Original Article

microRNA-218 Inhibits Oxygen-induced Retinal Neovascularization via Reducing the Expression of Roundabout 1

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Abstract

Background: The mechanisms of pathological retinal neovascularization (RNV) remain unknown. Several microRNAs were reported to be involved in the process of RNV. Oxygen-induced retinopathy (OIR) is a useful model to investigate RNV. Our present work explored the expression and the role of microRNA-128 (miR-218) in oxygen-induced RNV.
Methods: OIR was used to establish RNV model. The expression level of miR-218 in the retina from OIR mice was assessed by quantitative real-time reverse transcriptase polymerase chain reaction. Fluorescein angiography was performed in retinae of OIR mice, and RNV was quantified by hematoxylin and eosin staining to evaluate the effect of pCDH-CMV-miR-218 intravitreal injection on RNV in OIR mice. Roundabout 1 (Robo1) expression was detected by Western blotting in mouse retinal vascular endothelial cells expressing a high or low level of miR-218 and retinal tissues from OIR mice. Cell migration was evaluated by scratch wound assay.
Results: In OIR mice, the expression level of miR-218 was significantly down-regulated ($P = 0.006$). Retinal Robo1 expression was significantly increased at both mRNA and protein levels ($P = 0.001$, 0.008; respectively). miR-218 intravitreal injection inhibited retinal angiogenesis in OIR mice, and the restoration of miR-218 in retina led to down-regulation of Robo1.
Conclusions: Our experiments showed that restoration of miR-218 inhibited retinal angiogenesis via targeting Robo1. MiR-218 contributed to the inhibition of retinal angiogenesis and miR-218 might be a new therapeutic target for preventing RNV.

Key words: miR-218; Oxygen-induced Retinopathy; Retinal Neovascularization; Roundabout 1

Introduction

Retinal neovascularization (RNV) plays a crucial role in many ocular diseases, such as age-related macular degeneration, proliferative diabetic retinopathy, and retinopathy of prematurity. Endothelial cell (EC) proliferation and migration lead to the angiogenic growth of new blood vessels sprouting from the retinal veins, which resulting in vitreous hemorrhage, retinal detachment, even blindness.[1-4] However, the pathological mechanisms of RNV remain unknown and need to be explored.

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MicroRNAs (miRNAs), small noncoding RNAs of 21–24 nucleotides, negatively regulate gene translation by pairing with

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3'-UTR of a specific mRNA,[5] and had multiple physiological and pathological functions.[6-8] Several angiogenic factors that contribute to neovascular growth are mediated by miRNAs such as miR-155, -21, and -132.[9] Many studies pointed out that miRNAs also played a key role in ocular neovascularization.[7,10-12]

Previous studies on miRNA-218 mainly focused on tumor invasion,[13] metastasis and vascular development.[14] miR-218 was reported to exerts function role in regulating angiogenesis.[15]

Slit-roundabout (Robo) signaling consists of three Slit (Slit1-3) of secreted proteins and their four corresponding receptors (Robo1-4). Slit-Robo signaling was first discovered as a repulsive cue on axonal guidance.[16,17] However, the role of Robo1 in angiogenesis is still elusive. Han and Zhang[18] showed that Robo1 mediate inhibitory effect on corneal neovascularization. However, there was also evidence that Robo1 was pro-angiogenic. Wang *et al.*[19] reported that Robo1 mediated human umbilical vein EC (HUVEC) migration on Slit2 stimulation, indicating that Slit2-Robo1 signaling promoted cancer angiogenesis. Simultaneously, Robo1 was expressed in fibrovascular membranes, showing that Robo1 contributed to the development of diabetic retinopathy.[20] Whether Robo1 stimulated or inhibited RNV of oxygen-induced retinopathy (OIR) mice remains to be elucidated.

Previous studies indicated that Robo1 was one of the targets of miR-218, Small *et al.*[21] suggested that miR-218 contributed to retinal angiogenesis in mouse embryos. Thus, we put forward the hypothesis that miR-218 might also play a key role in retinal angiogenesis by inhibiting the expression of Robo1 in ocular disease. In our current study, mouse retinal vascular ECs and OIR mice were used to investigate the regulation of miR-218 in RNV.

