

MiR-375-3p regulates rat pulmonary microvascular endothelial cell activity by targeting Notch I during hypoxia

Journal of International Medical Research

48(7) 1–14

© The Author(s) 2020

Article reuse guidelines:

sagepub.com/journals-permissions

DOI: 10.1177/0300060520926851

journals.sagepub.com/home/imr



Yuan An^{1,2}, Ziquan Liu³, Hui Ding⁴, Qi Lv³,
Haojun Fan³, Shike Hou³ , Wei Cai⁵ and
Sanli Liu⁵

Abstract

Objective: Pulmonary microvascular endothelial cells (PMECs) exhibit specific responses in adaptation to hypoxia. However, the mechanisms regulating PMEC activities during hypoxia remain unclear. This study investigated the potential involvement of a microRNA, miR-375-3p, in the regulation of PMEC activities.

Methods: Primary PMECs were isolated from rats. The expression levels of miR-375-3p and Notch I in the PMECs were detected by quantitative PCR and western blotting. Luciferase reporter assays were performed to explore the transcriptional regulation of Notch I by miR-375-3p. The proliferation and chemotaxis of the PMECs were measured with the Cell Counting Kit-8 and Transwell invasion assays, respectively. Additionally, the capacity of hypoxia-treated PMECs for angiogenesis and inflammatory response was determined with tube formation assays and ELISA, respectively.

Results: The expression of miR-375-3p and Notch I in the PMECs was significantly down-regulated and up-regulated during hypoxia, respectively. The results demonstrated that miR-375-3p directly targets Notch I in PMECs, thereby suppressing the transcriptional expression of *Notch I*. It was further revealed that miR-375-3p regulates the proliferation, chemotaxis, angiogenesis, and inflammatory response of PMECs.

Conclusions: Our findings revealed the important role of miR-375-3p in the regulation of PMEC function and suggest the potential involvement of miR-375-3p in the development of lung diseases.

¹Tianjin Medical University, Tianjin, P. R. China

²Qinhuangdao Haigang Hospital, Hebei, P. R. China

³Institute of Disaster Medicine, Tianjin University, Tianjin, P. R. China

⁴The Second Affiliated Hospital of Shaanxi University of Chinese Medicine, Tianjin, P. R. China

⁵Logistics College of Chinese People's Armed Police Forces, Tianjin, P. R. China

Corresponding author:

Shike Hou, Institute of Disaster Medicine, Tianjin University, Tianjin, 300072, P. R. China.
Email: houshike@126.com



Keywords

Pulmonary microvascular endothelial cells, proliferation, chemotaxis, angiogenesis, inflammatory response, miR-375-3p, microRNA

Date received: 25 July 2019; accepted: 24 April 2020

Introduction

Pulmonary microvascular endothelial cells (PMECs) help to maintain normal gas exchange during breathing and exhibit specific responses toward hypoxia adaptation.¹ Under low oxygen pressure, the activity of PMECs changes significantly.² Hypoxia can lead to cell injury, apoptosis, and exfoliation due to the release of oxygen free radicals.³ Exfoliation occurs when endothelial cells on the surface of the endothelium lose their adherence and slough off. Hypoxia can also lead to the development of pulmonary hypertension (PH).⁴ PH is an important pathological process in the occurrence and development of various cardiopulmonary diseases, including chronic pulmonary disease, chronic obstructive pulmonary disease (COPD), pulmonary embolism, and sleep apnea syndrome (SAS).^{5,6} The activity of PMECs is critical in maintaining lung function. Therefore, the activities of PMECs need to be explored in studies of the pathogenesis and treatment of lung diseases such as PH.^{7,8} Under the pathological conditions of PH, PMECs adapt to and influence the unbalanced microenvironment through angiogenesis and the reparation of tissue damage.⁵⁻⁷ Chronic hypoxia is an important inducer of PH,⁸ and chronic hypoxia-induced endothelial dysfunction and angiogenesis disorders are considered important components of PH.⁸

Tumoral PH is a form of primary PH in which an endothelial tumor results from the rapid growth of endothelial cells in the pulmonary artery after malignant transformation.

However, secondary PH often indicates long-term micro-inflammatory injury of the endothelial cells of small pulmonary vessels, which can lead to remodeling of the pulmonary vascular structure over time. PMEC function/dysfunction likely plays a key role in the pathogenesis of PH through the proliferation, migration, and micro-inflammatory response of these cells.

