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Original Article

miR-132 promotes retinal neovascularization under anoxia and reoxygenation conditions through up-regulating Egr1, ERK2, MMP2, VEGFA and VEGFC expression

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Abstract: Retinal neovascularization (RNV) is a prominent pathological angiogenesis, which causes detrimental outcomes in visual functions. Previous literature represents that miR-132 induces angiogenesis in tumor development and ischemic diseases. Considering the important role in angiogenesis, we hypothesized that miR-132 might be involved in RNV. In this study, human retinal microvascular endothelial cells were maintained in hypoxia for indicated time, followed by further incubation in normoxic conditions to establish hypoxia/reoxygenation (H/R) models *in vitro*. mRNA microarray analysis was undertaken to detect alterations in gene profiles in the cells. qRT-PCR and Western blotting were performed to evaluate expression of genes that are closely associated to neovascularization. Results showed that miR-132 expression was increased under hypoxic conditions. Reoxygenation for a limited time (6 h) failed to restore miR-132 expression to basal level. Interference of miR-132 expression via its inhibitor suppressed the cell proliferation under H/R conditions, increasing the apoptosis rate. mRNA microarray analysis revealed that miR-132 is involved in the regulation of vasculature development, blood vessel morphogenesis, and proliferation and migration of microvascular endothelial cells through regulating genes such as early growth response gene 1 (Egr1), extracellular signal-regulated kinase (ERK), metal matrix proteinase (MMP2), vascular endothelial growth factor (VEGF)-A and VEGF-C. qRT-PCR and Western blotting further demonstrated that miR-132 up-regulated their gene and protein expression under H/R conditions. In summary, miR-132 was involved in the development of RNV under H/R conditions, at least partly, through up-regulating Egr1, ERK2, MMP2, VEGFA and VEGFC expression. This finding facilitates the understanding of pathogenic mechanisms of RNV.

Keywords: Hypoxia, microvascular endothelial cells, miR-132, retinal neovascularization, VEGF

Introduction

Retinal neovascularization (RNV) is regarded as a pathological angiogenesis in retina. It is associated to various diseases including diabetic retinopathy, retinopathy of prematurity, retinal vein occlusions, and age-related macular degeneration [1, 2]. Development of RNV impairs visual function, even leading to blindness, because RNV is commonly accompanied with vitreous hemorrhage, macular edema and tractional retinal detachment [3]. Several lines of evidence indicate that ischemia and hypoxia are major causes of RNV, but the underlying mechanism remains largely undefined [4, 5]. It is generally believed that hypoxia triggers activation of hypoxia inducible factor (HIF). Activated HIF promotes expression of vascular endothelial growth factor (VEGF), thereby facilitating the occurrence and development of RNV [6, 7]. However, RNV is a multi-step process that involves complex interactions of a variety of angiogenic actors, inflammatory cytokines, chemokines and growth factors, as well as variation of the extracellular matrix that is substrates for endothelial migration [8]. That is the reason why agents solely against VEGF functions are unable to effectively heal RNV in the clinical settings.

MicroRNAs (miRNAs) is a class of 17-25 nucleotide small noncoding RNA. miRNAs modulates mRNA stability and/or translation through binding to the 3' untranslated region (3'-UTR) of mRNAs, thus it can regulate the expression of their target genes [9]. Through affecting gene

expression, miRNAs participates in regulation of many physical and pathological processes, such as cell development, differentiation, proliferation and tumorigenesis [10, 11]. miRNAs affecting development of RNV has been revealed in a few of recent researches. Shi et al. [12] found that reduction in miR-150 serum is an important cause of retinal vascular overgrowth in high-fat-diet induced diabetic mice. Besides, Han et al. [13] uncovered that microRNA-218 exerts inhibitory effects on oxygen-induced retinal neovascularization via reducing the expression of roundabout 1. There are also *in vitro* and *in vivo* studies indicating that down-regulation of microRNA-155 attenuates VEGF-triggered retinal neovascularization via the modulation of PI3K/Akt pathway [14].

miR-132 has emerged as an important factor involved in vascular formation. miR-132 was reported to enhance arteriogenesis after hindlimb ischaemia through modulation of the Ras-MAPK pathway [15]. Moreover, it has been confirmed that miR-132 plays a critical role in angiogenesis in human breast carcinoma by suppressing endothelial p120RasGAP expression and then leading to Ras activation [16]. Considering the important role in vascular formation, miR-132 might be involved in the pathogenesis of RNV. This study was performed to identify the hypothesis.

Materials and methods

Cell culture

Human retinal microvascular endothelial cells (HRMECs) were obtained from Cell Systems (Kirkland, WA, USA). HRMECs were cultured in endothelial basal medium-2 (EBM-2; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and endothelial cell growth supplements (EGM SingleQuots; Lonza, Basel, Switzerland). The cells were maintained in a humidified incubator with 5% CO_2. Culture medium was switched every 3 days and changed to culture medium without the fetal bovine serum and growth supplements 24 h before HRMECs were subjected to any treatment.

Cell treatments

HRMECs were exposed to hypoxia by culturing in a sealed chamber that created a hypoxic condition (0.5% O_2). Then, the cells were cultured under normoxic conditions (reoxygenation) for indicated time periods. Hereafter, these processes were referred as hypoxia/reoxygenation (H/R). The miR-132 inhibitors and negative control molecules, which were synthesized by GenePharma Co., Ltd. (Shanghai, China), were added to the culture media of HRMECs at a final concentration of 100 nM and transfected into cells using Lipofectamine™ 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from HRMECs using Trizol reagent (TaKaRa, Japan). The expression of miR-132 was quantified by qRT-PCR using TaqMan microRNA assays (Applied Biosystems, Carlsbad, CA, USA) and normalized to U6 expression. The expression of other indicated genes was quantified by qRT-PCR using SYBR-Green assays (Applied Biosystems) and normalized to that of β-actin. Gene expression was calculated using the $2^{-\Delta Ct}$ method as previously described by Yang et al. [17]. The primers were as follows: miR-132 forward primer, 5'-GGCAACCGTGGCTTTCGA-3'; reverse primer, 5'-TTTGGCACTAGCACATT-3'; U6 forward primer, 5'-CTCGCTTCGGCAGCACA-3'; reverse primer, 5'-AACGCTTCACGAATTTGCGT-3'; early growth response gene 1 (Egr1) forward primer, 5'-TGACCGCAGAGTCTTTTCCT-3'; reverse primer, 5'-TGGGTTGGTCATGCTCACTA-3'; extracellular signal-regulated kinase (ERK) forward primer, 5'-CCAGACCATGATCACACAGG-3'; reverse primer, 5'-CTGGAAAGATGGGCCTGTTA-3'; chemokine (C-X-C motif) ligand 1 (CXCL1) forward primer, 5'-AGGGAATTCACCCCAAGAAC-3'; reverse primer, 5'-CACCAGTGAGCTTCCTCCTC-3'; ephrin A1 (EFNA1) forward primer, 5'-GAGACAGTCCTTTCCCACCA-3'; reverse primer, 5'-CTGGCTTCCAAGCAAGAAAC-3'; metal matrix proteinase 2 (MMP2) forward primer, 5'-ACAGCAGGTCTCAGCCTCAT-3' reverse primer, 5'-TGAAGCCAAGCGGTCTAAGT-3'; VEGFA forward primer, 5'-AAGGAGGAGGGCAGAATCAT-3'; reverse primer, 5'-ATCTGCATGGTGATGTTGGA-3'; VEGFC forward primer, 5'-GGAAAGAAGTTCCACCACCA-3'; reverse primer, 5'-ATCTGCATGGTGATGTTGGA-3'; β-actin forward primer, 5'-CATTAAGGAGAAGCTGTGCT-3'; reverse primer, 5'-GTTGAAGGTAGTTTCGTGGA-3'.