Methods

Cell culture

Mouse retinal vascular endothelial cells (ECs) were purchased from Pricells (Wuhan, China) and were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/L streptomycin (Gibco, Carlsbad, USA) under 5% CO_2 at 37°C in a humidified incubator.

Small interfering RNA (siRNA) design

Robo1 siRNA (sense strand: 5′-CGGGAAAGG CCGAGGAACAAAGGCAGC-3′; antisense strand: 5′-GCUGCCUUUGUUCCUCGGC CUUUCCCG-3′) was synthesized by RiboBio Co., Ltd. (Guangzhou, China).

Cells were trypsinized and harvested from a monolayer to a cell suspension in the antibiotics-free medium. Cells (1.4×10^6/ml) were placed on 24-well ultra-low attachment plate (Corning Inc., Corning, NY, USA) in 1.5 ml of particular medium. Complexes of siRNA duplexes and Lipofectamine 2000 (Invitrogen, USA) were prepared as follows: 5 μl of 20 μmol/L siRNA was diluted in 500 μl Opti-MEM I medium (Gibco, USA) to which 5 μl of Lipofectamine 2000 was added. After 15 min incubation of this mixture, the complexes were added to each well. Cells were incubated at 37°C in a 5% CO_2 humidified incubator for 5 h and then plated on Petri dishes of diameter 10 cm for further analysis.

Plasmid construct

miR-218 was amplified using the following primers: forward primer, 5′-TTCTGAGGATCCGTGGA GGCACCTTTTCCATA-3′; reverse primer, 5′-ATTCTAAGATCTTT CACAGCTAGTCACACAATGG-3′. Plasmid vector pCDH-CMV-miR-218 was constructed by inserting pri-miR-218 polymerase chain reaction (PCR) fragment into plasmid vector pCDH-CMV-MCS-EF1-Puro (SBI, Norwood, USA) through *Eco*RI and *Not*I digestion. Vector pCDH-CMV-mock was used as the negative control.

Transfection

The cells in the exponential growth phase were seeded in 6-well plates (Corning Inc., Corning, NY, USA) at a density of 5×10^4 cells/ml. The cells were transfected with miR-218 mimics, miR-218 inhibitor, and the negative control (Shanghai GenePharma Co., Ltd.) using the lipofectamine 2000 (Invitrogen, USA) at a 5:1 volume/mass ratio of reagent to oligodeoxynucleotide in serum-free M199 for 6 h. After transfection, the cells were incubated in a complete medium (DMEM). Transfection of the miR-218 mimics, miR-218 inhibitor, and the negative control was performed using 1 μg of DNA per transfection. All transfections were performed according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells and retina tissues using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized using the cDNA Synthesis Kit (TaKaRa, Otsu, Japan). Real-time PCR reactions with SYBR Green qPCRKit (TaKaRa, Japan) were performed with specific primers. The cycle threshold (Ct) indicates the fractional cycle number at which the PCR product was first detected above a fixed threshold. Changes in the expression of target genes were calculated with the $2^{-\Delta Ct}$ method, where ΔCt = (Ct [target sample] − Ct [U6 sample] − (Ct [target calibrator] − Ct [U6 calibrator]).

The primer sequences used in our research were as follows:

- miR-218 primer, 5′-TTCTGAGGATCCGTGGA GGCACCTTTTCCATA-3′
- Robo1 forward primer, 5′-GAGGTAGCTA TACTACGGGATGAC-3′;
- Robo1 reverse primer, 5′-CAGATGTAGT AGCCGACATCAGAC-3′;
- U6 forward primer, 5′-CGCTTCGGCA GCACATATAC-3′;
- U6 reverse primer, 5′-AAAATATGGA ACGCTTCACGA-3′.

Western blotting

Total proteins of cell and retinae were isolated and separated on 10% SDS-PAGE gels (Bio-Rad, USA). Western blotting (WB) was performed according to the standard protocol. α-Tubulin

was used as a loading control. The antibodies used were anti-Robo1 (Abcam, ab7279, UK) and anti-α-Tublin (Sigma, St. Louis, USA). Bands were quantified by Image J software (National Institutes of Health, NIH, USA).