MicroRNAs (miRNAs) are endogenous, short, noncoding RNAs (19–22 nucleotides) that function as regulators of gene transcription.⁹ MiRNAs suppress gene expression by pairing with their 3'-UTR, thereby affecting multiple cellular processes and physiological contexts such as cell proliferation, apoptosis, differentiation, and angiogenesis.¹⁰ Notably, miRNAs are dysregulated in multiple cancers.¹¹ MiR-375 was previously reported as a key regulator of pancreas development, and its depletion results in defects of the endocrine pancreas.¹² MiR-375 also affects the migration of pancreatic cancer cells. Indeed, the target genes of miRNA-375 appear to be mainly related to pancreatic development, cell proliferation, and insulin secretion.¹³ However, the roles of miRNA-375 in lung disease are still unclear.

The Notch signaling pathway is widely involved in cell differentiation, organism development, and homeostasis regulation.¹⁴ Recently, Notch has been found to affect the proliferation and differentiation of lung microvascular endothelial cells.¹⁵ Notch is activated through the inhibition of VEGFR2, which regulates endothelial cell proliferation and differentiation, thus promoting vascular development and

maintaining physiological function.¹⁶ Multiple miRNAs are reported to affect the function of endothelial cells via the Notch pathway.¹⁷

Herein, we identified a miRNA, miR-375-3p, that was down-regulated in pulmonary microvascular endothelial cells (PMECs) in response to hypoxia. We further demonstrated that miR-375-3p transcriptionally inhibited Notch1 and mediated PMEC activities including proliferation, chemotaxis, angiogenesis, and inflammatory response. The findings of our study may provide new insights into the pathogenesis of lung disease.

Materials and methods

Hypoxia treatment of postnatal rats

One-day-old rats were exposed to normal air (control) or hypoxia for 0, 24, 48, or 72 hours using a chamber containing 5% O₂ and 95% N₂ (Sanyo Electric Biomedical Co., Ltd., Tokyo, Japan). The rats were euthanized immediately after being exposed to hypoxia and their PMECs were isolated. Six rats were used in each control and treatment group (48 rats in total). All hypoxic rats survived the hypoxia treatment. This study was approved by the Ethics Committee of Tianjin University. The study was approved by the Institutional Animal Care and Use Committee. Every effort was made to reduce the number of rats used and their suffering.

Quantitative PCR assays

TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) was used to extract total RNA from the rat PMECs. The total RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega) to produce cDNA. Quantitative real-time PCR was then performed for the quantification of miR-375-3p and *Notch1* using the SYBR

Premix Ex Taq kit (Takara Bio, Inc., Shiga, Japan), and the relative expression levels were normalized to the expression of GAPDH.

Immunoblot assays

Lung tissues or PMECs were lysed in RIPA Buffer (Cell Signaling Technology, Danvers, MA, USA) to extract the total protein. The samples were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. After transmembrane transfer for 2 hours, the polyvinylidene fluoride membranes were blocked with 5% milk buffer and then incubated with a primary antibody against Notch1 (Abcam, Cambridge, MA, USA) or GAPDH (Abcam) for 2 hours. Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. The immunoblot signals were detected with an ECL kit. Image-Pro software was used to calculate the intensity of the signals on each blot.

Immunohistochemical assays

Sample sections of rat tissues were fixed with 4% paraformaldehyde and then blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS) for 30 minutes. Subsequently, the sections were incubated with an antibody against Notch1 at room temperature for 2 hours. After washing with PBS, the slides were incubated with a biotinylated secondary antibody at room temperature for 1.5 hours, and a chromogenic substrate was then added for detection.

Primary pulmonary microvascular endothelial cell (PMEC) culture

Pulmonary veins were collected aseptically and incubated with collagenase II (1 mg) for 45 minutes. The PMECs were

maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with endothelial growth factor and 10% (w/v) fetal bovine serum. The cells were cultured under normoxia or hypoxia (2% O₂) in a three-gas incubator. Cells were harvested for RNA, biochemical, and immunofluorescence studies.

