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Original Article

MicroRNA-410 alleviates retinopathy of prematurity in a mouse model by inhibiting vascular endothelial growth factor expression

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Abstract: Objective: We aimed to investigate the role of microRNA-410 (miR-410) in retinopathy of prematurity (ROP) in a mouse model by regulating vascular endothelial growth factor (VEGF) expression. Methods: Over- and low-expressed miR-410 plasmid, over-expressed VEGF plasmid, and an empty plasmid (pLKO.1) were constructed and transferred into human umbilical vein endothelial cells (HUVEC) using the Liposome method. The mouse were randomly divided into a normal control group and seven ROP model groups, including the blank model group (with pLKO.1 empty plasmid), the over-expressed miR-410 group, the low-expressed miR-410 group, the miR-410 empty plasmid group, the over-expressed miR-410 + over-expressed VEGF group, the miR-410 empty plasmid + over-expressed VEGF group, and the over-expressed VEGF group. Real time polymerase chain reaction (RT-PCR) was performed to determine the miR-410 and VEGF expressions, and Western blotting was used to measure the changes in VEGF expression. ADP enzyme histochemistry and HE staining were applied for observing the morphology changes and hyperplasia of retinal vessels. Results: The HUVEC transfected with over-expressed miR-410 plasmid showed significantly down-regulated VEGF while the HUVEC transfected with low-expressed miR-410 plasmid showed up-regulated VEGF (all $P < 0.05$). Compared with the normal control group, mice in the other groups were lighter in weight from the 15th day after birth, and a greater number of vascular endothelial nuclei breaking through the retinal inner limiting membrane was observed in the model groups (both $P < 0.05$). Compared with the normal control group, VEGF expression increased, retinal vessels were distorted and dilated, and the quantity of new vessels formed and endothelial nuclei increased in the model groups except for the over-expressed miR-410 group (all $P < 0.05$). Compared with the blank model group, VEGF expression declined ($P < 0.05$), and retinal vessels showed reduced circuity, new vessels regressed, and endothelial nuclei decreased in the over-expressed miR-410 group, while the changes were not significantly different when compared with the normal control group. Conclusion: MiR-410 could inhibit retinal neovascularization in ROP mouse model via down-regulating VEGF expression.

Keywords: MicroRNA-410, retinopathy, prematurity, vascular endothelial growth factor, mouse model, human umbilical vein endothelial cell

Introduction

Retinopathy of prematurity (ROP), previously known as retrolental fibroplasia (RLF), is categorized as a retinal vascular developmental disease and appears suddenly in premature infants [1]. In most cases, ROP begins at 31~32 weeks in post-menstrual age with peak severity developing over the next 2~5 weeks [2]. It has been reported that ROP involves a series of pathologies that affect vision, from mild diseases that spontaneously lead to severe diseases which result in retinal detachment and subsequent blindness [3]. Statistics show that the prevalence of ROP varies from 5~8% in developed countries with sufficient neonatological facilities to 30% in middle-income developing countries [4]. Approximately 20,000 infants become blind or suffer from severe visual impairment because of ROP in 2010 [5]. The pathogenesis of ROP is reported to encompass two discrete phases. Phase one is induced by relative hyperoxia, which causes vasoattenuation, or the cessation of retinal vascular devel-

opment, and phase two is involved in relative hypoxia and increased vascular endothelial growth factor (VEGF) expression [6]. Of the various factors that are related to the development of ROP, gestational age, birth weight, oxygen therapy, infection, sepsis, anemia, and relative hyperoxia are the most influential [7]. Furthermore, previous evidence showed that miRNA expression had potential functional specificity of retina and eye cells and tissues [8].

MiRNAs are endogenous, non-coding, regulatory RNAs, approximately 22 nucleotides (nts) in size, and regulate the activities of target mRNAs by binding at sites in the 3' untranslated region of the mRNAs [9]. MiRNAs have been acknowledged as main factors of post-transcriptional regulation of the fine-tuning of gene expression, playing crucial roles in cell proliferation, differentiation, and cell death [10, 11]. The human microRNA-410 (miR-410), one of more than 40 miRNAs, clusters at the conserved 14q32 locus [12]. Previous evidence demonstrated that the miRNAs within this cluster were most abundant in fetal and brain tissues [13, 14]. To be specific, miR-410 expression has been verified in endothelial, adipose, neuronal cell lines as well as others [15-18]. Several studies also found a relationship between miR-410 expression and the regulation of insulin secretion and neuroblastoma in breast cancer and prostatic carcinoma [13, 19, 20]. Furthermore, a study conducted by Chen *et al.* found the relationship between miR-410 and retinal neovascularization [21]. Thus, we conducted this study to investigate the role of miR-410 in ROP mouse models by regulating VEGF.