Scratch wound assay

Cell migration ability was assessed by scratch wound assay. Transfected cells were cultured in 6-well plates. When cells reached 90% confluence, a scratch wound was created using a pipette tip. Wound edges were photographed with Nikon Eclipse TE 2000-U (Nikon, Japan) and scratch widths were analyzed using ImageJ software (NIH). Three trials were used for each condition.

Oxygen-induced retinopathy mice

Neonate CB57BL/6J mice were obtained from the Animal Institute of Chinese Academy of Medical Sciences. Neovascularization was induced as described by Kong *et al*.[22] Briefly, at postnatal day 7 (P7), CB57BL/6J mice were exposed to hyperoxia (75% O_2) for 5 days (P12) and then returned to room air to induce RNV.

This research was approved by the Ethics Committee of Tianjin Eye Hospital (No. TJYYLL-2015-09). All animal experiments were conducted in accordance with the ARVO Statement for *the Use of Animals in Ophthalmic and Vision Research* and *the Guide for the Care and Use of Laboratory Animals*.

Intravitreal injection

Twelve-day-old OIR mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (30 mg/kg). Intravitreal injections were performed using a 10-μl Hamilton syringe fitted with a 32-G needle.

miR-218 injection group

Among the six mice, each animal received intravitreous injections of 0.4 μl (2 μg) pCDH-CMV -218 in one eye and 0.4 μl (2 μg) control plasmid in contralateral eye as the negative control.

Roundabout 1 siRNA injection group

Among the six mice, one eye was intravitreally injected with 0.4 μl (2 μg) Robo1 siRNA (RiboBio Co., Ltd., USA) and the other eye received intravitreous injections of 0.4 μl (2 μg) control siRNA (RiboBio) as the negative control.

Fluorescein angiography and quantification of preretinal neovascular nuclei were performed on postnatal day 17 (P17).

Fluorescein angiography

On P17, mice (n = 6/each group) were anesthetized and perfused with fluorescein via retro-orbital injection of 2.5 mg/50 μl of FITC-dextran (Sigma, USA) as described previously.[23] The eyes were enucleated and fixed with 4% paraformaldehyde in phosphate buffered saline for 1 h. Retinae were then separated from the eyecup. Four incisions were made, which was flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescent microscope (Nikon Eclipse TE 2000-U, Nikon, Japan). Images were analyzed by Photoshop 8.0 software (Adobe, USA). Neovascularization was calculated by the ratio of the number of pixels in the neovascular area to the total number of pixels in the retina.

Quantification of retinal neovascularization

On P17 the eyes of mice (n = 6/each group) were enucleated and fixed with 10% formaldehyde and embedded in paraffin. Six micrometers thick sagittal sections were made through the cornea parallel to the optic nerve and then stained with hematoxylin and eosin. The nuclei of vascular cells on the vitreal side of the retina were counted under a light microscope. Ten noncontinuous sections from each eye were examined, and cell numbers were averaged in each group of animals. The average number of preretinal vascular nuclei was compared.

Statistical analysis

Data were expressed as a mean ± standard deviation (SD) and analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). To compare multiple sets of data, one-way analysis of variance (ANOVA) test was used. For paired data sets, LSD t-test was used. Bivariate correlations were calculated by Spearman rank correlation coefficients. A $P < 0.05$ was considered statistically significant.

Results

Inhibition of cell migration by miR-218 was mediated by roundabout 1

Cell migration ability was tested and shown in Figure 1. Overexpression of miR-218 by miR-218 mimic dramatically reduced ECs migration, whereas inhibition of miR-218 expression using miR-218 inhibitor markedly promoted EC migration [Figure 1a and 1b].