Plasmid construction and dual-luciferase reporter assays

Plasmids for promoter activity measurements were constructed with the pmirGLO vector (Promega, Madison, WI, USA). The region upstream of the *Notch1* gene (i.e., the putative Notch1 promoter region) containing the predicted miR-375-3p binding site was generated by PCR using the forward primer, 5'-GAAGATCTTGAGGTA CATC GCAGAGGCCAG-3', the reverse primer, 5'-CATGCCATGGGGGCCGGA GCGGAAGACCC-3', and rat genomic DNA as the template, and then inserted into the pmirGLO vector (pmirGLO/Notch1-UTR). A plasmid bearing several mutations of the *Notch1* promoter region (pmirGLO/Notch1-mUTR) was generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Transfections were performed with the transfection reagent, jetPEI (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. The cells were harvested 24 hours after transfection and their relative firefly luciferase activity (normalized to *Renilla* luciferase activity) was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Cell proliferation assays

Approximately 1×10^3 PMECs were seeded into each well of 96-well plates and incubated for 4 days. Cells were then incubated with the Cell Counting Kit-8 reagent for

3 hours followed by removal of the medium. The cells were washed with PBS, followed by the addition of 200 μ L dimethyl sulfoxide to each well and measurement of the absorbance with a microplate reader at 490 nm.

Cell chemotaxis assays

PMECs were maintained for 48 hours and then resuspended in serum-free medium. The upper chamber filters of the chemotaxis chamber were coated with 20% Matrigel in serum-free medium by incubation at 37°C for 30 minutes. The PMECs (5×10^3 cells in 150 μ L of serum-free culture medium) were then added to the upper chambers and induced to migrate toward the bottom chambers, which contained medium supplemented with 10% fetal bovine serum. After 24 hours, the cells in the top chamber were removed, and those on the underside were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet for 10 minutes. Cell numbers were manually counted with the use of a microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Tube formation assays

PMECs were seeded into six-well plates pre-coated with 50% Matrigel. To detect tube formation, the wells were photographed 4 hours later with a microscope, and the levels of tube formation were quantified by measuring the overall tube length with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

ELISA

An enzyme-linked immunosorbent assay (ELISA) kit was purchased commercially (Abcam) and used to detect the expression of IL-6, IL-8, and TNF- α in accordance with the manufacturer's instructions. The plates were read with the CLARIOstar microplate reader (BMG Labtech, Cary,

NC, USA), and the results were normalized according to the protein concentration in each well.

Statistical analysis

All results in this study are presented as the mean \pm standard deviation (SD). Student's t-test was used for statistical comparisons between groups. $P < 0.05$ was considered statistically significant. GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Hypoxia induces low expression of miR-375-3p and high expression of Notch1 in rats

The expression of miR-375-3p and Notch1 was quantified in lung sections from postnatal rats exposed to different durations of hypoxia (0, 24, 48, or 72 hours) by quantitative PCR and immunoblotting, respectively. The level of miR-375-3p significantly decreased ($P < 0.05$)

within the first 24 hours of hypoxia treatment (Figure 1a), whereas the protein expression of Notch1 dramatically increased (Figure 1b). Immunohistochemical assays similarly showed increased Notch1 protein levels after hypoxia induction (Figure 1c).

Negative correlation between miR-375-3p and Notch1 expression in hypoxia-induced PMECs

Quantitative PCR assays were performed to detect the miR-375-3p and *Notch1* expression levels in PMECs under conditions of normoxia or hypoxia for 0, 24, 48, and 72 hours. PMECs were successfully isolated from rats and their identity was confirmed through CD31 staining (Figure 2a). MiR-375-3p transcripts were significantly down-regulated at all tested time points (all $P < 0.001$) relative to the 0-hour control (Figure 2b). In contrast, *Notch1* mRNA expression increased over two-fold in response to 24 hours of hypoxia ($P < 0.001$) but then declined to levels lower than that of the control after 48