mRNA microarray analysis

mRNA microarrays (HOA 7.1) were used for the analysis of differentially expressed genes. After

indicated treatments, total RNA was extracted from HRMECs using Rneasy MiNi Kit (Qiagen, CA, USA) according to the manufacturer's instruction. The test sample and the reference were labelled with Cy5, and co-hybridized to the mRNA arrays. The hybridized arrays were scanned by Agilent 0.1 XDR (Phalanx Biotech, Taiwan) and subsequently analyzed using Rosetta Resolver 7.2, a specialized microarray data storage and analysis software package developed by Rosetta Biosoftware (Merck & Co., Inc, NJ, USA). Expression profile clustering and visualization were preformed with unsupervised hierarchical clustering analysis (Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA, USA).

MTT assay

Cell viability of HRMECs after treatments was assessed by MTT assay. HRMECs were exposed to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 5 mg/ml, and incubated for 4 h at 37°C. The formazan generated in each well was dissolved in 150 ml of DMSO (dimethyl sulfoxide) (Sigma-Aldrich). Absorbance of each well at 490 nm was read using a microplate reader.

Apoptotic rate measurement

After indicated treatments, HRMECs were dual-stained with Alexa Fluor 488-Annexin V and propidium iodide (PI) using an Annexin V-fluorescein isothiocyanate/PI apoptosis kit (Kaiji Biological Inc., Nanjing, China) according to the manufacturer's instructions. The apoptotic rate was measured using flow cytometry (FC 500 MPL system; Beckman Coulter Inc., Miami, FL, USA).

Wound healing assay

Migration ability of HRMECs was evaluated by wound healing assay. HRMECs were maintained in 6-well plates to 80% confluence formed. 1-mL pipette tip was used to scratch a line on the cell monolayer. Then the cells were subjected to indicated treatments. Microscopic images of the same area were captured immediately after the scratching as well as treatments. Cell migration rate was calculated using the following equation: (initial distance - final distance/initial distance) × 100.

Western blot assay

RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) was used to extract protein from HRMECs. BCA Protein Assay Kit (Thermo Scientific, USA) was used to measure the protein concentration. Total 20 μg of proteins were separated on 10% or 12% SDS-PAGE and blotted onto 0.22 μm nitrocellulose membranes. After blocking with 5% non-fat milk for 2 h, the membranes were incubated with the primary antibodies including anti-Egr1 antibody (Dilution 1:800, ab191441, Abcam), anti-ERK2 antibody (Dilution 1:1000, ab32081, Abcam), anti-CXCL1 antibody (Dilution 1:400, ab86436, Abcam), anti-EFNA1 antibody (Dilution 1:1000, ab199-697, Abcam), anti-MMP2 antibody (Dilution 1:500, ab124294, Abcam), anti-VEGFA antibody (Dilution 1:500, ab1316, Abcam), anti-VEGFC antibody (Dilution 1:500, ab9546, Abcam) and anti-β-actin antibody (Dilution 1:800, sc-47778, SANTA) at 37°C for 1 h. The membranes were then washed with tris-buffered saline containing 0.1% Tween20 (TBST), and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:2000; goat anti-mouse, 1:2000; Santa Cruz, USA) for 1 h at 37°C. Enhanced chemiluminescence reagent (Merck Millipore, Germany) was used to detect the signal on the membrane. The data were analyzed via densitometry using Image-Pro plus software 6.0 (BIO-RAD, MD, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA) and the data are presented as the mean ± standard deviation. One way analysis of variance (ANOVA) with Bonferroni *t* post-test was used to analyze the data. $P < 0.05$ indicates a statistically significant difference.

Results

Level of miR-132 expression in HRMECs under H/R conditions

HRMECs were incubated in hypoxic conditions for different time periods (2, 4, 6, 8 and 10 h). Using qRT-PCR assay, we found that miR-132 was up-regulated during hypoxia, with the highest expression at time point of 6 h ($P < 0.05$,