Materials and methods

Construction of miR-410 and VEGF plasmid vector

Based on the gene sequences of miR-410 and VEGF in mice, the sequences of miR-410 mimics, miR-410 inhibitors, and VEGF were sent to Invitrogen Inc. (USA) for synthesis. Plasmid pLKO.1 was chosen as the vector to construct the over-expressed pLKO.1-miR-410 plasmid, low-expressed pLKO.1-miR-410 plasmid, and over-expressed pLKO.1-VEGF plasmid.

Cell grouping and transfection

Cell transfection was performed with the constructed over-expressed pLKO.1-miR-410 plasmid, low-expressed pLKO.1-miR-410 plasmid, over-expressed pLKO.1-VEGF plasmid, and pLKO.1 empty plasmid in accordance with the specifications of the liposome 2000 reagent (Invitrogen Inc., USA). Human umbilical vein endothelial cells (HUVEC) [American Type Culture Collection (ATCC), VA, USA] in good condition and in the logarithmic growth phase were seeded in a 6-well plate at 5×10^5 cells in each well. After overnight incubation, the cells were washed twice with serum-free Dulbecco's Modified Eagle Medium (DMEM) (Grand Island Biological Company, Grand Island, NY, USA), and 1.5 ml DMEM was added. Each plasmid (4 μg) was diluted with 250 μl of DMEM. Lipofectamine 2000 reagent (10 μl) was diluted with 250 μl of serum-free DMEM, and reserved for 5 min at room temperature. The diluted plasmid and lipofectamine 2000 reagent were gently mixed and reserved at room temperature for 20 min. Each tube of liposome-plasmid complex (500 μl) was slowly added into the cells, followed by gentle mixing. After a 4~6 h culture in an incubator at 37°C and with 5% CO_2, the cells were cultured with DMEM medium containing 10% fetal bovine serum (FBS) (Grand Island Biological Company, Grand Island, NY, USA) at 37°C and with 5% CO_2. Forty-eight hours later, a real time quantitative polymerase chain reaction (qRT-PCR) was carried out to determine the mRNA expressions of miR-410 and VEGF.

Experimental animals and grouping

A total of 80 clean and healthy C57BL/6J mice (7 days old) were selected from the Linyi People's Hospital. Mice with ocular inflammation and other abnormalities were excluded. All the mice were selected in accord with the standards for the construction of the ROP mouse model and fed in a standard feeding room with nursing rats. The feed, water supply, light, temperature and other conditions were kept consistent. Our study complied with animal feeding management and animal experiment operation regulations in accordance with the *Regulations of Beijing Municipality on the administration of experimental animals*, and the animal feeding conditions were in accordance with the China National Standard *Conditions and facilities for laboratory animals* (GB14925-2001).

In the 80 C57BL/6J mice, 10 mice who were fed in a normal oxygen environment were

Table 1. Primer sequence for real time quantitative polymerase chain reaction

Target genes	Primer sequences
MiR-410	F 5'-AGGTTGTCTGTGATGAGTTCG-3'
	R 5'-TGGTGTCGTGGAGTCG-3'
U6	F 5'-CTCGCTTCGGCAGCACA-3'
	R 5'-AACGCTTCACGAATTTGCGT-3'
VEGF	F 5'-ATCTTCAAGCCGTCCTGTGT-3'
	R 5'-GCATTCACATCTGCTGTGCT-3'
β-actin	F 5'-GAATCAATGCAAGTTCGGTTCC-3'
	R 5'-TCATCTCCGCTATTAGCTCCG-3'

Note: F, forward; R, reverse; VEGF: Vascular endothelial growth factor.

selected as the normal control group. The remaining 70 mice lived in a high oxygen environment, and 5 days later returned to the normal oxygen environment. The 70 mice were randomly divided into 7 groups with 10 mice in each group: blank model group (no drug treatment), over-expressed miR-410 group (the mice were injected with 0.4 μl of over-expressed miR-410 plasmid), low-expressed miR-410 group (the mice were injected with 0.4 μl of low-expressed miR-410 plasmid), miR-410 empty plasmid group (the mice were injected 0.4 μl miR-410 empty plasmid), over-expressed miR-410 + over-expressed VEGF group (the mice were injected with 0.4 μl of over-expressed miR-410 plasmid and 0.4 μl of over-expressed VEGF plasmid), miR-410 empty plasmid + over-expressed VEGF group (the mice were injected with 0.4 μl of miR-410 empty plasmid and 0.4 μl of over-expressed VEGF plasmid), and over-expressed VEGF group (the mice were injected with 0.4 μl of over-expressed VEGF plasmid). All injection was performed in the vitreous cavity of the right eye in the day the mice get out from the oxygen chamber. The left eye o of mice in the 7 groups were not exogenously treated.