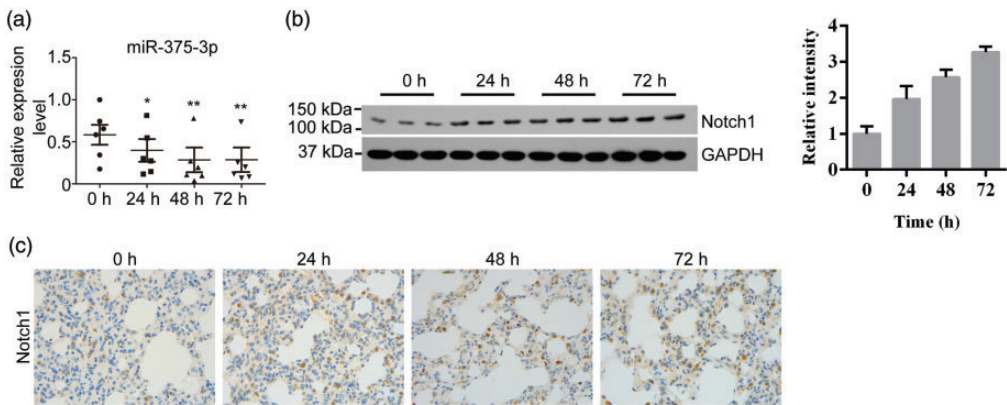


Figure 1. MiR-375-3p levels were negatively correlated with the protein expression of Notch1 in rat lung tissues following hypoxic exposure for 0, 24, 48, and 72 hours. (a) Quantitative PCR of miR-375-3p in lung tissues following hypoxic treatment. Data represent the mean \pm SD, $n = 6$. * $P < 0.05$, ** $P < 0.01$ versus the 0-hour group. (b) Immunoblot assays of Notch1 protein expression in lung tissues following hypoxic treatment. (c) Representative images showing immunohistochemical staining of Notch1 expression in rat lung tissues following hypoxic treatment. Scale bar, 100 μ m.

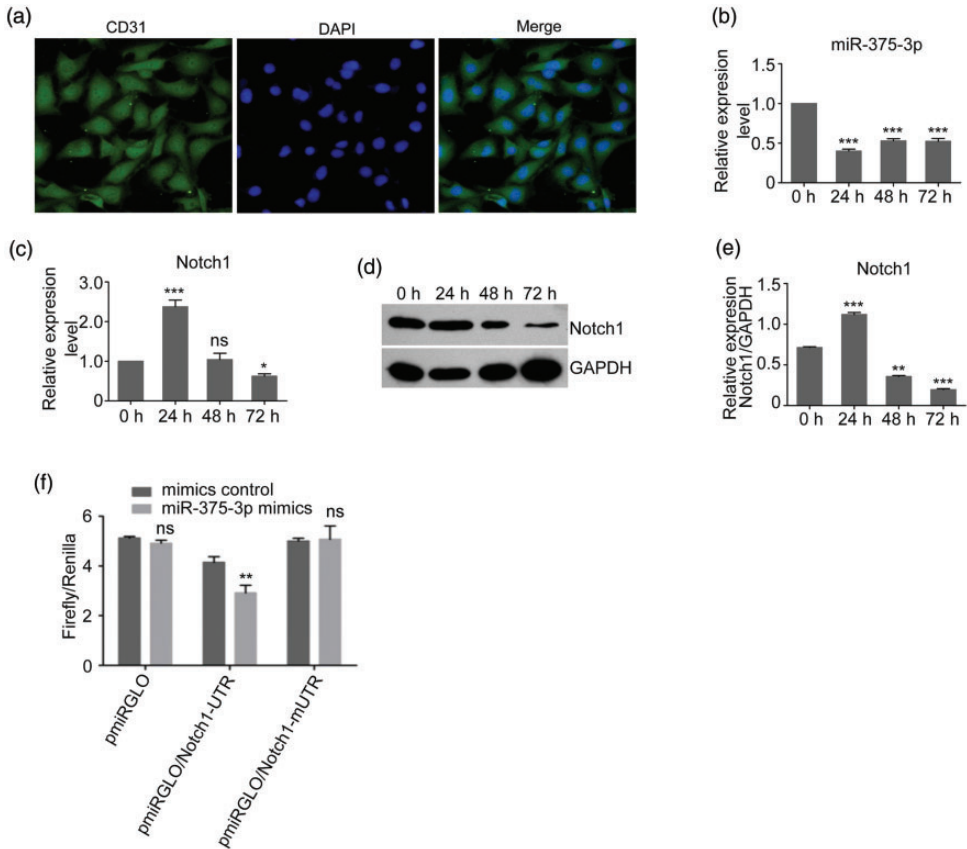


Figure 2. MiR-375-3p levels were negatively correlated with the expression of Notch1 following hypoxic exposure for 0, 24, 48, or 72 hours in primary PMECs. (a) Immunohistochemical staining of CD31 expression, a marker of PMECs. (b, c) Quantitative PCR of the miR-375-3p and *Notch1* expression levels in PMECs following hypoxic treatment. (d) Immunoblotting of the Notch 1 protein expression in PMECs following hypoxic treatment. (e) The relative expression of Notch1 in (d) was quantified and normalized to that of GAPDH. (e) Dual-luciferase reporter assay illustrating the effect of miR-375-3p on the expression of Notch1. Data represent the mean \pm SD, $n = 6$. ** $P < 0.01$, *** $P < 0.001$ versus the control group (0 hours or mimics control); ns, no significant difference.

and 72 hours of hypoxia treatment, with the 72-hour time point level being significantly lower ($P < 0.05$; Figure 2c). Immunoblotting of the PMECs similarly showed that Notch1 protein expression significantly increased after hypoxic exposure for 24 hours ($P < 0.001$) and then decreased to significantly lower levels than that of the control after 48 hours ($P < 0.01$) and 72 hours ($P < 0.001$) of hypoxia (Figure 2d).