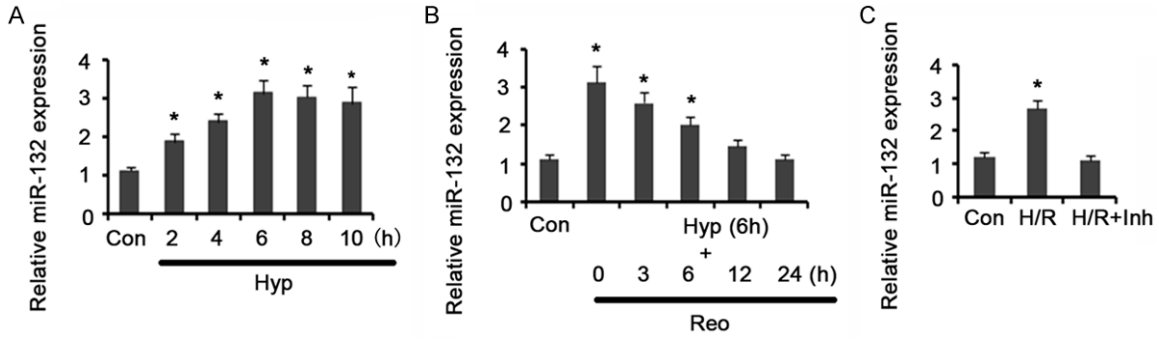


Figure 1. miR-132 expression in HRMECs following indicated treatments. A. HRMECs were incubated in hypoxic conditions for different time periods (2, 4, 6, 8 and 10 h). B. After maintaining in hypoxia for 6 h, HRMECs were cultured in a normoxic condition for additional time periods (3, 6, 12 and 24 h). C. HRMECs were incubated under hypoxia for 6 h, followed by culture under normoxia conditions for additional 6 h. These processes were referred as hypoxia/reoxygenation (H/R). Before the H/R, HRMECs were transfected with miR-132 inhibitor or the negative control. qRT-PCR assay was performed to detected miR-132 expression in HRMECs. The untreated cells were used as control. *$P < 0.05$ *vs.* control (n = 4). Hyp: hypoxic conditions; Reo: Reoxygen to normoxic conditions. Con: control. H/R: hypoxia with following reoxygen; Inh: miR-132 inhibitor.

Figure 1A). After maintaining in hypoxia for 6 h, HRMECs were cultured in a normoxic condition for additional time periods (3, 6, 12 and 24 h). Restoration of the oxygen level gradually reversed miR-132 expression, but miR-132 expression level was still higher than control level after incubation in normoxia for 3 and 6 h ($P < 0.05$, **Figure 1B**). To inhibit miR-132 expression, miR-132 inhibitor was transfected into HRMECs prior to the incubation under H/R conditions. qRT-PCR assay demonstrated that miR-132 inhibitor effectively suppressed miR-132 up-regulation under H/R conditions.

Regulatory effects of miR-132 on HRMECs' proliferation, apoptosis and migration under H/R conditions

HRMECs showed an increased proliferation rate under H/R conditions, compared to control cells that were cultured under normal condition ($P < 0.05$, **Figure 2A**). However, inhibition of miR-132 expression abolished the increase in cell proliferation. Apoptosis assay showed that apoptosis rate of HRMECs was decreased under H/R conditions ($P < 0.05$, **Figure 2B**), whereas inhibition of miR-132 expression reversed the apoptosis rate. As shown in **Figure 2C**, migration rate of HRMECs was dramatically increased under H/R conditions ($P < 0.05$). Suppressing miR-132 expression just moderately decreased the migration rate ($P < 0.05$ vs. control).

Involvement of miR-132 in vasculature development

mRNA microarray analysis was undertaken to detect alterations in gene profiles in HRMECs under H/R conditions. **Figure 3A** shows heatmap of the genes differentially expressed in the three groups. Red represents up-regulated mRNAs, but blue indicates down-regulated mRNAs. **Figure 3B** exhibits volcano plots for the visualization of differentially expressed mRNAs with significance cut off $P < 0.005$ and fold-change > 1.5 symmetrically in the cells. The mRNA microarray test screened more than 30 thousand of mRNAs that were involved in diverse kinds of physiological and pathological processes. It was detected that 2207 genes were up-regulated in HRMECs under H/R conditions, while 2482 genes were down-regulated (**Figure 3C**). Suppressing miR-132 expression increased levels of 2410 gene expression, but decreased levels of 2316 gene expression, compared to control. **Figure 3D** expresses the fold changes of genes between each groups. In the horizontal axis, positive numbers indicate the folds of gene up-regulation, while negative numbers indicate the folds of gene down-regulation. Our data showed a normal distribution (also known as Gaussian distribution) of gene fold changes between groups.

GO (Gene Ontology) analysis for differentially expressed genes showed the top 10 altered biological processes under H/R conditions. Vas-

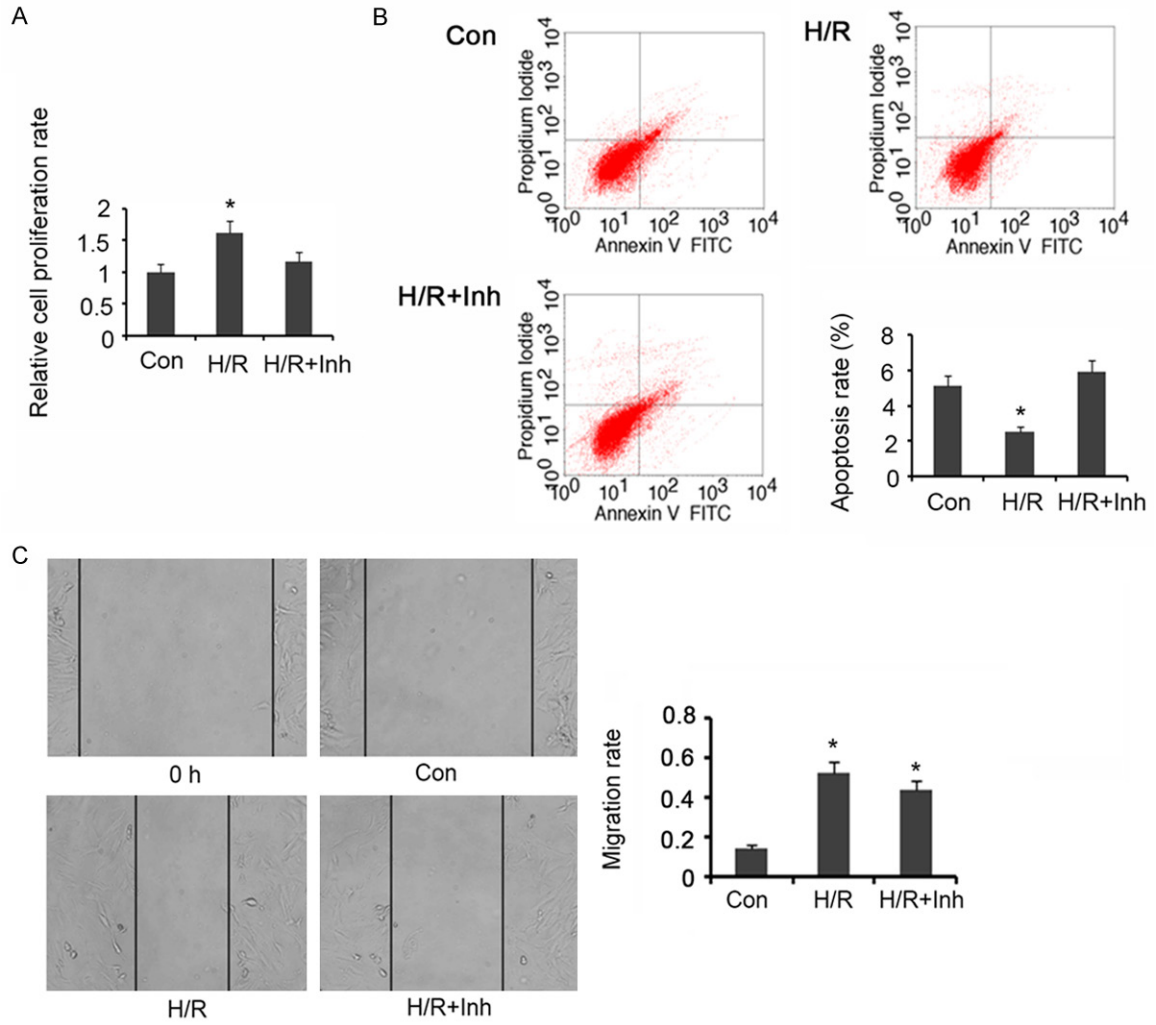
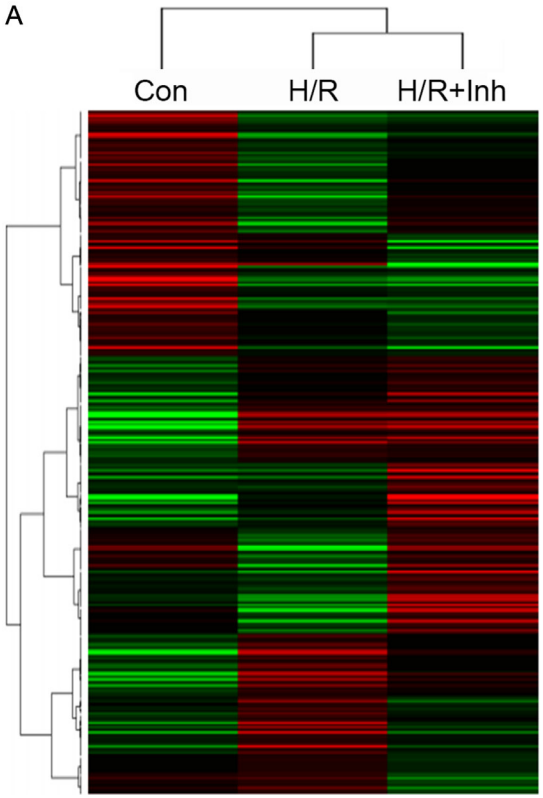