Construction of retinopathy of prematurity mouse model

The mice in the normal control group were fed in a normal oxygen environment, while the mice in the model groups were fed with their nursing rats in a closed chamber, where 100% moist, medically pure oxygen was present. The oxygen concentration in the closed chamber was monitored by an oxygen analyzer and kept relatively stable with an oxygen flow of 1.0 L/min and an oxygen partial pressure of (75 ± 2)%. The chamber was opened daily for bedding replacement and the addition of feed. After being fed in a high oxygen environment for 5 days, the mice in the model groups were fed in a normal environment with natural illumination and temperature at (23 ± 5)°C until they were 17 days old.

qRT-PCR assay

Total RNA of the cells and tissues was extracted using the one-step Trizol method following Trizol (Invitrogen company, Carlsbad, CA, USA) reagent specification and was dissolved by diethylpyrocarbonate (DEPC)-treated, ultra-pure water. The ND-1000 ultraviolet/visible light spectrophotometer (Nanodrop Inc., USA) was applied to measure the absorbance at 260 nm and 280 nm. The quality of the total RNA was identified and its concentration was adjusted for qRT-PCR. The RNA was reverse transcribed according to the High Capacity cDNA Reverse Transcription Kit (Fermentas Company, Hanover, MD, USA) with the reaction conditions: 70°C for 10 min, ice-bath for 2 min, 42°C for 60 min, 70°C for 10 min. The cDNA from the reverse transcription was temporarily conserved in a refrigerator at -80°C. The TaqMan probe method was applied for qRT-PCR following the manufacturer instructions of the Kits (Fermentas Company, Hanover, MD, USA), with the primer sequence shown in **Table 1**. The reaction conditions were: 95°C pre-denaturation for 30 s, 95°C denaturation for 10 s, 60°C annealing for 20 s, 70°C extension for 10 s, for a total of 40 cycles. qRT-PCR (Bio-Rad iQ5 version, Bio-Rad Company, USA) was used as a measurement tool with U6 as an internal reference for miR-410 and β-actin as an internal reference for VEGF. A relative quantitative method was used for calculations and $2^{-\Delta\Delta Ct}$ was used to represent the relative expression of each target gene. Each experiment was performed 3 times.

Western blotting

The total protein samples of the cells and tissues separately had 1 × sodium dodecylsulphate (SDS) lysate added for protein extraction. The samples were then heated at 100°C for 5 min. Electrophoresis of a 20 μl sample was performed in a 12% polyacrylamide gel for transferring the membrane, and was followed by sealing the sample in an adecolorizing shaking table with Tris-buffered saline (TBST) containing 5% BSA at room temperature for 1 h. After

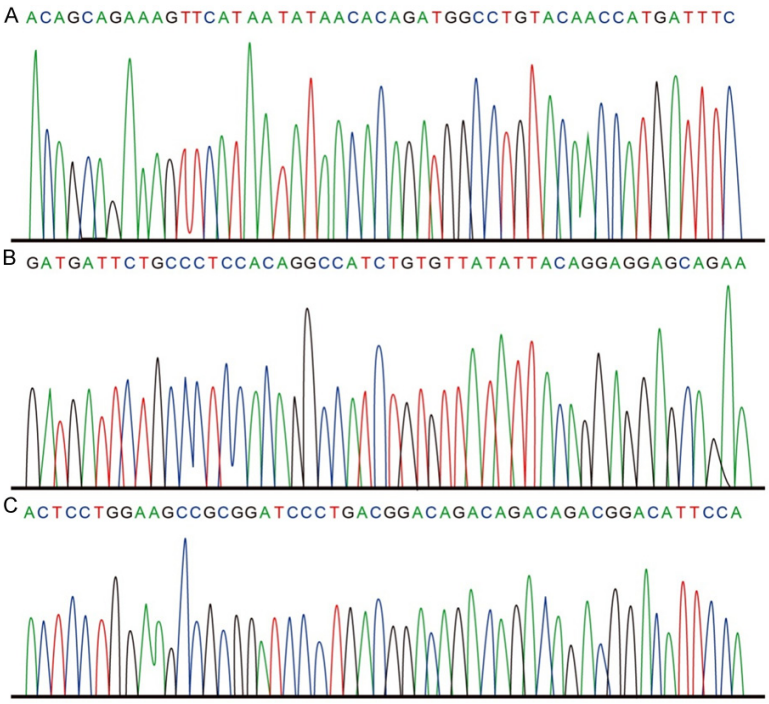

Figure 1. Sequences of three eukaryotic expression vectors. Note: A. MiR-410 mimics sequence of recombinant plasmid; B. MiR-410 inhibitors sequence of recombinant plasmid; C. VEGF sequence of recombinant plasmid.

discarding the sealing fluid, the membrane was placed into a plastic groove, added with 5% BSA to make up VEGF and β-actin antibodies [Cell Signaling Technologies (CST), Beverly, MA, USA] of corresponding concentration. The membrane was placed face up in a refrigerator set at 4°C with oscillation overnight and washed the next 3 × 10 min with TBST. The membrane was added with the diluted second antibody (Abcam company, Cambridge, UK) in the same way, followed by 4-6 h incubation at 4°C and was then washed 3 × 15 min with TBST. The nitrocellulose (NC) membrane was evenly added with drops of the mixture of chemiluminescence reagent A and B solutions (1:1) and developed by a developer. Relative optical density analysis was performed for all the Western blotting strips.