To further confirm our hypothesis that miR-375-3p down-regulates the mRNA levels of *Notch1*, we performed dual-luciferase assays. We found that when PMECs were co-transfected with the empty pmirGLO plasmid (no insert control) and miR-375-3p, the resulting fluorescence signal was similar to that for the control alone. However, co-transfection of pmirGLO/Notch1-UTR and miR-375-3p

significantly decreased the fluorescence output compared with the control ($P < 0.01$). In contrast, co-transfection of pmirGLO/Notch1-mUTR and miR-375-3p did not yield a significant difference in fluorescence relative to the control (Figure 2e). We used the online server, TargetScan (www.targetscan.org), to predict the target genes of miR-375-3p, which identified a binding site for miR-375-3p on the *Notch1* 3'UTR (data not shown). These results confirmed that miR-375-3p directly suppressed the mRNA levels of *Notch1* in PMECs.

MiR-375-3p inhibits the transcription of *Notch1* in PMECs

To explore the effects of miR-375-3p on PMEC activities, we constructed the lentivirus fusion constructs, GFP-NC and GFP-miR-375-3p, and transfected these constructs into primary PMECs. The transfection efficiency of GFP-miR-375-3p was then validated by quantitative PCR (Figure 3a). Immunoblotting showed that the expression level of *Notch1* mRNA was significantly decreased ($P < 0.001$) in PMECs transfected with GFP-miR-375-3p (Figure 3b, c).

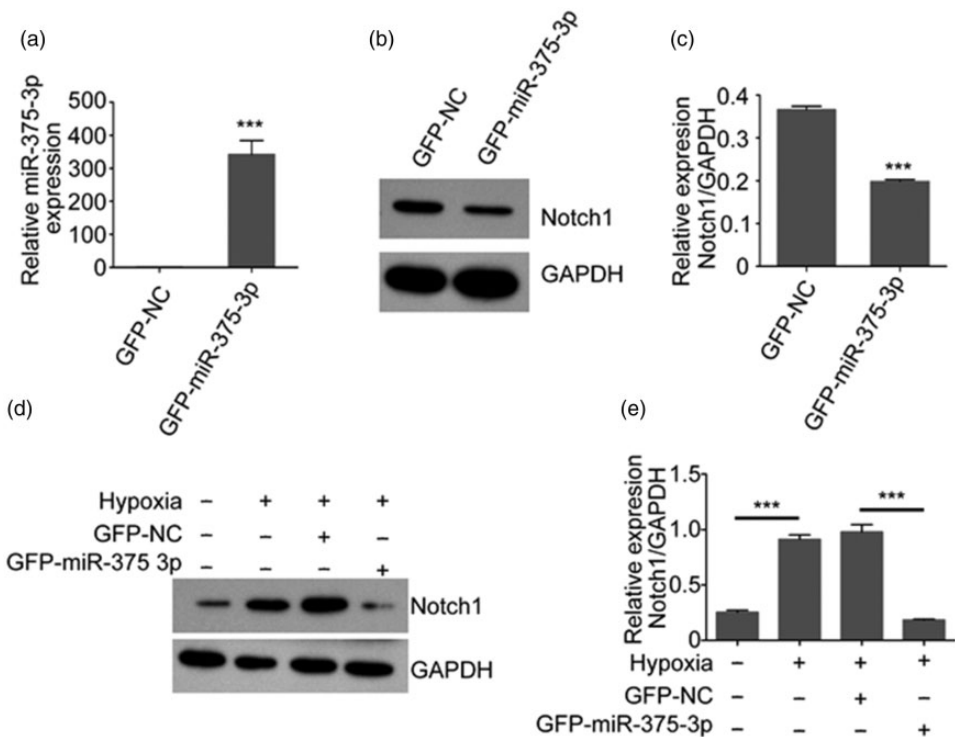


Figure 3. MiR-375-3p negatively regulated the expression of Notch1 upon hypoxia in PMECs. (a) Quantitative PCR of miR-375-3p expression in PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus. (b) Immunoblotting of Notch1 protein expression in PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus. (c) The relative expression of Notch1 in (b) was quantified and normalized to that of GAPDH. (d) Immunoblotting of Notch1 expression in PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus and subjected to normoxic or hypoxic conditions. (e) The relative expression of Notch1 in (d) was quantified and normalized to that of GAPDH. Data represent the mean \pm SD, $n = 6$. *** $P < 0.001$ versus the indicated group.