The Robo1 expression was shown in Figure 1c. The results indicated that up-regulation of miR-218 expression decreased Robo1 expression and reduced EC migration. Conversely, down-regulation of miR-218 expression by miR-218 inhibitor increased Robo1 expression and promoted EC mobility. However, miR-218 inhibitor did not promote the migratory ability of EC after Robo1 knockdown by siRobo1 [Figure 1a and 1b]. These observations suggested that miR-218 suppressed EC migration by inhibiting Robo1 expression.

miR-218 and roundabout 1 expression in retinal neovascularization of oxygen-induced retinopathy mice

The expression level of miR-218 in the retinae of OIR mice was detected. As shown in Figure 2a, quantitative real-time polymerase chain reaction (qRT-PCR) results demonstrated that the expression level of miR-218 was significantly decreased (P = 0.006) in retinae of OIR mice at P17. Then, we compared gene and protein expression of Robo1 in retinae of OIR mice with control mice. The mRNA and protein level of Robo1 were up-regulated at P17 in OIR mice (P = 0.001, 0.008; respectively) [Figure 2].

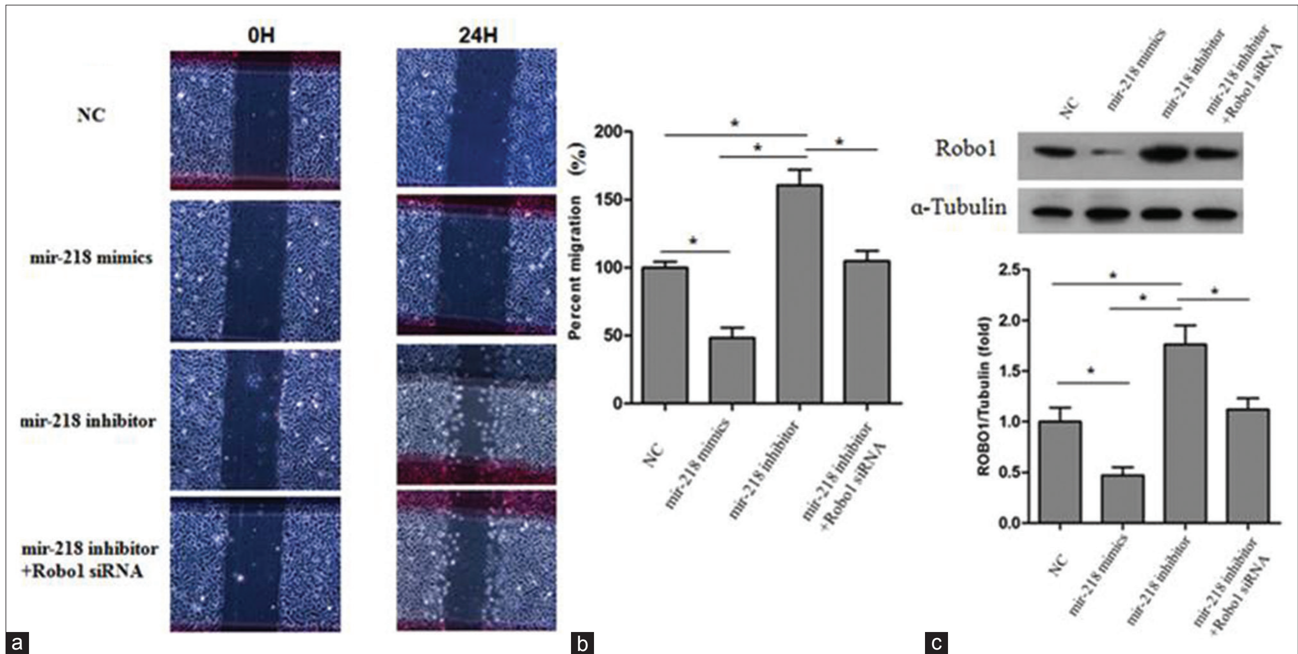

Figure 1: miR-218 inhibited EC migration by suppressing Robo1 expression. (a) Representative images of cell migration in the wounding area were indicated; (b) Quantification analysis of wound-healing assay; (c) Robo1 expression was determined by Western blotting assay and normalized by internal control (α-Tubulin). *$P < 0.05$. EC: Endothelial cell; Robo: Roundabout; NC: Negative control.