Enzyme-histochemical staining

Each group were given an intraperitoneal injection of 10% hydration chloric aldehyde (0.3 ml/100 g) for anaesthesia and were then fixed on a board. Their right eyes were washed with physiological saline, fixed using a 4% paraformaldehyde perfusion for 15 min and then removed. The eyes were opened with a small window (a small hole made by a 20 ml syringe needle) in the sclera, and then placed in 10% neutral formalin for 12-24 h fixation. The retinas were removed in sections, which were then washed 5 × 15 min with Tris-maleic acid buffer, incubated for 15 min in the reaction solution at 37°C, and washed again 5 × 15 min with Tris-maleic acid buffer, coloration for 5 min with the addition of ammonium sulfide (1:10), washed 5 × 15 min with Tris-maleic acid buffer, and finally mounted in glycerol gelatin. The morphology and distribution of stained retinas were observed under the optical microscope.

Hematoxylin-eosin (HE) staining

The fixed eyes were dehydrated successively in 70%, 80%, and 95% ethanol for 8 h and 100% ethanol for 3 h, transparentized for 15 min in xylene at 60°C, immersed in paraffin at 60°C for 6 h, and embedded for further use. The prepared paraffined sections were placed into distilled water for a moment and then into hematoxylin solution for 5 min for staining, followed by color separation for several seconds in acid water and ammonia, water flushing for 1 h, and were placed again into distilled water for a while. The following procedures included dehydration successively in 70% and 90% ethanol for 10 min, staining for 2-3 min in ethanol eosin staining solution, dehydration with pure alcohol, transparentization by xylene, adding with neutral gum drops, sealing by coverslips, and tagging. Five sections were extracted from each eye, 30 um between every two extractions. The number of the endothelial nucleus breaking through the inner limiting membrane was calculated, as well as the average number per eye for statistical analysis.

Statistical methods

SPSS 20.0 software (SPSS Inc, Chicago, IL, USA) was performed for all the data analysis.