Figure 2. Association between miR-132 and hallmarks of HRMECs including proliferation, apoptosis and migration. HRMECs were maintained in hypoxia for 6 h, followed by incubation in a normoxic condition for additional 6 h. These processes were referred as hypoxia/reoxygenation (H/R). Before the H/R, HRMECs were transfected with miR-132 inhibitor or the negative control. MTT (A), apoptosis rate (B) and wound healing assays (C) were performed to evaluated cell proliferation, apoptosis and cell migration ability respectively. The untreated cells were used as control. *P < 0.05 *vs.* control (n = 4). Con: control. H/R: hypoxia with following reoxygen; Inh: miR-132 inhibitor.

culature development, blood vessel development and blood vessel morphogenesis were ranked as the first, the second and the fifth places respectively (**Table 1**). Cell proliferation, cell death, programmed cell death (apoptosis and autophagy), cell motion and cell migration were also included in the top 10 altered biological processes. These processes have been confirmed to be closely associated with vasculature development. GO analysis after the inhibition of miR-132 expression under H/R conditions indicated that miR-132 expression intervention mainly affects cell cycle and cell proliferation (**Table 2**).

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis showed that pathway governing focal adhesion, ECM-receptor interaction and actin cytoskeleton were notably impacted under H/R conditions (**Table 3**). The pathway may be involved in the regulation of cell migration. Besides, H/R dramatically influenced signaling pathway regulating steroid biosynthesis, adipocytokine production and secretion, fatty acid metabolism. Inhibition of miR-132 expression under H/R conditions also notably affected the pathway regulating focal adhesion and ECM-receptor interaction (**Table 4**). Moreover, miR-132 expression intervention influenced the signaling pathway controlling DNA replication, cell cycle and p53 pathway, which suggests that miR-132 is associated to cell proliferation and apoptosis.

A



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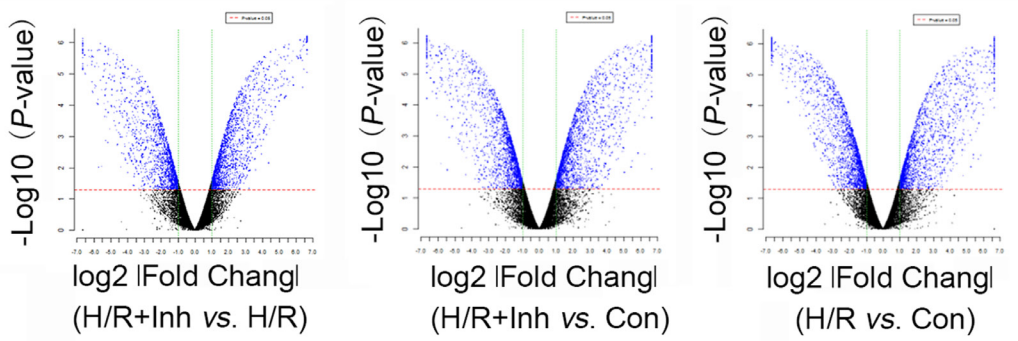

C

Contrast group	Numbers of up-regulated genes	Numbers of down-regulated genes
H/R *vs.* Con	2207	2482
H/R+Inh *vs.* Con	2410	2316
H/R+Inh *vs.* H/R	1989	1894