Furthermore, the enhancement of *Notch1* induced by hypoxia was significantly reversed by GFP-miR-375-3p transfection (all $P < 0.001$, Figure 3d–f).

MiR-375-3p suppresses PMEC proliferation in vitro

Activities of PMECs such as proliferation and chemotaxis are crucial in the pathogenesis of a variety of diseases. Because our findings here indicated that miR-375-3p transcriptionally inhibited the expression of *Notch1* in PMECs, we hypothesized that PMEC activities regulated by the *Notch1* pathway, such as cell proliferation, migration, and angiogenesis, may be affected by this microRNA. We, therefore, investigated the potential effects of miR-375-3p on the activities of PMECs *in vitro*.

We first evaluated the possible influence of miR-375-3p on cell proliferation through Cell Counting Kit-8 assays. PMECs were transfected with the lentivirus constructs GFP-NC (control) or GFP-miR-375-3p and then subjected to normoxia or hypoxia (2% O₂). MTT assays were performed at 24, 48, and 72 hours after transfection. Interestingly, we noticed that under normoxia, the proliferation of GFP-miRNA375-3p-transfected cells decreased compared with the control group, suggesting a strong inhibitory effect on cell proliferation by miR-375-3p (data not shown). Additionally, GFP-miRNA375-3p treatment significantly inhibited the proliferation of PMECs under hypoxia, and there was a tendency toward cell death from 48 hours of hypoxic exposure (Figure 4a, b). Collectively, these results suggest that miRNA375-3p has a strong anti-proliferation effect on PMECs.

MiR-375-3p inhibits the chemotaxis of PMECs

Subsequently, the chemotaxis of PMECs upon GFP-miR-375-3p transfection was assessed by Transwell invasion assays in

the presence of Matrigel. We found that GFP-miR-375-3p transfection prevented the invasion of PMECs through the membranes, with cell numbers dropping significantly under normoxia (data not shown) and hypoxia ($P < 0.01$, Figure 5a, b). These results confirmed the inhibition of PMEC chemotaxis by miR-375-3p transfection.

MiR-375-3p transfection suppresses the angiogenesis of PMECs

Angiogenesis is the main feature of endothelial cells including PMECs, and this process affects the development of a variety of diseases and is of great significance in the growth, metastasis, and prognosis of tumors. On this basis, the effect of miRNA375-3p on angiogenesis in normoxic and hypoxic PMECs was studied through tube formation assays. Under normoxic conditions, a tubular network of interconnecting branches was observed in the control group after 3 hours of PMECs deposition, whereas fewer tubes were observed in the GFP-miRNA375-3p-transfected group (data not shown). We further noticed that the angiogenesis of PMECs in the hypoxic control group was significantly enhanced ($P < 0.01$), while miRNA375-3p significantly reduced the number of PMECs tubes compared with the control group ($P < 0.01$), which was consistent with the results under the normoxic condition (Figure 5c, d). Thus, miRNA375-3p significantly inhibited the angiogenesis of PMECs under hypoxia and normoxia *in vitro*.

MiR-375-3p contributes toward the inflammatory response of PMECs

We next used ELISA to analyze the potential effects of miR-375-3p on the inflammatory response of PMECs. The levels of the inflammatory cytokines, IL-6, IL-8, and TNF- α , were determined in PMECs

