Research Article

Effects of long non-coding RNA myocardial infarction-associated transcript on retinal neovascularization in a newborn mouse model of oxygen-induced retinopathy

https://doi.org/10.4103/1673-5374.306098

Date of submission: July 6, 2020

Date of decision: September 23, 2020

Date of acceptance: November 17, 2020

Date of web publication: January 25, 2021

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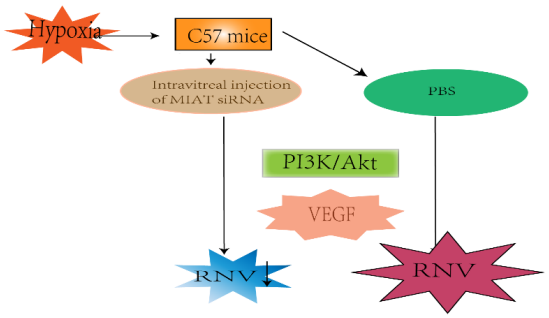
Graphical Abstract *MIAT siRNA may serve as a clinical treatment method for preventing retinopathy of prematurity*



Abstract

Whether long non-coding RNA myocardial infarction-associated transcript is involved in oxygen-induced retinopathy remains poorly understood. To validate this hypothesis, we established a newborn mouse model of oxygen-induced retinopathy by feeding in an oxygen concentration of 75 ± 2% from postnatal day 8 to postnatal day 12, followed by in normal air. On postnatal day 11, the mice were injected with the myocardial infarction-associated transcript siRNA plasmid via the vitreous cavity to knockdown long non-coding RNA myocardial infarction-associated transcript. Myocardial infarction-associated transcript siRNA transcription significantly inhibited myocardial infarction-associated transcript mRNA expression, reduced the phosphatidylinositol-3-kinase, phosphorylated Akt and vascular endothelial growth factor immunopositivities, protein and mRNA expression, and alleviated the pathological damage to the retina of oxygen-induced retinopathy mouse models. These findings suggest that myocardial infarction-associated transcript is likely involved in the retinal neovascularization in retinopathy of prematurity and that inhibition of myocardial infarction-associated transcript can downregulate phosphatidylinositol-3-kinase, phosphorylated Akt and vascular endothelial growth factor expression levels and inhibit neovascularization. This study was approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University, China (approval No. 2016PS074K) on February 25, 2016.

Key Words: long non-coding RNA; myocardial infarction-associated transcript; neovascularization; neurovascular; prematurity; retinopathy; vascular development; vascular endothelial growth factor

Chinese Library Classification No. R453; R774; Q522

Introduction

The mammalian genome contains numerous long non-coding RNA (lncRNA) genes. lncRNAs play biological functions through gene imprinting, cell cycle regulation and splicing regulation. They are also associated with the development of several human diseases (Kumar et al., 2016; Arslan et al., 2017; Raut and Khullar, 2018; Ding et al., 2020). Increasing evidence indicates that lncRNAs are related with the development of nervous and neovascular diseases (Xu et al., 2014; Chen et al., 2017; Wang et al., 2020c). The physiological function of the nervous system is closely related to that of the vascular system, and both systems may share pathological mechanisms. Quaegebeur et al. (2011) previously reported the interaction between these two systems.

Vascular endothelial cells promote the proliferation of neural precursor cells, microglia, and monocytes. Furthermore, they are involved in retinal vascular diseases, and microglia activation has been shown to prevent retinal degeneration (Alves et al., 2020; Cao et al., 2020; Chumsakul et al., 2020; Yu et al., 2020a). Myocardial infarction-associated transcript (MIAT), also known as retina noncoding RNA 2, is expressed in mitotic progenitor cells and post-mitotic retinal precursor cells, including human and mouse retinal pigment cells, in the outer and inner nuclear layers and the retinal nerve cell layer (Almnaseer and Mourtada-Maarabouni, 2018; Yu et al., 2020b). MIAT is of great significance in the treatment