D

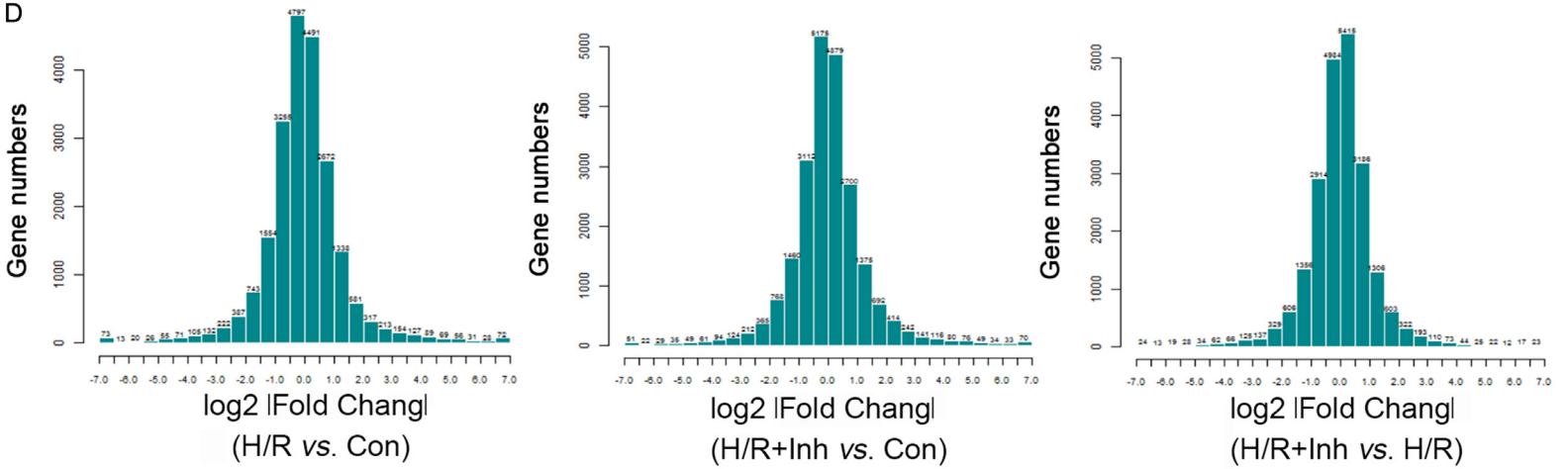

Figure 3. Association between miR-132 and vasculature development. mRNA expression profile microarray screening was performed, which identified more than 30 thousand of mRNAs that involves diverse kinds of biological processes, to understand regulatory effect of miR-132 in HRMECs in hypoxia. HRMECs were incubated under hypoxia for 6 h, followed by culture under normoxia conditions for additional 6 h. These processes were referred as hypoxia/reoxygenation (H/R). Before the H/R, HRMECs were transfected with miR-132 inhibitor or the negative control. A. Heatmap of the genes differentially expressed in the three groups cells. Red represents upregulated mRNAs, but blue indicates downregulated mRNAs. B. A volcano plot for the visualization of differentially expressed mRNAs with significance cut off $P < 0.005$ and fold-change > 1.5 symmetrically in the cells (n = 4). C. Numbers of genes that were changed among groups. D. Fold changes of genes between each groups. In the horizontal axis, positive numbers indicate the folds of gene up-regulation, while negative numbers indicate the folds of gene down-regulation.

Table 1. Top 10 altered biological processes in GO analysis after H/R treatments (B *vs.* A)

Geneset name	Genes in overlap (k)	*p* value
GO:0001944~vasculature development	96	2.75E-11
GO:0001586~blood vessel development	94	3.68E-11
GO:0042127~regulation of cell proliferation	232	4.89E-10
GO:0010941~regulation of cell death	236	2.14E-10
GO:0048514~blood vessel morphogenesis	82	3.57E-10
GO:0043067~regulation of programmed cell death	234	4.23E-10
GO:0010033~response to organic substance	212	4.73E-10
GO:0042981~regulation of apoptosis	231	7.52E-10
GO:0051270~regulation of cell motion	76	8.36E-10
GO:0030334~regulation of cell migration	68	2.51E-09

A: control; B: culture under H/R conditions. $P < 0.05$ indicates a statistically significant difference.

Table 2. Top 10 altered biological processes in GO analysis after the inhibition of miR-132 expression under H/R conditions (C *vs.* B)

Geneset name	Genes in overlap (k)	*p* value
GO:0007049~cell cycle	237	7.90E-21
GO:0022403~cell cycle phase	148	7.38E-20
GO:0000279~M phase	122	6.33E-18
GO:0022402~cell cycle process	175	5.68E-16
GO:0000278~mitotic cell cycle	127	2.08E-15
GO:0048285~organelle fission	90	4.95E-15
GO:0007067~mitosis	86	3.04E-14
GO:0000280~nuclear division	86	3.04E-14
GO:0000087~M phase of mitotic cell cycle	87	3.28E-14
GO:0051301~cell division	103	3.59E-13

B: culture under H/R conditions; C: silencing miR-132 prior to culture under H/R conditions. $P < 0.05$ indicates a statistically significant difference.

In the mRNA microarray analysis, we found that expression profiles of genes such as *Egr1*, *ERK2*, *CXCL1*, *EFNA1*, *MMP2*, *VEGFA* and *VEGFC* are changed under H/R conditions compared to the normoxic conditions (**Table 5**). These genes are closely related to vasculature development, blood vessel morphogenesis, and proliferation and migration of microvascular endothelial cells. Inhibition of miR-132 expression changed their expression levels.

Interruption of miR-132 expression under H/R conditions led to changes of signaling pathways related to angiogenesis, as indicated by KEGG pathway enrichment analysis. VEGF pathway is critical for sustained angiogenesis. VEGF is regulated by HIF-α and HIF-β. Results showed that suppressing miR-132 expression impaired VEGF expression compared to H/R group, although HIF-α and HIF-β were up-regulated (**Figure 4A**). H/R is known as a leading cause for oxidative stress. Oxidative stress transcriptionally regulates genes and further impacts endothelial proliferation and migration (through degradation of extracellular matrix and chemoattraction of endothelial cells), thus promoting angiogenesis. Suppressing miR-132 expression impaired up-regulation of VEGF, MMPs, and IL-8 that were induced by oxidative stress (**Figure 4B**). VEGF, MMPs, and IL-8 are also regulated by ERK pathway. **Figure 4C** shows that down-regulated ERK was companied with reduction in VEGF, MMPs, and IL-8. ERK pathway also regulates proliferation and survival of endothelial cells. The down-regulation after miR-132 expression interruption probably inhibited cell proliferation and caused cell death (**Figure 4D**).

