervention from PD 7, delaying the intervention to PD 22, or delaying the intervention (from PD 17) to make it more relevant to clinical practice.

The safety of propranolol treatment will be a focus of future research. Studies have found that the systemic use of propranolol

has been associated with side effects,<sup>52</sup> and multiple vitreous injections of the VEGF antagonist bevacizumab can cause apoptosis of retinal neurons.<sup>53</sup> In this study, retinal vascular development was not fully restored to normal using a dose of 2 mg/kg propranolol thrice a day by intraperitoneal injection; therefore, more studies are needed to determine the appropriate delivery methods and drug concentrations of propranolol to treat ROP.

In conclusion, intraperitoneal injection of propranolol may reduce the abnormal proliferation of blood vessels in OIR, improve vascular integrity, and have a therapeutic effect on ROP in OIR. Propranolol intervention improves OIR, and the underlying mechanism appears to be through the PI3K/Akt and ERK/p-ERK pathways instead of VEGFR-2 to inhibit HIF-1 $\alpha$  overexpression.

## DATA AVAILABILITY

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

## REFERENCES

- Lad, E. M., Hernandez-Boussard, T., Morton, J. M. & Moshfeghi, D. M. Incidence of retinopathy of prematurity in the United States: 1997 through 2005. *Am. J. Ophthalmol.* **148**, 451–458 (2009).
- Gilbert, C. Retinopathy of prematurity: a global perspective of the epidemics, population of babies at risk and implications for control. *Early Hum. Dev.* **84**, 77–82 (2008).
- Wolforth, L. M., Loo, S. W. & Sood, S. L. Retinopathy of prematurity and ethnicity in Hawai'i: a retrospective study (1996 - 2006) of medical records from Kapi'olani Medical Center for Women and Children. *Hawaii J. Med. Public Health* **75**, 68–72 (2016).
- Good, W. V. et al. The incidence and course of retinopathy of prematurity: findings from the Early Treatment for Retinopathy of Prematurity Study. *Pediatrics* **116**, 15–23 (2005).
- Dhingra, D. et al. Change in the incidence and severity of retinopathy of prematurity (ROP) in a neonatal intensive care unit in Northern India after 20 years: comparison of two similar prospective cohort studies. *Ophthalmic. Epidemiol.* **26**, 169–174 (2019).
- Liu, Q. et al. Incidence of retinopathy of prematurity in Southwestern China and analysis of risk factors. *Med. Sci. Monit.* **20**, 1442–1451 (2014).
- Luo, X. Q. et al. Prevalence of retinopathy of prematurity in some tertiary hospitals in Guangdong Province. *Chin. J. Ocul. Fundus Dis.* **26**, 273–275 (2010).
- Zhu, L. *Incidence Rate and Risk Factors of Retinopathy of Prematurity from a Multicenter Study*. (Fudan University, 2006).
- Tian, N. *The Screening and Results in the Analysis of Premature Retinopathy in the Panyu District of Guangzhou City*. (Southern Medical University, 2013).
- Blencowe, H., Lawn, J. E., Vazquez, T., Fielder, A. & Gilbert, C. Preterm-associated visual impairment and estimates of retinopathy of prematurity at regional and global levels for 2010. *Pediatr. Res.* **74**, 35–49 (2013).
- Solebo, A. L., Teoh, L. & Rahi, J. Epidemiology of blindness in children. *Arch. Dis. Child* **102**, 853–857 (2017).
- Mercurio, A. M. VEGF/neuropilin signaling in cancer stem cells. *Int. J. Mol. Sci.* **20**, 490 (2019).
- Wang, H. Anti-VEGF therapy in the management of retinopathy of prematurity: what we learn from representative animal models of oxygen-induced retinopathy. *Eye Brain* **8**, 81–90 (2016).
- Semenza, G. L. Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. STKE* **2007**, cm8 (2007).
- Miwa, Y. et al. Pharmacological HIF inhibition prevents retinal neovascularization with improved visual function in a murine oxygen-induced retinopathy model. *Neurochem. Int.* **128**, 21–31 (2019).
- Wang, P. et al. Protein Kinase B (Akt) promotes pathological angiogenesis in murine model of oxygen-induced retinopathy. *Acta Histochem. Cytochem.* **44**, 103–111 (2011).
- Di, Y., Zhang, Y., Nie, Q. & Chen, X. Ccn1/Cyr61-Pi3k/Akt signaling promotes retinal neovascularization in oxygen-induced retinopathy. *Int. J. Mol. Med.* **36**, 1507–1518 (2015).
- Di, Y., Zhang, Y., Yang, H., Wang, A. & Chen, X. The mechanism of Ccn1-enhanced retinal neovascularization in oxygen-induced retinopathy through Pi3k/Akt-Vegf signaling pathway. *Drug Des. Devel. Ther.* **9**, 2463–2473 (2015).
- Fukuda, R. et al. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on map kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J. Biol. Chem.* **277**, 38205–38211 (2002).
- Ristori, C. et al. Role of the adrenergic system in a mouse model of oxygen-induced retinopathy: antiangiogenic effects of beta-adrenoreceptor blockade. *Invest. Ophthalmol. Vis. Sci.* **52**, 155–170 (2011).