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Funding: *This study was supported by the National Natural Science Foundation of China, No. 81600747 (to YD) and the Start-Up Foundation for Doctors of Liaoning Province, China, No. 201501020 (to YD).*

How to cite this article: *Di Y, Wang Y, Wang X, Nie QZ (2021) Effects of long non-coding RNA myocardial infarction-associated transcript on retinal neovascularization in a newborn mouse model of oxygen-induced retinopathy. Neural Regen Res 16(9):1877-1881.*

of neurovascular diseases, and it can effectively reduce the development of neovascularization (Eichmann and Thomas, 2013; Jiang et al., 2016; Yu et al., 2020b). These findings suggest that MIAT may play an important role in angiogenesis and pathogenesis of the nervous and retinal system. However, its role and mechanism in retinopathy of prematurity (ROP) remain unclear. The present study investigated the effect of MIAT small interfering RNA (siRNA) on retinal neovascularization (RNV) in oxygen-induced retinopathy (OIR).

Materials and Methods

Animals

Twenty C57BL/6J timed-pregnant mice were purchased from Shenyang Changsheng Biological Technology Co., Ltd. [Shenyang, China; license No. SCXK (Liao) 2015-0001]. The study was approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University, China (approval No. 2016PS074K) on February 25, 2016.

OIR induction and intravitreal injection in mice

The 7-day-old mice were fed in the oxygen concentration of 75 ± 2% until P12, then returned to normal air (21 ± 2% oxygen), as previously described (Smith et al., 1994). The mice were randomly assigned into the hyperoxia control siRNA and hyperoxia MIAT siRNA groups (n = 90/group). On P11, the mice were injected with the empty vector plasmid (1 μL, 20 μM) or malat1 siRNA plasmid (1 μL, 20 μM) designed by GenePharma (Shanghai, China) with lipofectamine (ThermoFisher, Waltham, MA, USA) or the polarization beam splitter into the vitreous cavity (1 μL). On P17, the eyes were removed after anesthesia by isoflurane.

Preparation of MIAT siRNA

MIAT siRNA and scrambled MIAT siRNA sequences were designed by GenePharma (Shanghai, China). The sequences were as follows: MIAT siRNA forward, 5′-GGU GUU AAG ACU UGG UUU CUU-3′ and reverse, 5′-ACA UAC UCA UAA AGG CCA CUU-3′; and scrambled MIAT siRNA forward, 5′-UUC UCC GAA CGU GUG UCA CGU UU-3′ and reverse, 5′-ACG UGA CAC GUU CGG AGA AUU-3′.

Quantitative reverse transcription polymerase chain reaction

On P12, total RNA was extracted from mouse retina by Trizol (Takara, Tokyo, Japan) and subsequently transcribed into complementary DNA. The sequences were as follows: MIAT, forward: 5′-TGG AAC AAG TCA CGC TCG ATT-3′ and reverse: 5′-GGT ATC CCA AGG AAT GAA GTC TGT-3′; phosphoinositide 3-kinase (PI3K), forward: 5′-GGC TTG GAC CGA ATG CT-3′ and reverse: 5′-TTG TTG AAG GCT GTG GC-3′; AKT, forward: 5′-AGC AAA CAG GCT CAC AGG TT-3′ and reverse: 5′-TAA GTC CTC CCC ATC TCC CT-3′; vascular endothelial growth factor (VEGF), forward: 5′-CCC GAC AGG GAA GAC AAT-3′ and reverse: 5′-TCT GGA AGT GAG CCA ACG-3′; and β-actin, forward: 5′-CCT CCT CCT GAG CGC AAG TA-3′ and reverse: 5′-GAT GGA GGG GCC GGA CT-3′. The thermocycling conditions were as follows: preheating at 95°C for 30 seconds, and the two-step method consisting of 95°C 5 seconds and 60°C 31 seconds, for 50 cycles. Electrophoresis was performed on a 1.5% agarose gel, and the results of electrophoresis were observed under ultraviolet light. The $2^{-\Delta\Delta CT}$ value was used for result analysis (Livak and Schmittgen, 2001).