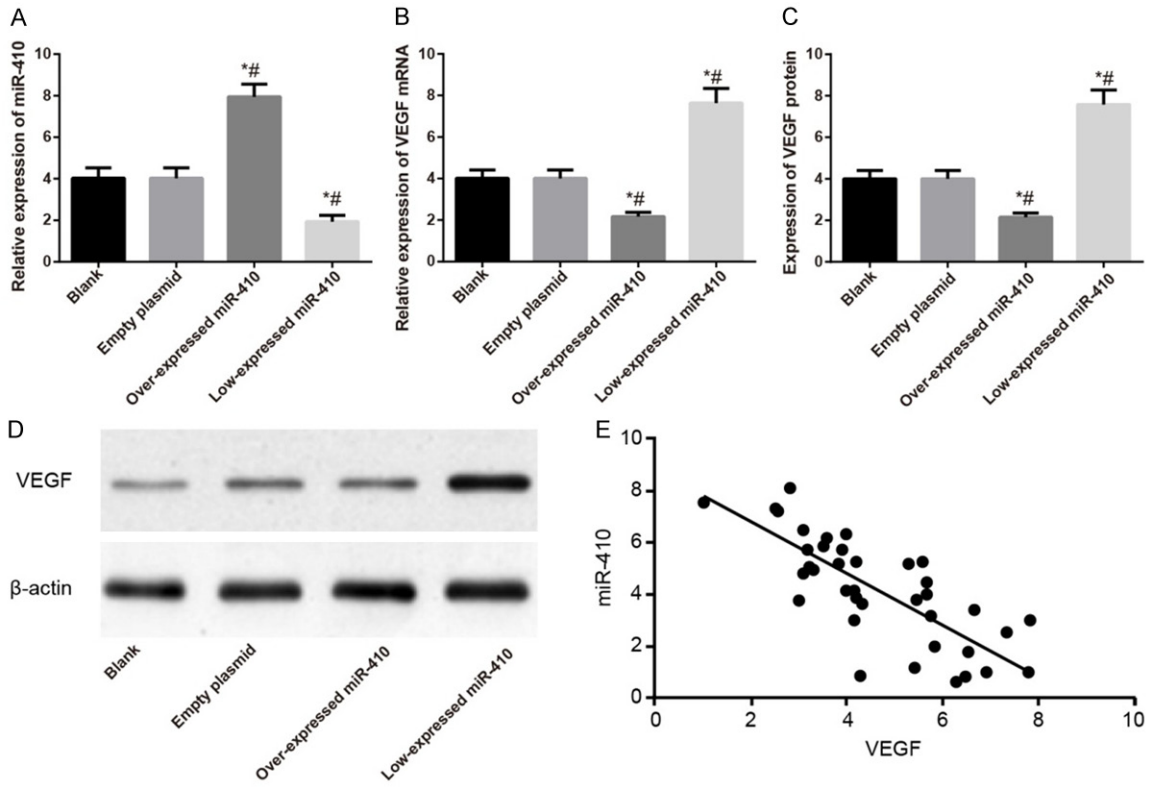

Figure 2. MiRNA-410 and VEGF expression of transfected cells in each group and the correlation between miR-410 and VEGF. Note: A. Histogram of miR-410 expression in each group; B. Histogram of VEGF mRNA expression in each group; C. Histogram of VEGF protein expression in each group; D. Histogram of VEGF protein expression immunoblotting; E. Correlation analysis chart between miR-410 and VEGF; *, Compared with blank plasmid-transfected group, $P < 0.05$; #, Compared with empty plasmid-transfected group, $P < 0.05$.

Measurement data were represented as mean ± standard deviation ($\bar{x} \pm s$). The expressions of miR-410 and VEGF in different transfected cells and groups were compared by using one-way analysis of variance (ANOVA), and comparison between groups was verified by LSD (*t*-test). The correlation between the miR-410 and VEGF was analyzed by the Pearson method. The weight of the mice at different time points in the model groups and the control group were compared by *t* test (LSD). The numbers of nuclei in vascular endothelial cells of mice among multiple groups were compared by ANOVA. A *P* value of less than 0.05 was considered statistically significant.

Results

Construction of miR-410 and VEGF plasmid vector

Constructed over-expressed pLKO.1-miR-410 plasmid, low-expressed pLKO.1-miR-410 plasmid, and over-expressed pLKO.1-VEGF plasmid were transformed into competent DH5 α cells. Positive cells were cloned and then sequenced, and the results showed that the insertion sequence was consistent with the designed and synthesized sequence (**Figure 1**), proving the three eukaryotic expression vectors were successfully constructed.

Correlation between miR-410 and VEGF

MiR-410 expressions of the transfected cells in each group could be seen in **Figure 2A**. Compared with HUVEC cells and HUVEC transfected with pLKO.1, the miR-410 expression in HUVEC transfected with over-expressed miR-410 plasmid significantly increased ($P < 0.05$). However, the miR-410 expression in HUVEC transfected with low-expressed miR-410 plasmid was greatly decreased ($P < 0.05$). Compared with HUVEC cells and HUVEC transfected with pLKO.1, VEGF expression evidently decreased in HUVEC transfected with over-

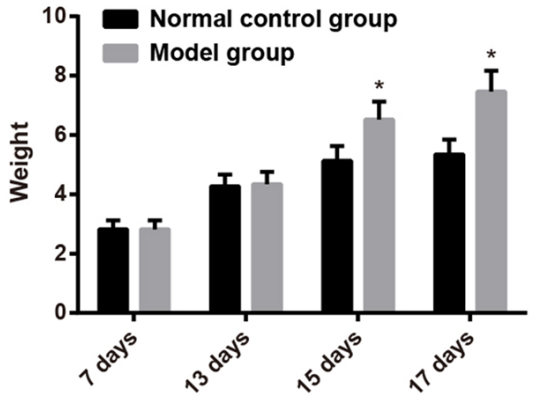

Figure 3. Comparison of state and weight between the mice in the normal control group and model groups. Note: *, compared with the control group, $P < 0.05$.

expressed miR-410 plasmid ($P < 0.05$), but greatly increased in HUVEC transfected with low-expressed miR-410 plasmid ($P < 0.05$) (**Figure 2B-D**). Pearson correlation analysis showed r = -0.633 and $P < 0.01$, which indicated that miR-410 expression was negatively associated with VEGF mRNA expression (r = -0.633, $P < 0.01$) (as shown in **Figure 2E**).

Growth status of experimental mouse

Mother and juvenile mice in the normal control group grew well and had no casualties. During the model construction, all mice survived and the survival rate was 100%. The mice in the normal control group had thick hair, significantly increased weight, were sensitive to stimulation, ate more, and were able to do continuous activities. Compared with the normal control group, at the age of 7 days, there was no difference in the vitality or state of the mice in the model groups. At the age of 12 days (in a closed oxygen tank for 5 days), the model group's motility decreased, reactions became sluggish, hair turned sparse, and skin became rough. Until the age of 17 days, their symptoms continued to significantly decline. At first, there was no difference in weight between the mice in each group. However from the 15th day of birth, compared with the mice in the normal control group, the mice in the model groups were smaller and lighter ($P < 0.05$), revealing that mice grew more slowly in a high oxygen environment (**Figure 3**).