- Dal Monte, M. et al. Eye drop propranolol administration promotes the recovery of oxygen-induced retinopathy in mice. *Exp. Eye Res.* **111**, 27–35 (2013).
- Yun, J. H. et al. Propranolol increases vascular permeability through pericyte apoptosis and exacerbates oxygen-induced retinopathy. *Biochem. Biophys. Res. Commun.* **503**, 2792–2799 (2018).
- Filippi, L. et al. Oral propranolol for retinopathy of prematurity: risks, safety concerns, and perspectives. *J. Pediatr.* **163**, 1570–1577.e1576 (2013).
- Filippi, L. et al. Study protocol: safety and efficacy of propranolol 0.2% eye drops in newborns with a precocious stage of retinopathy of prematurity (DROP-ROP-0.2%): a multicenter, open-label, single arm, phase II trial. *BMC Pediatr.* **17**, 165 (2017).
- Friedman, L. M. et al. Effect of propranolol in patients with myocardial infarction and ventricular arrhythmia. *J. Am. Coll. Cardiol.* **7**, 1–8 (1986).
- Huang, X. R., Wang, Y. J., Yang, G. R., Yang, Z. X. & Zhang, J. S. The effect of propranolol on oxygen-induced retinal neovascularization. *Chin. J. Pediatr.* **54**, 131–136 (2016).
- Ying, G. S., Bell, E. F., Donohue, P., Tomlinson, L. A. & Binenbaum, G. Perinatal risk factors for the retinopathy of prematurity in postnatal growth and ROP study. *Ophthalmic. Epidemiol.* **26**, 270–278 (2019).
- Gao, H.-C., Chen, C., Zhang, Y.-Q. & Zhang, J.-M. Progress on study of the risk factors of retinopathy of prematurity. *Int. Eye Sci.* **18**, 80–83 (2018).
- Weber, M. A., Drayer, J. I. & Kaufman, C. A. The combined alpha- and beta-adrenergic blocker labetalol and propranolol in the treatment of high blood pressure: similarities and differences. *J. Clin. Pharm.* **24**, 103–112 (1984).
- Andersson, O. K., Widgren, B. & Berglund, G. A 10-year follow-up of men with mild hypertension. bendroflumethiazide and propranolol give equal effect in treatment of high blood pressure. *Lakartidningen* **82**, 1159–1162 (1985).
- Chandraratna, P. A. Comparison of acebutolol with propranolol, quinidine, and placebo: results of three multicenter arrhythmia trials. *Am. Heart J.* **109**, 1198–1204 (1985).
- Huang, J., Jiang, D., Zhao, S. & Wang, A. Propranolol suppresses infantile hemangioma cell proliferation and promotes apoptosis by upregulating Mir-125b expression. *Anticancer Drugs* **30**, 501–507 (2019).
- Jamshidian-Tehrani, M. et al. Clinical and ultrasonographic evaluation of infantile periorbital hemangioma treated with oral propranolol. *Ophthalmic. Plast. Reconstr. Surg.* **35**, 484–486 (2019).
- Osada, A., Araki, E., Yamashita, Y. & Ishii, T. Combination therapy of propranolol, levothyroxine, and liothyronine was effective in a case of severe consumptive hypothyroidism associated with infantile hepatic hemangioma. *Clin. Pediatr. Endocrinol.* **28**, 9–14 (2019).
- Huang, X., Wang, Y., Yang, G., Yang, Z. & Zhang, J. Effects of propranolol on oxygen-induced retinal neovascularization in mouse. *Zhonghua Er Ke Za Zhi* **54**, 131–136 (2016).
- Cammalleri, M. et al. The beta adrenergic receptor blocker propranolol counteracts retinal dysfunction in a mouse model of oxygen induced retinopathy: restoring the balance between apoptosis and autophagy. *Front. Cell Neurosci.* **11**, 395 (2017).
- Villalvilla, A. et al. Circulating endothelial progenitor cells are reduced in rat oxygen-induced retinopathy despite a retinal Sdf-1/Cxcr4 and VEGF proangiogenic response. *Life Sci.* **91**, 264–270 (2012).
- Pisani, F. et al. Potential role of the methylation of VEGF gene promoter in response to hypoxia in oxygen-induced retinopathy: beneficial effect of the absence of Aqp4. *J. Cell Mol. Med.* **22**, 613–627 (2018).
- Dong, L. et al. PTB-associated splicing factor inhibits IGF-1-induced VEGF upregulation in a mouse model of oxygen-induced retinopathy. *Cell Tissue Res.* **360**, 233–243 (2015).
- Stevens, M. & Oltean, S. Modulation of receptor tyrosine kinase activity through alternative splicing of ligands and receptors in the VEGF-A/VEGFR axis. *Cells* **8**, 288 (2019).
- Li, G. et al. VEGFR-2 inhibitor apatinib hinders endothelial cells progression triggered by irradiated gastric cancer cells-derived exosomes. *J. Cancer* **9**, 4049–4057 (2018).
- Mao, Y., Liu, X., Song, Y., Zhai, C. & Zhang, L. VEGF-A/VEGFR-2 and FGF-2/FGFR-1 but not PDGF-BB/PDGFR-B play important roles in promoting immature and inflammatory intraplaque angiogenesis. *PLoS One* **13**, e0201395 (2018).
- McLeod, D. S. & Lutty, G. A. Targeting VEGF in canine oxygen-induced retinopathy – a model for human retinopathy of prematurity. *Eye Brain* **8**, 55–65 (2016).