Fluorescein isothiocyanate staining

On P17, 15 mice from each group were anesthetized and fluorescein isothiocyanate-dextran (2×10^6 Da, 50 mg/mL, 500 μL; Sigma, San Francisco, CA, USA) was circulated through the body for 3 minutes. The retinas were dissected after the eyeball was removed and each retina was divided into four equal sections. The clock hour scores of neovascularization and non-perfusion area were counted by Photoshop CS6 (Adobe, San Francisco, CA, USA) as previously described (Chikaraishi et al., 2007).

Hematoxylin-eosin staining

On postnatal day 17 (P17), 15 mice from each group were anesthetized. The eyes were fixed and serial sections (6-μm in thickness) were prepared. Ten pieces of each eyeball were selected for hematoxylin-eosin staining (Mitchell et al., 2018). After conventional dewaxing, the slices were stained with hematoxylin for 3 minutes, then stained with eosin for 2 minutes, dehydrated by conventional machine, and sealed with neutral resin. The vascular cell nuclei getting into the vitreous humor were counted under a light microscope (Eclipse, NI, Nikon, Tokyo, Japan) (Park et al., 2009; Arachchi et al., 2018).

Immunohistochemistry

Immunohistochemistry was performed using a Streptavidin Biotin Complex immunohistochemistry kit (Boster Bioengineering Co., Wuhan, China). The paraffin sections were dewaxed and antigen was repaired and sealed with goat serum. Then the sections were incubated overnight at 4°C with primary antibodies [phospho-PI3K (p-PI3K; 1:2000; mouse; Cat# sc-12929; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), phospho-Akt 1/2/3 (1:2000; mouse; Cat# sc-101629; Santa Cruz Biotechnology Inc.), and vascular endothelial growth factor (1:2000; mouse; Cat# sc-365578; Santa Cruz Biotechnology Inc.)], followed by incubation at 37°C for 30 minutes with horseradish peroxidase-labeled goat anti-mouse IgG(H+L) (1:2000; Cat# ZB-5305; Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) on the next day. After 3,3′-diaminobenzidine staining and hematoxylin staining, photographs were taken under the light microscope.

Western blot analysis

Total protein from each sample from retinas was extracted using radioimmunoprecipitation assay (RIPA) buffer (Solarbio Science, Beijing, China). The bicinchoninic acid (BCA) method was used to determine the protein concentrations (ThermoFisher). A total of 50 μg of each sample was electrophoresed (80 V) and subsequently transferred (at 4°C, 350 mA) to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies [p-PI3K (1:2000; mouse; Cat# sc-12929; Santa Cruz Biotechnology Inc.), phospho-Akt 1/2/3 (1:2000; mouse; Cat# sc-101629; Santa Cruz Biotechnology Inc.), and vascular endothelial growth factor (1:2000; mouse; Cat# sc-365578; Santa Cruz Biotechnology Inc.)] for 16 hours at 4°C after blocking with 5% non-fat milk. The membranes were then incubated with horseradish peroxidase-labeled goat anti-mouse IgG(H+L) (1:2000; Cat# ZB-5305; Zhongshan Jinqiao Biotechnology Co. Ltd.) for 1 hour at room temperature. Chemiluminescence reagents (Millipore, Waltham, MA, USA) and an imaging system (GE AI680, Boston, MA, USA) were used to visualize the bands. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to calculate gray value.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD), and were analyzed by Mann-Whitney U test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MIAT expression is decreased in hyperoxia MIAT siRNA mouse retinas

Quantitative reverse transcription polymerase chain reaction results showed that the expression level of MIAT in the retinas of the hyperoxia MIAT siRNA group was reduced to 67.52% of that in hyperoxia control siRNA group at 1 day after transfection ($P < 0.05$; **Figure 1**).