Construction of ROP mouse model

Retinal vascular pattern of the mice aged 17 days: retinal vessels in the normal control group were mature, and a few large vessels with uniform diameter grew from the optic disc and were distributed evenly and radially to the periphery part of the retina. All retinal capillaries were divided into two layers, superficial vessels were thin and branching, deep blood vessels were thick and distributed like a network, and the vessels in the two layers were connected by a spiral communicating artery. In the model groups, retinal vessels growing from the optic disc dilated, distorted, and leaked. Also, large non-perfusion areas were seen in the posterior pole, peripheral capillary density increased, and a large number of new blood vessels grew from the junction of vascular and nonvascular areas.

Retinal vascular proliferation by HE staining (**Figure 4**): In the normal control group, vascular endothelial nuclei breaking through the retinal inner limiting membrane were not found in the sections of ocular tissue of the juvenile mice at the age of 17 days, and each section had a mean thickness of 1.09 ± 0.38. In the model groups, the number of vascular endothelial nuclei breaking through the retinal inner limiting membrane was 24.23 ± 2.39 in each section. There were significant differences between the normal control group and the model groups ($P < 0.05$).

Correlations of miR-410 and VEGF with retinopathy of prematurity

MiR-410 expression in each group can be seen in **Figure 5A**. Compared with the normal control group, miR-410 expression slightly decreased in the over-expressed miR-410 group and the over-expressed miR-410 + over-expressed VEGF group (all $P > 0.05$), but greatly declined in the other five groups ($P < 0.05$). Compared with the blank model group, miR-410 expression in the over-expressed miR-410 group and the over-expressed miR-410 + over-expressed VEGF group evidently increased but decreased in the low-expressed miR-410 group (all $P < 0.05$). VEGF mRNA and protein expressions can be seen in **Figure 5B-D**. Compared with the normal control group, there was no difference in the over-expressed miR-410 group ($P > 0.05$), but VEGF mRNA and protein expression of the other six groups significantly increased (all $P < 0.05$). Compared with the blank model group, VEGF mRNA and protein expression greatly increased in the low-expressed miR-410 group, miR-410 empty plasmid-transfected + over-

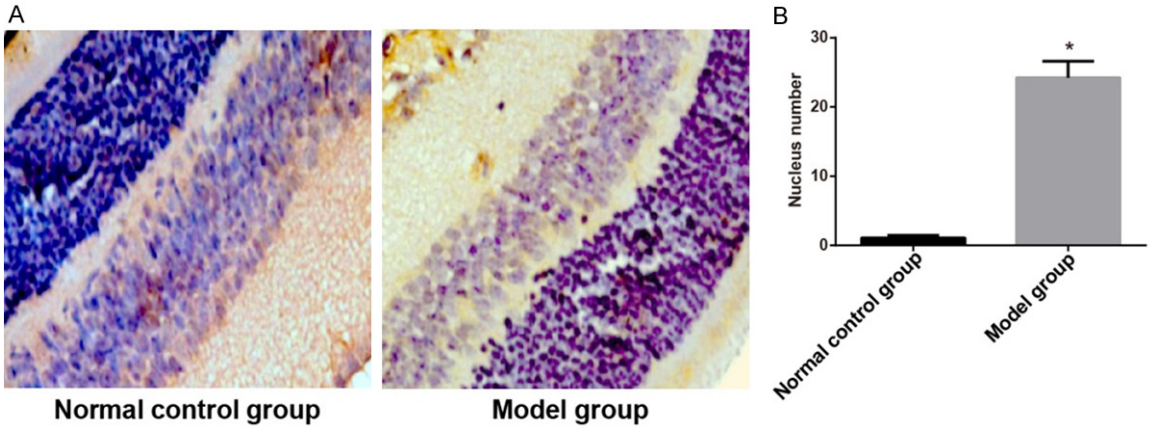

Figure 4. Quantitative study of retinal vascular proliferation by HE staining. Note: A. HE staining of the mice retinas in the two groups; B. The mean number of vascular endothelial nuclei breaking through the retinal inner limiting membrane between the two groups; *, compared with the normal control group, $P < 0.05$.