Table 3. Top 10 altered pathway in KEGG analysis after H/R treatments (B *vs.* A)

Geneset name	Genes in overlap (k)	*p* value
hsa04510:Focal adhesion	69	2.38E-05
hsa04920:Adipocytokine signaling pathway	29	1.27E-04
hsa04512:ECM-receptor interaction	34	1.40E-04
hsa00100:Steroid biosynthesis	12	1.44E-04
hsa05200:Pathways in cancer	97	3.56E-04
hsa00760:Nicotinate and nicotinamide metabolism	14	4.36E-04
hsa04530:Tight junction	45	1.31E-03
hsa05410:Hypertrophic cardiomyopathy (HCM)	31	2.17E-03
hsa04810:Regulation of actin cytoskeleton	64	3.52E-03
hsa00071:Fatty acid metabolism	17	5.90E-03

A: control; B: culture under H/R conditions. $P < 0.05$ indicates a statistically significant difference.

Table 4. Top 10 altered pathway in KEGG analysis after the inhibition of miR-132 expression under H/R conditions (C *vs.* B)

Geneset name	Genes in overlap (k)	*p* value
hsa03030:DNA replication	21	8.46E-07
hsa04110:Cell cycle	45	8.30E-06
hsa04115:p53 signaling pathway	25	8.97E-04
hsa05200:Pathways in cancer	85	9.79E-04
hsa00480:Glutathione metabolism	19	2.98E-03
hsa04512:ECM-receptor interaction	27	4.69E-03
hsa04114:Oocyte meiosis	32	9.85E-03
hsa00330:Arginine and proline metabolism	18	1.40E-02
hsa04510:Focal adhesion	51	1.75E-02
hsa05219:Bladder cancer	15	1.75E-02

B: culture under H/R conditions; C: silencing miR-132 prior to culture under H/R conditions. $P < 0.05$ indicates a statistically significant difference.

Effect of miR-132 on expression of genes related to RNV

To further understand the regulatory effects of miR-132 on expression of genes including *Egr1*, *ERK2*, *CXCL1*, *EFNA1*, *MMP2*, *VEGFA* and *VEGFC*, qRT-PCR and western blot assays were performed after indicated treatments. As shown in **Figure 5A**, Egr1, ERK2, CXCL1, MMP2, VEGFA and VEGFC were up-regulated in HRMECs under HR conditions ($P < 0.05$), but EFNA1 expression level was not affected (data not shown). Depletion of miR-132 expression level abrogated the up-regulation of genes including Egr1, ERK2, CXCL1, MMP2, VEGFA and VEGFC. Similar to qRT-PCR outcomes, Western blotting showed the up-regulation of proteins including Egr1, ERK2, CXCL1, MMP2, VEGFA and VEGFC under HR conditions ($P < 0.05$, **Figure 5B**). Inhibition of miR-132 expression reversed expression of Egr1, ERK2, MMP2, VEGFA and VEGFC, but not CXCL1.

Discussion

In response to hypoxia, signaling pathways are induced to modulate angiogenesis to improve local blood supply and remit the lack of oxygen. Previous studies focusing on tumor pathogenesis and ischemic diseases (e.g. stroke) provide mounting evidence that miRNAs plays critical role in the pro-angiogenic signaling [18-20]. As many genes that associate to angiogenesis are regulated by miRNAs, hypoxia modulating miRNAs expression becomes an important approach regulating angiogenesis. But it should be noted that, in some cases, there exists a feedback loop in which the target genes conversely impact on expression of miRNAs. For instance, HIF-1A was confirmed as a target of miR-429. miR-429 attenuates HIF-1 activity by decreasing HIF1A mRNA in human endothelial cells during the early stages of hypoxia. But HIF-1A can promote miR-429 expression during normoxic conditions [21]. This evidence show complicated interaction between miRNAs and their target genes. Hypoxia takes the major responsibility of RNV pathogenesis. Although over-activation of HIF/VEGF signaling by hypoxia has been confirmed to participate in the RNV pathogenesis by numerous studies [6, 7], other molecular mechanisms underlying hypoxia-induced RNV are largely unknown.

The present study provided novel evidence that miR-132 up-regulation is probably involved in

Table 5. Expression profiles of candidate genes involved in angiogenic function of miR-132

Gene symbol	Normalized intensity			log2 (Ratio)			*P*-value (Differentially expressed)		
	A	B	C	B/A	C/A	C/B	B/A	C/A	C/B
EGR1	2903.29	58255.04	14737.15	4.36	2.34	-2.08	2.49E-04	1.36E-04	2.33E-04
ERK2	5315. 90	10385.33	5619.38	1.09	0.08	-0.98	0.02	0.86	0.04
CXCL1	111.26	392240.8	103573.3	6.64	6.64	-1.92	0. 5E-04	0. 66E-04	3.71E-04
EFNA1	35442.27	8354.93	15882.76	-2.00	-1.12	0.82	9.24E-04	0.02	0.07
MMP2	280.96	6043.50	901.52	4.33	1.48	-2.85	1.18E-03	0.04	8.78E-03
VEGFA	30765.94	58802.92	25496.93	0.99	-0.23	-1.29	0.03	0.58	6.77E-03
VEGFC	529.11	3097.81	1244.08	2.45	1.06	-1.40	0.58E-03	0.08	0.01

The data were arisen from mRNA microarray analysis. HRMECs were incubated under hypoxia for 6 h, followed by culture under normoxia conditions for additional 6 h. These processes were referred as hypoxia/reoxygenation (H/R). Before the H/R, HRMECs were transfected with miR-132 inhibitor or the negative control. A: control; B: culture under H/R conditions; C: silencing miR-132 prior to culture under H/R conditions. $P < 0.05$ indicates a statistically significant difference.