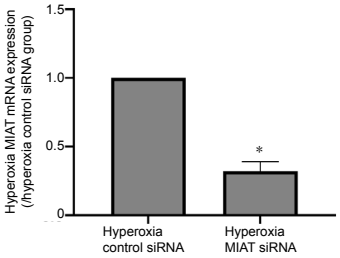

Figure 1 | **Quantitative reverse transcription polymerase chain reaction determination of MIAT mRNA expression in the retinas of mice at 1 day after transfection.**

Data are expressed as the mean ± SD. *P < 0.05, *vs*. hyperoxia control siRNA group (Mann-Whitney U test). MIAT: Myocardial infarction-associated transcript; siRNA: small interfering RNA.

Effect of lncRNA on the RNV of OIR mice

Results of fluorescein isothiocyanate staining showed that in the hyperoxia control siRNA group, the retina showed obvious vascular leakage and a large area of no perfusion area (**Figure 2A**). The pathological changes were alleviated in hyperoxia MIAT siRNA group compared with the hyperoxia control siRNA group (P < 0.05; **Figure 2B** and **C**).

Hematoxylin-eosin staining showed that the number of neovascular nuclei breaking through the inner limiting membrane was calculated to quantify the RNV (**Figure 3**). The number of preretinal neovascular cells in the hyperoxia MIAT siRNA group was lower than that in hyperoxia control siRNA group (Z = –4.427, P < 0.05). This result suggested that MIAT siRNA exhibited anti-neovascularizative effects in the retina.

Effect of lncRNA on the immunopositivities of p-PI3K, p-AKT, and VEGF in the OIR model mice

Immunohistochemical staining of retinal sections revealed that p-PI3K, p-AKT, and VEGF were highly expressed in the ganglion cell layer, inner plexiform layer, inner nuclear layer, and outer plexiform layer (**Figure 4**). However, their immunopositivities were lower in the hyperoxia MIAT siRNA group than those in hyperoxia control siRNA group (P < 0.05).

Effect of lncRNA on the PI3K/AKT/VEGF signaling pathway in the OIR model mice

Western blot analysis and quantitative reverse transcription polymerase chain reaction were performed to detect the expression levels of PI3K, AKT, and VEGF. Western blot results showed that the p-PI3K, p-AKT, and VEGF protein levels in the hyperoxia MIAT siRNA group were decreased by 40.94 ± 3.94%, 49.28 ± 4.16%, and 40.63 ± 4.03%, respectively (P < 0.05, *vs*. hyperoxia control siRNA group; **Figure 5A** and **B**). Quantitative reverse transcription polymerase chain reaction revealed that the p-PI3K, p-AKT, and VEGF mRNA levels in the hyperoxia MIAT siRNA group were decreased by 48.73 ± 3.98%, 46.79 ± 3.87%, and 55.09 ± 4.26%, respectively (P < 0.05, *vs*. hyperoxia control siRNA group; **Figure 5C**). These results demonstrated that MIAT is involved in the process of RNV in ROP, and inhibition of MIAT may effectively inhibit RNV through the PI3K/AKT/VEGF signaling pathway.

Discussion

LncRNAs are transcription products, 200–100,000 nucleotides in length, which structurally resemble mRNA and have little to no protein-coding potential (Li et al., 2020; Liu et al., 2020; Wang et al., 2020b). A number of studies have reported that lncRNAs play important roles in several biological processes, including stem cell maintenance and cellular phenotype differentiation (Ding et al., 2018; Sarropoulos et al., 2019; Yang et al., 2019; Qi et al., 2020). Furthermore, lncRNA MIAT has been implicated in the development of many diseases, including neurodegenerative diseases (Fanale et al., 2016), tumors (Li et al., 2017; Bai et al., 2019; Lin et al., 2019), and common eye diseases such as corneal neovascularization and diabetic retinopathy (Hutchinson et al., 2007; Yan et al., 2015; Zhang et al., 2017; Li et al., 2018). RNV is a hallmark of ROP, retinal vascular occlusion, and diabetic retinopathy. Anti-VEGF drugs have been used to decrease this RNV; however, their repeated injection may be problematic and current research is focused on overcoming this challenge (Satari et al., 2019; Sun et al., 2019b; Nagaraj et al., 2020). Retinal nerve cells are involved in the regulation of inflammation during neovascularization (Wang et al., 2020a). Microglia and macrophages play an important role in this process and transforming growth factor-β signaling and the retinoic acid receptor-related orphan receptor γ/interleukin17A axis may inhibit RNV through retinal microglia (Talia et al., 2016; Ma et al., 2019).