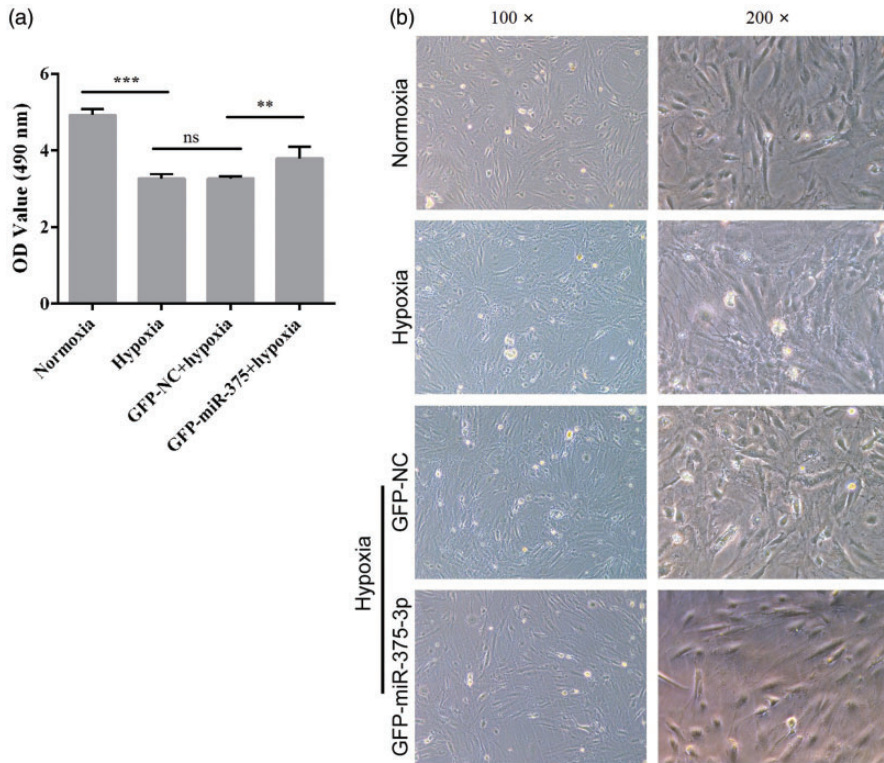


Figure 4. MiR-375-3p suppresses PMEC proliferation. (a) PMECs were transfected with GFP-NC or GFP-miR-375-3p lentivirus and subjected to normoxic or hypoxic conditions for 24, 48, or 72 hours, followed by MTT assays to determine cell proliferation. (b) Observation of PMEC proliferation by microscopy (100× or 200× magnification).

transfected with either GFP-NC or GFP-miR-375-3p lentiviral constructs and subjected to normoxia or hypoxia (Figure 6a–c). The levels of IL-6 ($P < 0.001$), IL-8 ($P < 0.01$), and TNF- α ($P < 0.001$), in the control cells were significantly increased by hypoxia treatment. In contrast, the levels of these inflammatory cytokines in PMECs transfected with GFP-miR-375-3p were significantly lower than those in the GFP-NC-transfected group under hypoxia (all $P < 0.001$). Our results, therefore, suggested that miR-375-3p significantly reduced the inflammatory response of PMECs *in vitro*.

Discussion

Pulmonary hypertension (PH) often results from hypoxia-induced vascular endothelial cell injury, which leads to an imbalance in various vasodilation factors synthesized and secreted by the vascular endothelium.¹⁸ This, in turn, leads to early pulmonary vasoconstriction and late pulmonary vascular reconstruction. PH has a high rate of disability and mortality and can cause a variety of serious conditions, such as heart failure.¹⁹ To understand the pathogenesis of pulmonary hypertension, it is important to explore the activity and regulatory

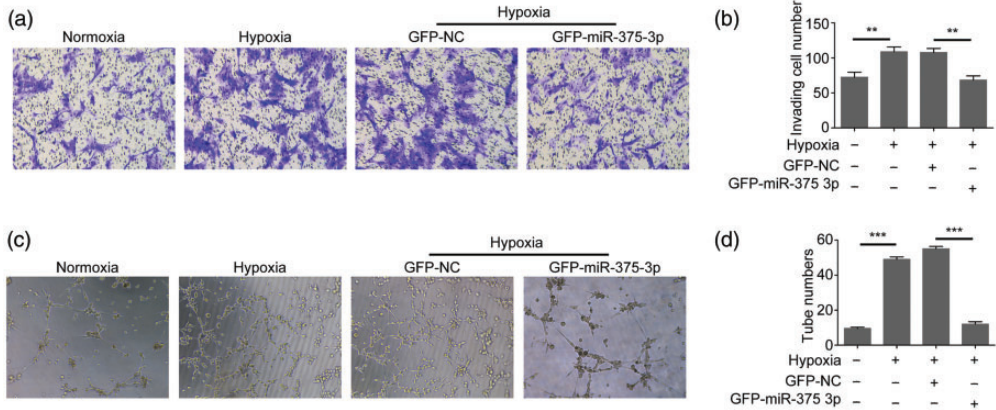


Figure 5. MiR-375-3p suppressed the chemotaxis and angiogenesis of PMECs. (a) Transwell invasion assays were performed on PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus and subjected to normoxic or hypoxic conditions. Cells on the upper surface of the Transwell filters were examined by crystal violet staining. (b) Invading cell numbers in each group were quantified. (c) Tube formation assays were performed on PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus and subjected to normoxic or hypoxic conditions. Cells on the upper surface of the Transwell filters were examined. (d) Tube numbers in each group were quantified. Data represent the mean ± SD, n = 6. ***P* < 0.01, ****P* < 0.01 versus the indicated group.