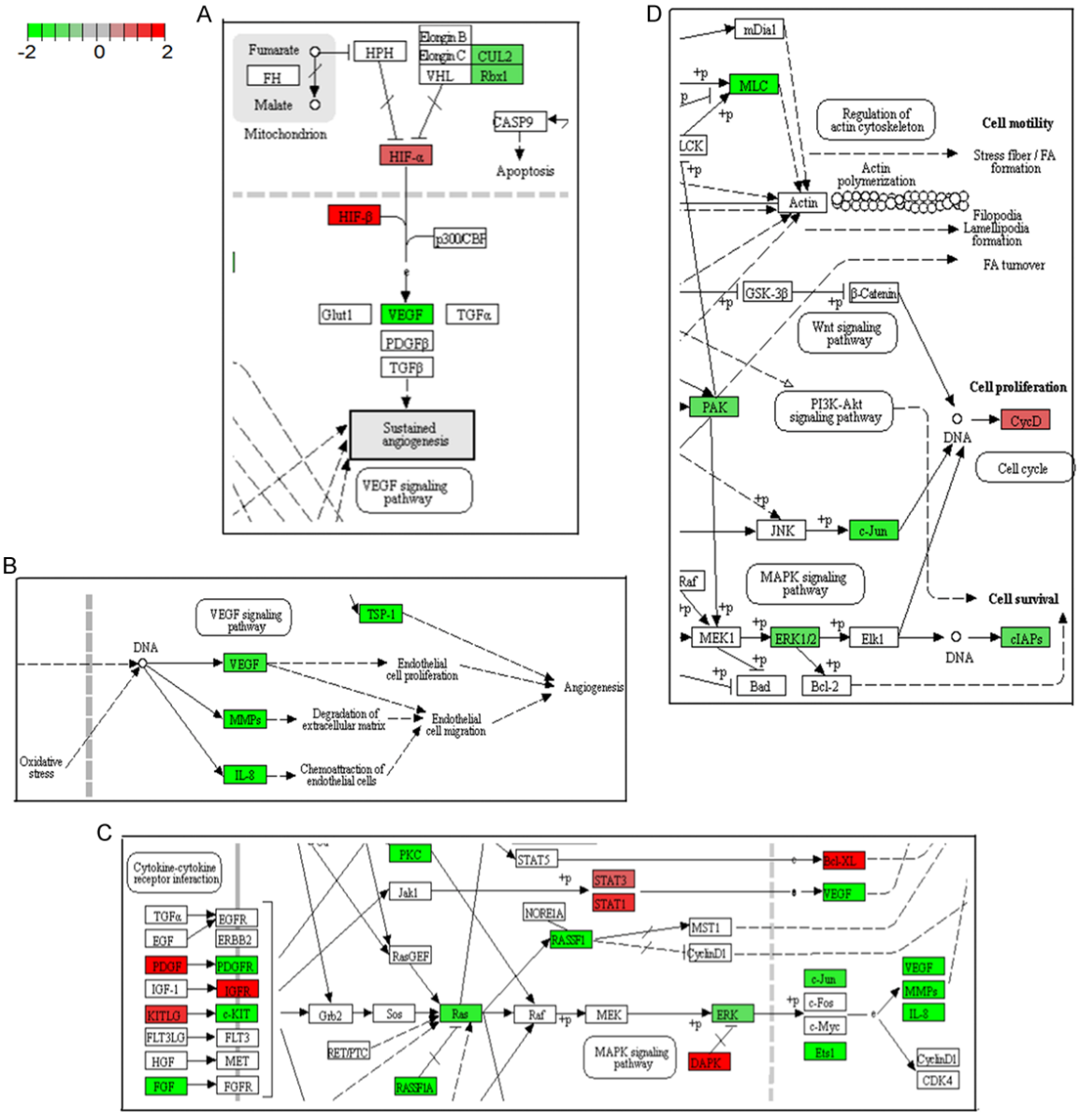

Figure 4. Changes of signaling pathways related to angiogenesis after miR-132 interruption. KEGG pathway analysis showed changes of a part of signaling pathways related to angiogenesis after interruption of miR-132 expression under H/R conditions. In comparison to control (H/R group), the red color represents the gene was up-regulated after the miRNA intervention; the green color represents the gene was down-regulated. A. HIF/VEGF pathway regulating sustained angiogenesis. B. Oxidative stress transcriptionally regulates genes and further impacts endothelial proliferation and migration, thus promoting angiogenesis. C. ERK pathway modulates expression of VEGF, MMPs, and IL-8, which are critical for angiogenesis. D. ERK pathway also regulates proliferation and survival of endothelial cells.