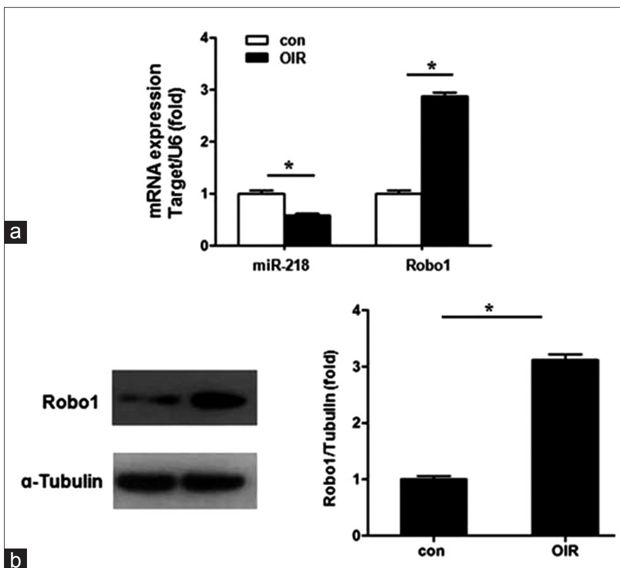

Figure 2: Identification of decreased miR-218 and increased Robo1 in the retina from OIR model. (a) qRT-PCR results showed that miR-218 was significantly decreased from OIR model compared to normal mice ($n = 5$). However, the expression of Robo1 mRNA was significantly increased ($n = 5$); (b) Western blotting analysis of Robo1 protein was highly expressed in the retina of OIR mice that in normal mice. *$P < 0.05$. OIR: Oxygen-induced retinopathy; Robo: Roundabout. qRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction.

Effects of roundabout 1 siRNA on retinal neovascularization

To evaluate the effect of Robo1 siRNA on RNV, the retinae were examined by fluorescein-dextrans perfusion on P17. The results showed that retinae from OIR group and negative control group developed a significant neovascularization [Figure 3a and 3b]. In contrast, fewer neovascular tufts were observed in the retinae from Robo1 siRNA group [Figure 3c].

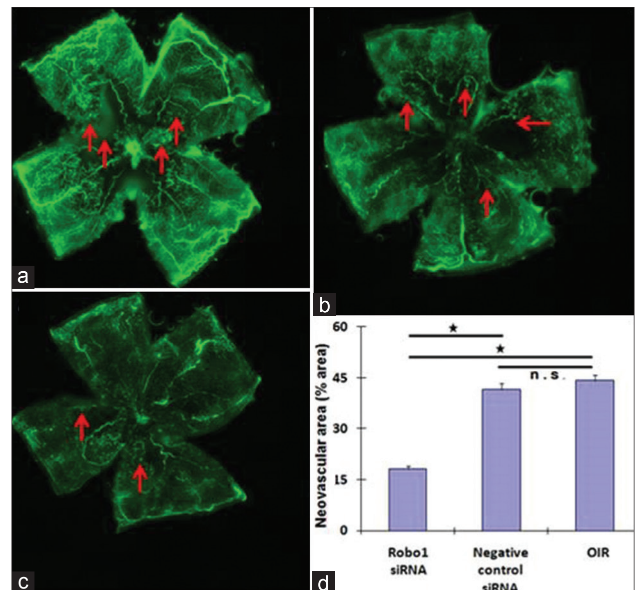

Figure 3: Inhibition of retinal neovascularization in OIR mice via intravitreous injections of Robo1 siRNA (mice were perfused with FITC-dextran, original magnification ×40). Fluorescein angiographs on postnatal day 17 (P17) were presented. (a) OIR mice, (b) OIR mice treated with control siRNA intravitreous injection, (c) OIR mice treated with Robo1 siRNA intravitreous injection, (d) Statistical analysis of retinal neovascularization. The results indicated that Robo1 siRNA effectively suppressed retinal neovascularization. Red arrows showed retinal neovascularization area. $n = 6$ mice/each group *$P < 0.05$. OIR: Oxygen-induced retinopathy; Robo: Roundabout.