44. Liu, Z. et al. Endothelial adenosine A2a receptor-mediated glycolysis is essential for pathological retinal angiogenesis. *Nat. Commun.* **8**, 584 (2017).
45. Jin, X. et al. Netrin-1 interference potentiates epithelial-to-mesenchymal transition through the Pi3k/Akt pathway under the hypoxic microenvironment conditions of non-small cell lung cancer. *Int J. Oncol.* **54**, 1457–1465 (2019).
46. Hu, F., He, Z., Sun, C. & Rong, D. Knockdown of Grhl2 inhibited proliferation and induced apoptosis of colorectal cancer by suppressing the Pi3k/Akt pathway. *Gene* **700**, 96–104 (2019).
47. Konstantinopoulos, P. A. et al. Olaparib and A-specific Pi3k inhibitor alpelisib for patients with epithelial ovarian cancer: a dose-escalation and dose-expansion phase 1b trial. *Lancet Oncol.* **20**, 570–580 (2019).
48. Zhang, Z., Yao, L., Yang, J., Wang, Z. & Du, G. Pi3k/Akt and Hif-1 signaling pathway in hypoxia-ischemia (review). *Mol. Med. Rep.* **18**, 3547–3554 (2018).
49. Jiang, B. H. & Liu, L. Z. Akt signaling in regulating angiogenesis. *Curr. Cancer Drug Targets* **8**, 19–26 (2008).
50. Gao, S. et al. PEDF mediates pathological neovascularization by regulating macrophage recruitment and polarization in the mouse model of oxygen-induced retinopathy. *Sci. Rep.* **7**, 42846 (2017).
51. Smith, L. E. et al. Oxygen-induced retinopathy in the mouse. *Invest. Ophthalmol. Vis. Sci.* **35**, 101–111 (1994).
52. Chen, J. et al. Propranolol inhibition of B-adrenergic receptor does not suppress pathologic neovascularization in oxygen-induced retinopathy. *Invest. Ophthalmol. Vis. Sci.* **53**, 2968–2977 (2012).
53. Romano, M. R. et al. Effects of bevacizumab on neuronal viability of retinal ganglion cells in rats. *Brain Res.* **1478**, 55–63 (2012).

## AUTHOR CONTRIBUTIONS

G.Y., .YW., and L.L. drafted the manuscript. Y.L. and J.Z. analyzed the data. G.Y. and Y.W. supervised the study. Y.W. and S.S. designed the study and revised the manuscript critically for important intellectual content. S.S., Y.D., and P.Z. performed experiments. All authors read and approved the final manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Correspondence** and requests for materials should be addressed to Yajuan Wang.

**Reprints and permission information** is available at <http://www.nature.com/reprints>

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.