Several lncRNAs, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), maternally expressed 3 (MEG3), MIAT, MANTIS, and PUNISHER, are involved in the regulation of angiogenesis and vascular disease (Shen et al., 2017; Lu et al., 2018; Yu and Wang, 2018). Research has shown that high glucose concentrations may significantly upregulate the expression of the lncRNA MIAT (Hanrahan et al., 2010). *In vivo* experiments have shown that the downregulation of MIAT may reduce RNV, vascular leakage, and the inflammatory response in diabetes (Meydan et al., 2020). In addition, downregulation of MIAT may reduce the proliferation, migration, and tube formation capacity of retinal vascular endothelial cells *in vitro* (Deng et al., 2020). MIAT may serve as a competing endogenous RNA during VEGF regulation and thus participate in RNV associated with diabetic retinopathy (Toraih et al., 2019). Additionally, MIAT knockdown inhibits the upregulation of tumor necrosis factor α and intercellular cell adhesion molecule-1, thereby reducing inflammation and vascular leakage (Roy et al., 2011).

The OIR mice in the hyperoxia MIAT siRNA group were administered MIAT siRNA via intravitreal injection on P11. One day after transfection, quantitative reverse transcription polymerase chain reaction confirmed that the relative expression of MIAT mRNA in mice in the hyperoxia MIAT siRNA group was significantly decreased, which confirmed the effectiveness of the transfection method used in this study. PI3K, AKT, and VEGF levels were markedly decreased in the hyperoxia MIAT siRNA mice compared with those in the hyperoxia control siRNA group. We have previously demonstrated that cellular communication network factor 1 and LY294002 can regulate RNV through the PI3K/Akt/VEGF signaling pathway (Di et al., 2015; Di and Chen, 2018). MIAT siRNA reduced the expression of VEGF at the protein and mRNA levels, which decreased the aberrant neovascularization in the OIR mouse model. These results indicated that MIAT plays an essential role in RNV, and that MIAT siRNA decreases RNV by inhibiting the PI3K/AKT/VEGF signaling pathway. Consistent with our results, previous studies have demonstrated that MIAT participates in angiogenesis through the PI3K/AKT pathway (Liu et al., 2018; Chen et al., 2019; Sun et al., 2019a).

The results of this study showed that although MIAT siRNA decreased the expression levels of PI3K, AKT, and VEGF at the peak of RNV on P17, their expression was not completely inhibited. Furthermore, MIAT siRNA could not completely inhibit the development of RNV from morphological and pathological aspects. We conclude that RNV is regulated by many factors. Additionally, the transfection efficiency of MIAT siRNA in the retina, and the dose and number of injections, may have important effects that require further observation and research.

In summary, we hypothesize that the relatively hypoxic environment to which OIR mice were exposed stimulated