Neovascularization was calculated by the ratio of the number of pixels in neovascular area to the total number of

pixels in the retina indicating that retinae of Robo1 siRNA group (18.2 ± 0.8% of whole retinal area) developed less neovascular area, 41.2% of that of OIR group (44.2 ± 1.6% of whole retinal area) (P = 0.048) and 43.8% of that of negative control siRNA group (41.6 ± 1.8% of whole retinal area), respectively (P = 0.069) [Figure 3d].

miR-218 inhibits retinal neovascularization through suppression of roundabout 1 expression

A plasmid pCDH-CMV-miR-218 expressing miR218 and a negative control plasmid pCDH-CMV-mock expressing mock sequence were constructed. Intravitreal injection of plasmids was performed at postnatal day 12 (P12).

On P17, we analyzed mRNA levels of miR-218 and Robo1 after intravitreal injection to verify whether the plasmids reached the retina. qRT-PCR results showed that pCDH-CMV-miR-218 significantly increased the expression of miR-218 (P = 0.006) and reduced that of Robo1 in retina (P = 0.012) [Figure 4a]. Western blotting assay indicated that intravitreal injection of pCDH-CMV-miR-218 significantly decreased the protein level of Robo1 within the retina of OIR mice (P = 0.026) [Figure 4b]. However, pCDH-CMV-mock had no effects on the expression of miR-218 and Robo1 (P = 0.14).

To examine the effects of miR-218 on RNV, we evaluated the retinal vasculature at P17 by fluorescein angiography in the flat-mounted retina. The retinae of normal mice showed a mature capillary network that extended from the optic to the periphery [Figure 5a]. The retinae of OIR and pCDH-CMV-mock group displayed more neovascular tufts [Figure 5b and 5c]. In contrast, the retinae of the pCDH-CMV-miR-218 group developed fewer neovascularization [Figure 5d]. The RNV was quantified by measuring areas of new blood tufts in the whole-mounted retina. The results showed that a large number of neovascular tufts appeared in OIR group (NV, 38.9% of the whole retina) and pCDH-CMV-mock group (NV, 36.8% of whole retina) (P = 0.127). However, in OIR group, neovascularization area significantly decreased in pCDH-CMV-miR-218 group (NV, 18.6% of whole retina) (P = 0.006) [Figure 5e]. No neovascularization was observed in the normal group. These results revealed an anti-neovascularization effect of miR-218 on RNV in OIR.

To further identify the inhibitory effect of miR-218 on angiogenesis, histological analysis was performed. We counted the vascular cell nuclei which broke through the inner limiting membrane, a marked feature of OIR.[24] There were no neovascular nuclei in the normal group [Figure 6a], but the average number of vascular cell nuclei was significantly increased in OIR group and pCDH-CMV-mock group by 61.48 ± 6.92 and 58.98 ± 6.48, respectively [Figure 6b and 6c]. However, pCDH-CMV-miR-218 group showed less neovascularization [Figure 6d] (12.64 ± 1.42 preretinal cells per section, (P = 0.004)) indicating that

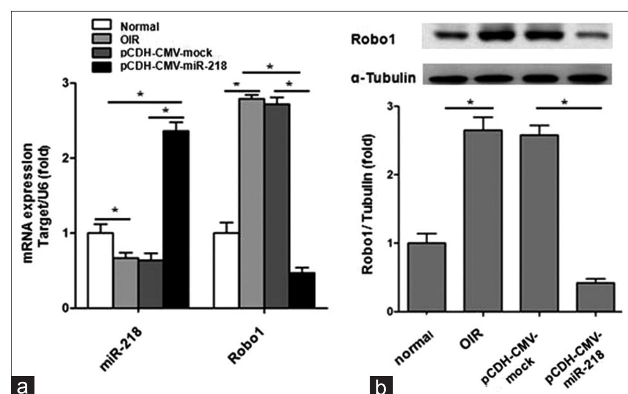

Figure 4: Down-regulation of Robo1 expression by intravitreal injection of miR-218 plasmid in OIR mice. (a) qRT-PCR results showed that relative Robo1 expression in the miR-218 group was significantly decreased compared to negative control group and OIR group; (b) Western blotting assay for Robo1 expression before and after miR218 intravitreal injection. Statistical analysis of WB indicated that intravitreal injection of miR218 could effectively inhibit Robo1 protein expression in retinae in OIR mice. n = 6 mice/each group. *P < 0.05. OIR: Oxygen-induced retinopathy; Robo: Roundabout. qRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction.