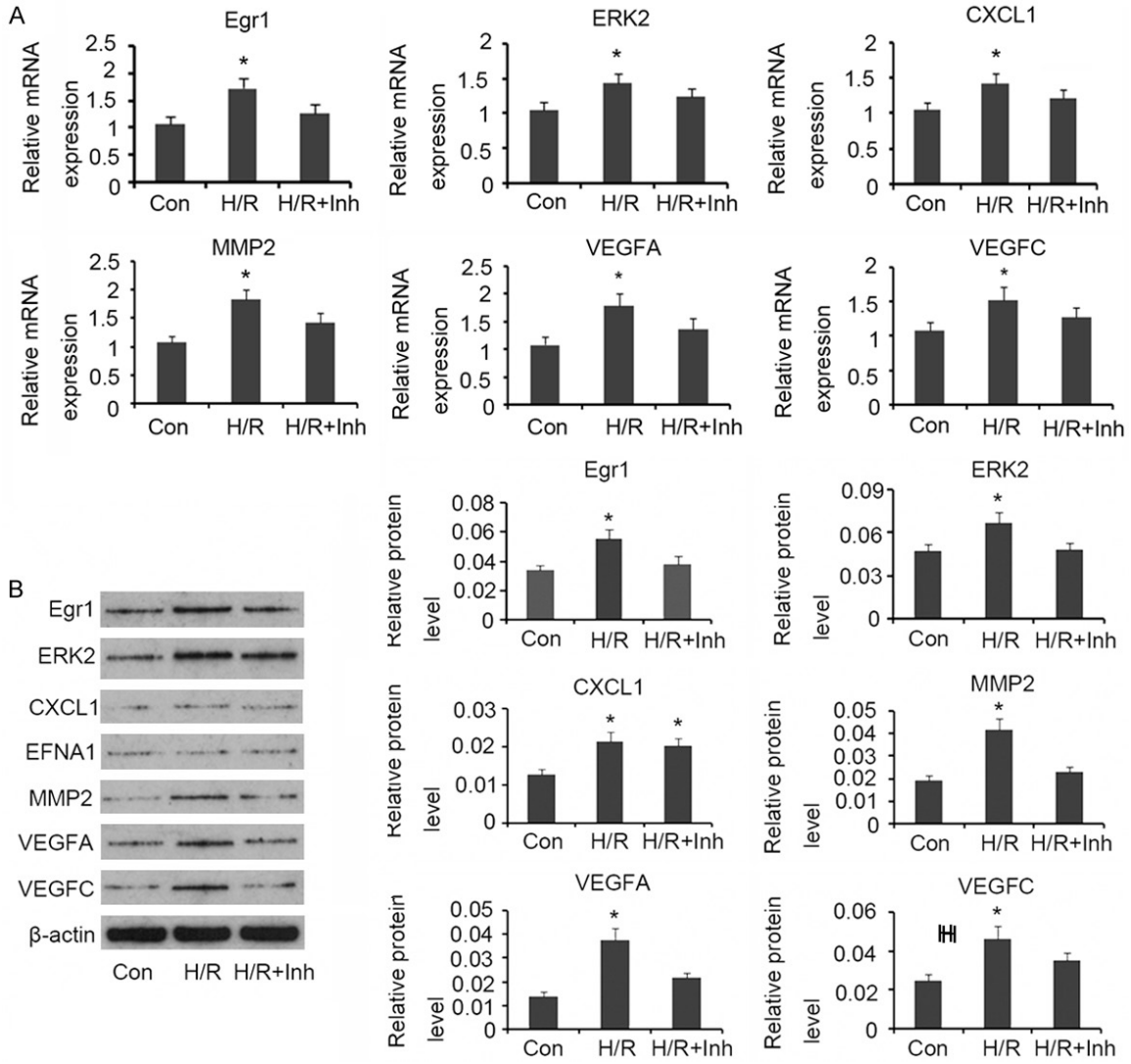

Figure 5. Regulatory effects of miR-132 on genes related to neovascularization. HRMECs were maintained in hypoxia for 6 h, followed by incubation in a normoxic condition for additional 6 h. These processes were referred as hypoxia/reoxygenation (H/R). Before the H/R, HRMECs were transfected with miR-132 inhibitor or the negative control. qRT-PCR (A) and Western blotting (B) were performed to evaluate expression of genes including Egr1, ERK2, CXCL1, EFNA1, MMP2, VEGFA and VEGFC. U6, a small nuclear RNA, was used an internal control for miR-132 in qRT-PCR. *$P < 0.05$ *vs.* control (n = 4). Con: control. H/R: hypoxia with following reoxygen; Inh: miR-132 inhibitor.

the pathogenesis of RNV. Up-regulated miR-132 in HRMECs under H/R conditions was accompanied with rapid cell proliferation and migration, but low apoptosis rate. However, interference of miR-132 expression with miR-132 inhibitor abolished the increase in cell proliferation and reduction in apoptosis rate. Increased proliferation of HRMECs is an important cause of RNV. mRNA microarray analysis manifested that miR-132 is associated to regu-

lation of blood vessel development and morphogenesis, in addition to the regulation cell proliferation and apoptosis, because numerous genes related to blood vessel development were altered by miR-132 (see KEGG analysis).

mRNA microarray analysis showed that expression profiles of genes such as *Egr1*, *ERK2*, *CXCL1*, *EFNA1*, *MMP2*, *VEGFA* and *VEGFC* are changed by miR-132 under H/R conditions. These genes are implicated in multiple processes of neovascularization, according to previous documents [22-25]. qRT-PCR and Western blotting further identified that these genes, except for *EFNA1*, were up-regulated in HRMECs under HR conditions, whereas interference of miR-132 expression via miR-132 inhibitor reversed their expression (miR-132 inhibition restored the mRNA expression of CXCL1, but not the protein level). Egr1, a zinc finger transcription factor, can be induced immediately in response to environmental stress, such as hypoxia, fluid shear stress, and vascular injury [22]. Egr-1 positively regulates the expression of proangiogenic genes, including VEGF, fibroblast growth factors, and IL-6 in endothelial cells or TNF-α in macrophages [22, 23]. ERK2 is associated to vascular endothelial cell proliferation and migration, thereby contributing to angiogenesis. Activated ERK2 was observed in accelerated neovascularization during ischemia and wound healing [24]. Embryos lacking ERK2 in endothelial cells died in utero due to reduced angiogenesis both in the yolk sac and embryo proper [26]. Besides, blockade of ERK2 pathway attenuates VEGF-induced proliferation of fetoplacental artery endothelial and tube formation, though the cell migration is unaffected [27]. MMP2, as a key enzyme responsible for degradation of extracellular matrix, is also involved in angiogenesis, because angiogenesis is dependent on focal degradation of the vascular basement membrane, which is essential for subsequent migration and proliferation of endothelial cells [25]. Our data demonstrated that Egr-1, ERK2 and MMP2 are regulated by miR-132, which suggests that miR-132 is involved in multiple processes of RNV.

The critical role of VEGFs in RNV has been identified by considerable studies *in vivo* and *in vitro* [8, 28, 29]. VEGFs are produced in the human eye by a variety of cells including Mueller cells, retinal pigment epithelial cells, retinal capillary pericytes, endothelial cells and ganglion cells [28]. VEGFs expression is induced by hyperglycemia and hypoxia, two major causes of RNV [8]. Expression of VEGFs levels is positively correlated to the occurrence rate and severity of RNV [29]. The present study verified that both VEGFA and VEGFC are positively regulated by miR-132. KEGG pathway analysis shows that VEGFs are regulated by ERK signaling, Jak1/STAT cascades, HIFs and oxidative stress. Although HIFs remained up-regulated, suppressing miR-132 impaired the up-regulation of VEGFs elicited by hypoxia, probably through inhibiting ERK signaling.

Although *EFNA1* expression was altered in mRNA microarray analysis, qRT-PCR and Western blot assays showed that EFNA1 expression was not changed by miR-132 under HR conditions. Thus mRNA microarray analysis might provide a false positive result in *EFNA1* expression. EFNA1 is a ligand of EPHA2. Their collectively regulates angiogenesis in tumor. Expression of CXCL1 in gene level was changed by miR-132 under H/R conditions, as evidenced by mRNA microarray analysis and qRT-PCR assays, but its protein level was not influenced by miR-132, based on Western blot assay. It was reported that CXCL1 induction by NADPH oxidase and NF-κB is through VEGF, because antagonist for VEGF receptor reduced the induction [30]. This report indicates that CXCL1 may be a downstream target of VEGF. Thus, there is the possibility that the regulation of CXCL1 gene expression by miR-132 is through VEGF. Further study is needed to identify the hypothesis. Accumulating evidence indicates that CXCL1 promotes angiogenesis through recruitment of monocyte into the peri-collateral space, regulation of stromal fibroblast senescence and interaction with VEGF [31-34].

In summary, this study uncovered that miR-132 is closely associated to RNV under H/R conditions. miR-132 increased proliferation and migration of HRMECs, inhibited the apoptosis, as well as modulated expression of genes involved in multiple processes of blood vessel development, thus promoted RNV development. This study promotes the better understanding of the pathogenic mechanisms of RNV. Further study *in vivo* is needed to identify whether miR-132 is a key target in the management of RNV.

Disclosure of conflict of interest

None.

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