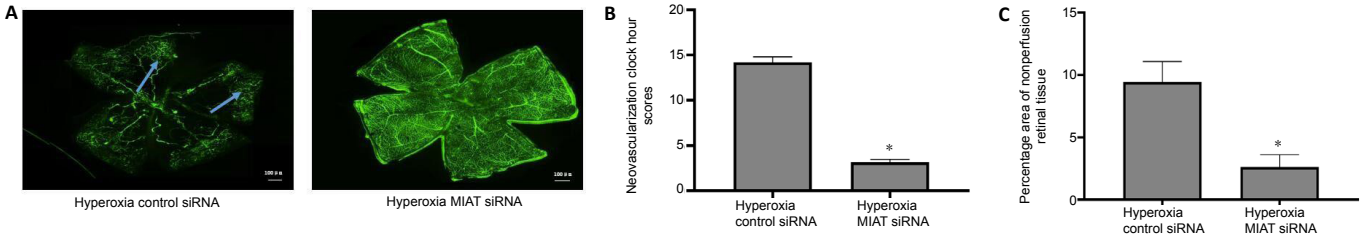

Figure 2 | **Effect of long non-coding RNA on the retina morphology of oxygen-induced retinopathy mice detected by fluorescein isothiocyanate staining.**
(A) Retina morphology on postnatal day 17. The blue arrows indicate neovascularization, the number of the neovascularization was lower in the hyperoxia MIAT siRNA group than hyperoxia control siRNA group. Scale bars: 100 μm. (B, C) Quantitative results of neovascularization clock hour scores (B) and percentage area of non-perfusion area (C). Data are expressed as the mean ± SD. *P < 0.05, *vs*. hyperoxia control siRNA group (Mann-Whitney U test). MIAT: Myocardial infarction-associated transcript; siRNA: small interfering RNA.

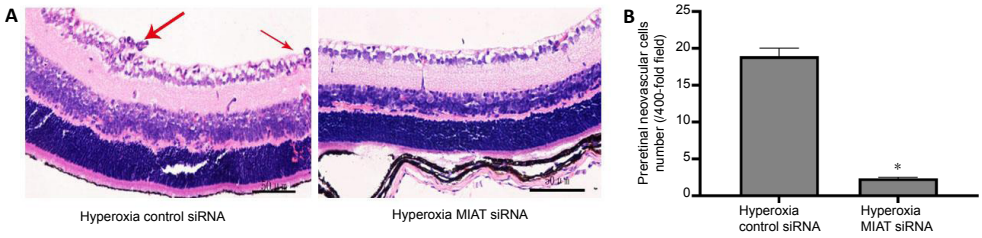

Figure 3 | **Effect of long non-coding RNA on the preretinal neovascular cells of oxygen-induced retinopathy mice detected by hematoxylin-eosin staining.**
(A) The retina morphology of P17 mice (original magnification 400×, scale bars: 50 μm). The red arrows indicate preretinal neovascular cells. The nucleus of neovascularization broke through the inner limiting membrane in hyperoxia group was more than the hyperoxia MIAT siRNA group. (B) Quantitative results of number of preretinal neovascular cells. Data are expressed as the mean ± SD. *P < 0.05, *vs*. hyperoxia control siRNA group (Mann-Whitney U test). MIAT: Myocardial infarction-associated transcript; P17: postnatal day 17; siRNA: small interfering RNA.