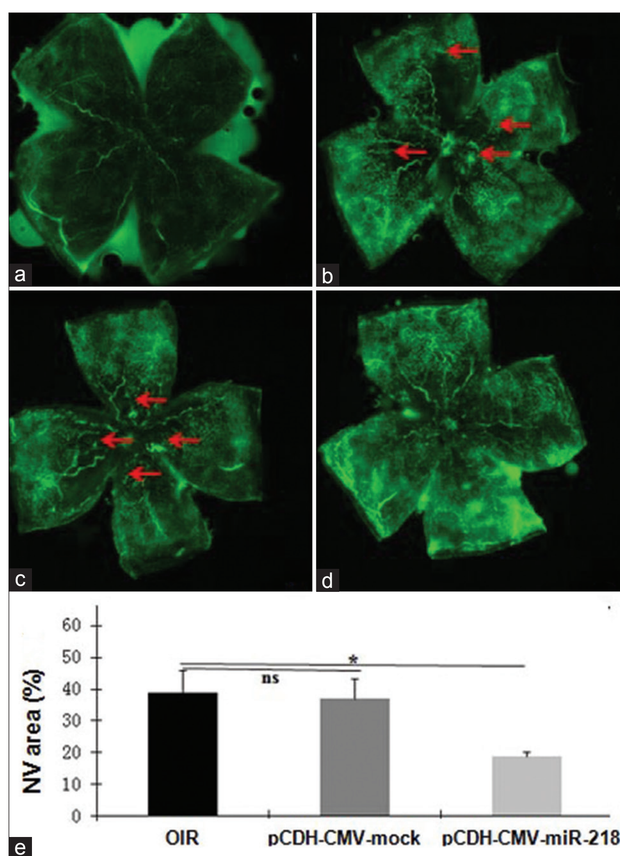

Figure 5: Representative retinal angiographs showed miR-218 inhibit retinal neovascularization, (mice were perfused with FITC-dextran, original magnification ×40). (a) Retinal vessels in normal mice; (b and c) In OIR group and pCDH-CMV-mock group (negative control), abnormal retinal vessels and neovascular tufts were presented at the junction between perfused and nonperfused region (red arrows); (d) After miR-218 injection, neovascular tufts area were reduced; (e) Quantification of retinal neovascularization from OIR group, pCDH-CMV-mock (negative control) group, and miR-218 injection group. n = 6 mice/each group. *P < 0.05. OIR: Oxygen-induced retinopathy; NV: Neovascularization.