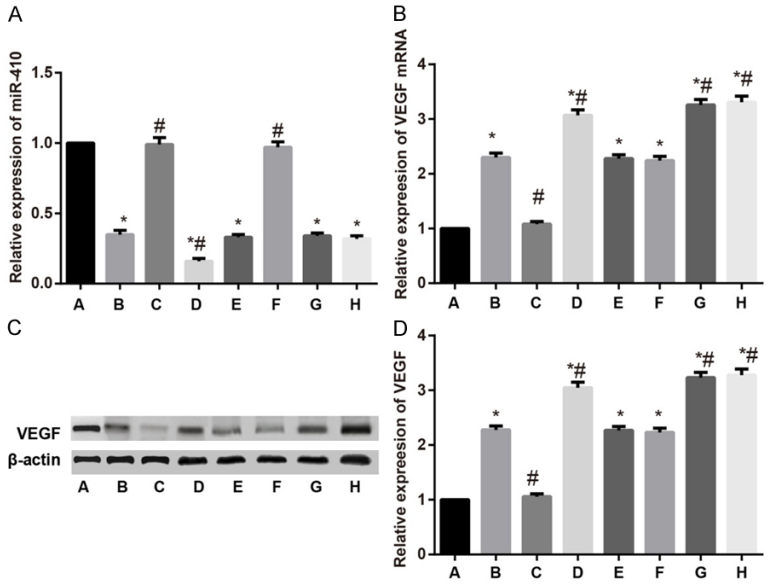

Figure 5. MiR-410 and VEGF expressions in each group. Note: A. The normal control group; B. The blank model group; C. The over-expressed miR-410 group; D. The low-expressed miR-410 group; E. The miR-410 empty plasmid-transfected group; F. The over-expressed miR-410 + over-expressed VEGF group; G. The miR-410 empty plasmid-transfected + over-expressed VEGF group; H. The over-expressed VEGF group; *, compared with the normal control group, $P < 0.05$; #, compared with blank model group, $P < 0.05$.

expressed VEGF group, and over-expressed VEGF group, but greatly declined in the over-expressed miR-410 group (all $P < 0.05$). No difference was found in the rest of the groups ($P > 0.05$).

Effects of miR-410 on ROP mouse model by regulating VEGF expression

Retinal vascular pattern of the mice aged 17 days: In the normal control group, the retinal vessels were mature and distributed evenly like a network, and all capillaries were recognizable and divided into two layers. Retinal vessels in the blank model group, miR-410 empty plasmid-transfected group and over-expressed miR-410 + over-expressed VEGF group were dilated and distorted, and a large number of new blood vessels grew from the edges of vascular and nonvascular areas. Compared with the above three groups, in the low-expressed miR-410 group, miR-410 empty plasmid-transfected + over-expressed VEGF group, and over-expressed VEGF group, vascular proliferation was worse, vascular density was higher, and the structure and distribution of new blood vessels were extremely disordered so the radial and polygon two-layer network structure was changed and most of the shallow and deep vessels were blocked. In the over-expressed miR-410 group, the degree of retinal vascular tortuosity was reduced, the new blood vessels gradually regressed, a small nonperfusion area was seen in the peripheral part of the retina, and retinal vessels changed to its normal morphology and distribution.

Quantitative study of retinal vascular proliferation by HE staining (**Figure 6**): A small amount of

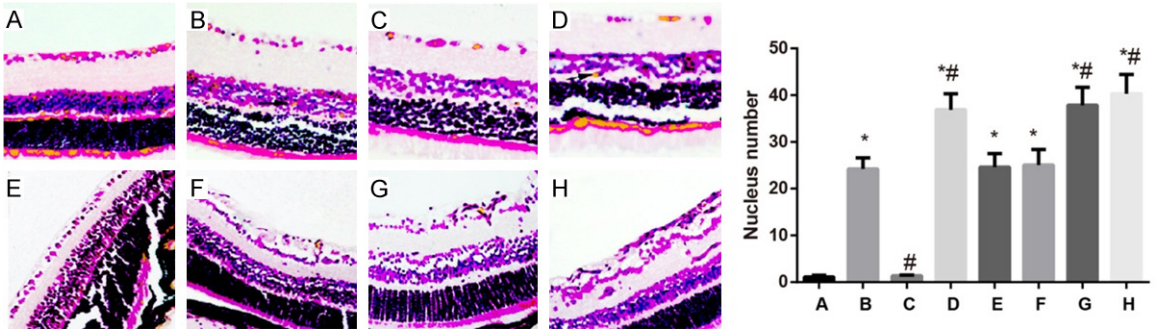

Figure 6. Quantitative study of retinal vascular proliferation by HE staining. Note: A. The normal control group; B. The blank model group; C. The over-expressed miR-410 group; D. The low-expressed miR-410 group; E. The miR-410 empty plasmid-transfected group; F. The over-expressed miR-410 + over-expressed VEGF group; G. The miR-410 empty plasmid-transfected + over-expressed VEGF group; H. The over-expressed VEGF group; A-H. HE staining of the mice retinas in each group; the histogram, the average number of vascular endothelial nuclei breaking through the retinal inner limiting membrane in each group; *, compared with the normal control group, $P < 0.05$; #, compared with blank model group, $P < 0.05$.