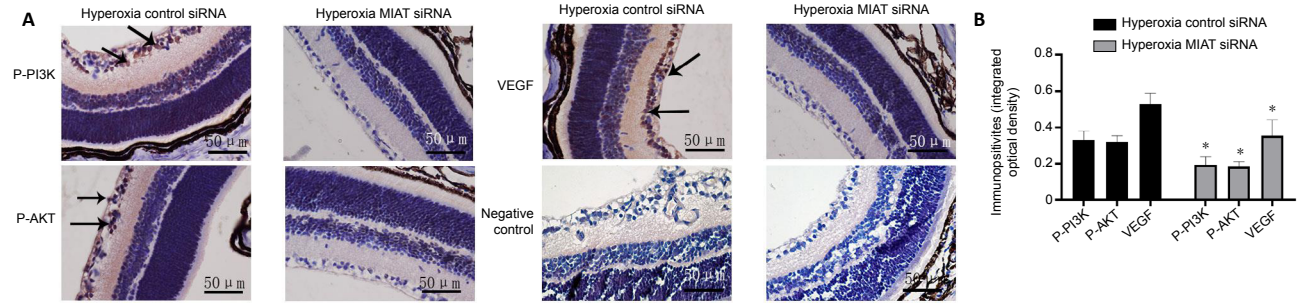

Figure 4 | **Effect of long non-coding RNA on the immunopositivities of p-PI3K, p-AKT, and VEGF in the oxygen-induced retinopathy model mice as determined by immunohistochemistry.**
(A) The images of immunohistochemical staining (magnification, 400×, scale bars: 50 μm). The arrows indicate the positive cells. (B) Quantitative results of p-PI3K, p-AKT, and VEGF immunopositivities. Data are expressed as the mean ± SD (n = 15). *P < 0.05, *vs*. hyperoxia control siRNA group (Mann-Whitney U test). MIAT: Myocardial infarction-associated transcript; p-AKT: phospho-Akt 1/2/3; p-PI3K: phospho-phosphatidylinositol 3-kinase; siRNA: small interfering RNA; VEGF: vascular endothelial growth factor.

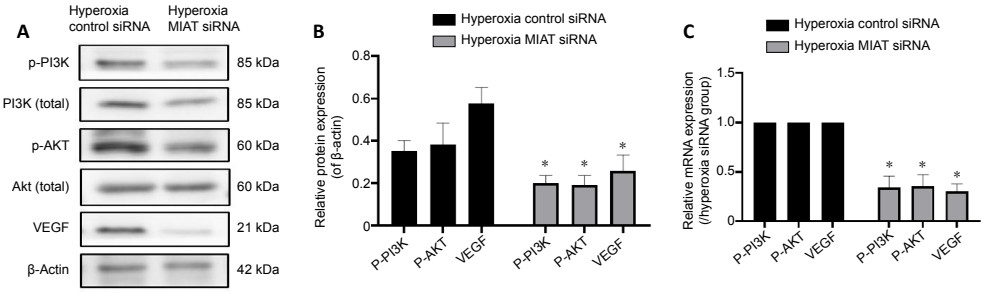

Figure 5 | **Effect of long non-coding RNA on the protein and mRNA expression in the PI3K/AKT/VEGF signaling pathway in the oxygen-induced retinopathy mouse model.**
(A) Bands of p-PI3K, p-AKT, and VEGF detected by western blot assay. (B) Quantification of p-PI3K, p-AKT, and VEGF protein levels. (C) Quantification of p-PI3K, p-AKT, and VEGF mRNA levels detected by quantitative reverse transcription polymerase chain reaction. Data are expressed as the mean ± SD (n = 15). *P < 0.05, *vs*. hyperoxia control siRNA group (Mann-Whitney U test). MIAT: Myocardial infarction-associated transcript; PI3K: phosphatidylinositol 3-kinase; p-AKT: phospho-Akt 1/2/3; p-PI3K: phosphatidylinositol 3-kinase; siRNA: small interfering RNA; VEGF: vascular endothelial growth factor.

endothelial cells to upregulate MIAT, which regulates the PI3K/AKT/VEGF signaling pathway, thereby promoting RNV. Silencing MIAT may effectively inhibit RNV in ROP.

Author contributions: *Study design: QZN; experiment implementation: YD, YW; data collection and analysis: XW; manuscript drafting: YD. All authors approved the final version of the manuscript.*

Conflicts of interest: *The authors declare that they have no competing interests.*

Financial support: *This study was supported by the National Natural Science Foundation of China, No. 81600747 (to YD) and the Start-Up Foundation for Doctors of Liaoning Province, China, No. 201501020 (to YD). The funding sources had no role in study conception and design, data analysis or interpretation, paper writing or deciding to submit this paper for publication.*

Institutional review board statement: *This study was approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University, China (approval No. 2016PS074K) on February 25, 2016.*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*

Plagiarism check: *Checked twice by iThenticate.*

Peer review: *Externally peer reviewed.*

Open peer reviewer: *Michel Desjarlais, Hopital Maisonneuve-Rosemont, Canada.*

Additional file: *Open peer review report 1.*

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P-Reviewer: Desjarlais M; C-Editor: Zhao M; S-Editors: Yu J, Li CH; L-Editors: Giles L, Yu J, Song CP; T-Editor: Jia Y