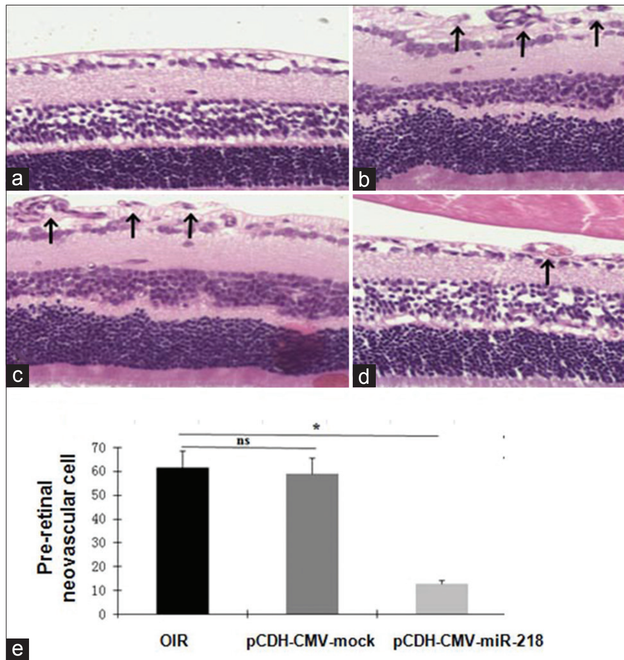

Figure 6: H and E staining of neovascularization in murine retinae, at postnatal day 17 (P17), the retinae were fixed and stained (Original magnification ×40). (a) Normal group; (b) OIR group; (c) pCDH-CMV-mock group (negative control); (d) miR-218 group. Arrow indicated preretinal vascular cell; (e) Vascular cell nuclei broke through ILM (arrows) were counted on ten sagittal sections and averaged. MiR-218 group showed less neovascularization. $n = 6$ mice/each group. $*P < 0.05$. OIR: Oxygen-induced retinopathy.

restoration of miR-218 played an inhibitory role in RNV in OIR [Figure 6e].

Taken together, these results indicated that miR-218 inhibited retinal angiogenesis in OIR by suppressing Robo1 expression.

Discussion

Robo1, one of Robo receptors-family, mediated negative signal in the nervous system.[25] However, Robo1 plays a positive role in the migration of ECs. Some studies demonstrated that decreased Robo1 markedly inhibited EC migration, such as in monkey choroidal retinal EC[26] and HUVEC.[19] This conclusion was further confirmed on mouse retinal EC in the present study.

The data presented in this study indicated that mRNA and protein expression level of Robo1 were significantly elevated in retinae of OIR. The result was consistent with that of previous study[26] and demonstrated the potential role of Robo1 in RNV. Meanwhile, retinal vascular formation data suggested that there was a significant positive correlation between Robo1 over-expression and RNV. The studies in tumor indicated that Robo1 plays a part in angiogenesis.[19] Huang *et al.*[26,27] pointed out that Robo1 might be involved in ocular neovascularization. Recently, genetic evidence by Rama *et al.*[28] supported a negative role for Robo1 in ocular pathological neovascularization. Our results also verified this point. In this study, we found that retinal neovascularization and ECs breaking into internal limiting membrane (ILM) in the retinae were both significantly reduced following the administrating of Robo1 siRNA. Therefore, down-regulation of Robo1 significantly suppressed RNV.

Important progress has been achieved in mapping the miRNA-gene target network. Previous data[14,21,29] supported that Robo1 could be a downstream target for miR-218, which binds to the 3'-UTR of Robo1 to down-regulate its expression. Thus, the research on miR-218/Robo1 has drawn intensive attention. Fish *et al.*[14] pointed out that miR-218/Robo1 controlled the migration of the heart fields to the midline in zebrafish. MiR-218 also modulated Slit-Robo signaling pathway to establish normal vascularization of the retina in mice.[21] Reports on tumor demonstrated that miR-218/Robo1 were involved in cancer progressions, such as gastric cancer[29] and nasopharyngeal cancer.[30] However, the role of interaction of miR-218 and Robo1 on RNV needs to be further clarified.

In *vitro*, cell migration was suppressed when miR-218 mimics was transfected into EC. Meanwhile, the expression of Robo1 was decreased in cells. In addition, an inverse expression pattern was found when miR-218 inhibitor was transfected into EC. Robo1 expression increased along with the decreasing of miR-218 expression and significant increasing of EC migration. However, miR-218 inhibitor had no effects on EC migration with knockdown of Robo1. It indicated that the inhibitory effect of miR-218 on EC migration was mediated by Robo1.

In OIR mice, the expression of miR-218 significantly decreased, and meanwhile the expression of Robo1 increased. The restoration of miR-218 by intravitreal injection of a plasmid expressing miR-218 tended to inhibit retinal angiogenesis, suggesting that miR-218 was an important regulator of RNV. Our results also proved that miR-218 might inhibit RNV mediated by down-regulation of Robo1 expression.

In summary, miR-218 was demonstrated as a modulator of oxygen-induced retinal neovascularization through suppressing the expression of Robo1. Restoration of miR-218 could be beneficial for the treatment on ocular neovascular disease.

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Conflicts of interest

There are no conflicts of interest.

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