endothelial nuclei (1.09 ± 0.38) breaking through the retinal inner limiting membrane was found in sections of the normal control group. Compared with the normal control group, the retinal inner limiting membrane was smoother, the number of endothelial nuclei breaking through the retinal inner limiting membrane was reduced in the over-expressed miR-410 group (1.34 ± 0.2). Also, there was no significant difference between the normal control group and the over-expressed miR-410 group ($P > 0.05$), but there were obvious differences in the other six groups ($P < 0.05$). A large amount of endothelial nuclei breaking through the retinal inner limiting membrane in sections and vascular angiectasis were found in the blank model group (24.23 ± 2.39), miR-410 empty plasmid-transfected group (24.62 ± 2.91), over-expressed miR-410 + over-expressed VEGF group (25.06 ± 3.32), low-expressed miR-410 group (36.89 ± 3.42), miR-410 empty plasmid-transfected + over-expressed VEGF group (37.85 ± 3.82), and over-expressed VEGF group (40.32 ± 4.12). Compared with the blank model group, there were more endothelial nuclei breaking through the retinal inner limiting membrane in the last three groups. This was statistically significant ($P < 0.05$). Evaluation of the safety of plasmid injections into the vitreous cavity of the eye: corneal lenses were transparent and there was no difference in eye weight in each group (all $P > 0.05$).

Discussion

In the present study, we focused on the role of miR-410 in ROP. We constructed a hyperoxia-induced ROP mouse model and confirmed that over-expressed miR-410 in the retina effectively prohibits high levels of VEGF, and thus significantly reduces ROP in this model.

According to our observations, we found that mice grew slowly in a high oxygen environment. In the model groups, retinal vessels growing from the optic disc were dilated and distorted, and leaked. Also, a large number of new blood vessels grew from the junction of vascular and nonvascular areas. Similar to our results, previous studies also demonstrated that neonatal rats exposed to a high oxygen environment can rapidly inhibit the expression of VEGF, resulting in the staling of vascular growth or degeneration. When the rats returned to a normal oxygen environment, while the non-vascularized retinal was found in the conditions of relative hypoxia, resulting in a high rate of VEGF production, promoting the growth of new vessels accompanied by bleeding and exudation [22, 23].

Besides, we also found that VEGF was highly expressed in the ROP mouse model. ROP remains a significant threat to extremely immature infants, chiefly due to the pathological characteristic of retinal neovascularization (RNV) [24, 25]. RNV has been demonstrated to lead to vision-threatening complications including neovascular glaucoma, traction retinal detachment, vitreous hemorrhage, and even blindness [26, 27]. It has been proposed that a wide variety of angiogenic growth factors may mediate RNV, and VEGF has been proven to be a main growth factor contributing to angiogen-

esis and mediating retinal neovascularization in the retina with ROP [28, 29]. VEGF was known as a signaling peptide produced by cells to stimulate both vasculogenesis and angiogenesis [30]. It was reported that the production of VEGF was primarily triggered by hypoxia, but could also be stimulated by other factors, such as vascular shear stress or tumor necrosis factor alpha [31]. Previous evidence also clarified two biological functions of VEGF. VEGF was found to be able to stimulate the proliferation and diabasis of vascular endothelial cells and then induce the formation of new vessels. Also, it could increase the permeability of vessels, thus leading to an increase of vascular exudation [32]. Furthermore, it was well known that VEGF plays a crucial role not only in retinal vascular development, but also in pathologic angiogenesis inischemic retinopathies and other forms of ocular neovascularization. Higher expression of VEGF was found in these experiments, and this is consistent with our results [33-35].

Mature miRNAs negatively regulate gene expression by either translational repression or mRNA degradation [36]. A study conducted by *Karali* et al. verified that engineering miRNA target sites into the 3'UTR of virally delivered transgenes can effectively modify the pattern of transgene expression in the mammalian retina [37]. Previous studies demonstrated that micro-RNA126 can effectively inhibit the formation of retinal neovascularization in mice via regulating VEGF, IGF-2, and HIF-1α [38]. In our study, we found a negative correlation between miR-410 and VEGF expression, and that miR-410 can reduce the vascular proliferation degree via inhibiting VEGF expression. Consistent with these results, *Chen* et al. also suggested that VEGF was a target gene of miR-410 using bioinformatics predictions, and that miR-410 can inhibit retinal neovascularization in oxygen-induced retinopathy mice via down-regulating VEGF expression [21].

Taken as a whole, our study revealed that miR-410 can inhibit VEGF expression at both the cellular and tissue levels. It also revealed that miR-410 has the potential to inhibit retinal neovascularization via down-regulating VEGF expression, suggesting a new approach to the treatment of vascular proliferative retinopathy. However, there are some limitations that need to be discussed. Our study had no discussion on the dose-effect relationship of miRAN-410 expression plasmid in the treatment of new vessels. This is important because a dose that is too small can have non-noticeable effects, and effective inhibition of retinal neovascularization. A dose that is too large can have side effects. Due to funding constraints, we did not especially divide the drug-treated groups into high concentration groups and low concentration groups, and consequently, the optimum drug concentration was not found.

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Disclosure of conflict of interest

None.

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