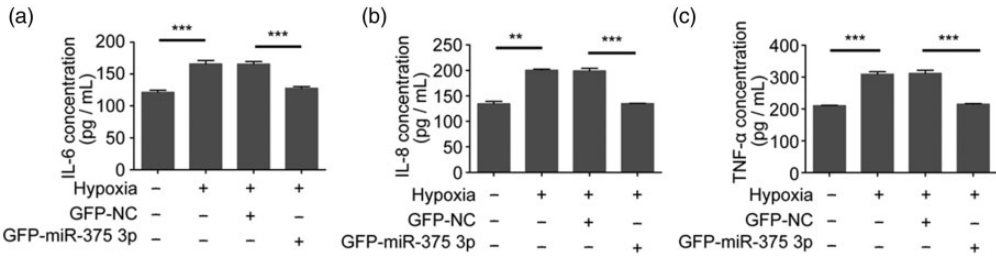


Figure 6. MiR-375-3p suppressed the inflammatory response of PMECs. (a–c) ELISA-based detection of IL-6, IL-8, and TNF-α protein expression, respectively, in PMECs transfected with GFP-NC or GFP-miR-375-3p lentivirus and subjected to normoxic or hypoxic conditions. Data represent the mean ± SD, n = 6. ***P* < 0.01, ****P* < 0.001 versus the indicated group.

mechanism of pulmonary microvascular endothelial cells.²⁰ Up to now, multiple proteins such as FGF7, and miRNAs such as miR-205-5p, have been found to be closely associated with the occurrence and development of PH, mainly through the regulation of endothelial cell activities.²¹ Notably, in the present study, we found that the miRNA, miR-375-3p, decreased during hypoxia and could mediate multiple

PMEC activities including proliferation, chemotaxis, angiogenesis, and inflammatory response. These data suggest that miR-375-3p may be involved in the pathogenesis of pulmonary diseases such as PH.

MiRNAs are considered important regulators of PMEC activities and tumorigenesis, exerting their effects via the direct targeting of multiple pathways.²² Several miRNAs have also been identified as

potential prognostic biomarkers of cancer.²² Although targeted therapies, such as targeted liposome therapy for highly metastatic tumors, have achieved good results,^{23–25} the discovery of new targets and markers is urgently needed, and miRNAs are promising prospects. MiR-375-3p is a member of the miR-375 family and a tumor suppressor regulator of colorectal cancer, breast cancer, and esophageal squamous cell carcinoma (ESCC).^{26,27} A previous study has shown that miR-375-3p is also involved in myocardial repair, thus affecting the pathophysiology of cardiomyocytes.²⁸ However, the role of miR-375-3p in the regulation of PMEC cellular function is still unclear. Here, we identified the involvement of miR-375-3p in the regulation of PMEC proliferation, chemotaxis, angiogenesis, and inflammatory response. Several other studies have confirmed the important cellular functions of the miR-375 family. For example, it has been shown that circFAT1 promotes the expression of YAP1 in osteosarcoma cells by acting as a sponge for miR-375,²⁹ while miR-375 can block the proliferation and invasion of glioblastoma via targeting of WNT5A.³⁰ In ovarian cancer, the high expression of serum exosomal miR-375 enhances the prognostic capacity of CA-125.³¹ These studies, together with our findings, confirm the critical roles of miR-375 in human diseases.

In this study, we successfully isolated PMECs from rats and studied their function. Our results showed that the expression level of miR-375-3p was significantly decreased following 24 hours of hypoxic exposure, with no further reductions observed with increasing duration of exposure. Interestingly, however, the expression of Notch1 increased significantly after 24 hours of hypoxia, suggesting a negative regulatory effect of miR-375-3p on Notch1, but this effect diminished with increasing duration of exposure. This suggests that

the influence of miR-375-3p gradually weakened over time, with the expression of Notch1 being restored through other pathways. This aspect requires further study. Indeed, Notch1 is known to be widely regulated by multiple proteins and miRNAs. The Notch pathway is considered an effective “switch” for the control of multiple cellular processes including cell apoptosis, proliferation, and differentiation during cancer progression,³² and multiple miRNAs may regulate tumor development and other cellular activities through the Notch signaling pathway. For example, miR-34a may be involved in epileptiform discharges via the Notch signaling pathway in hippocampal neurons.³³ Additionally, miR-30 has a regulatory effect on pulmonary fibrosis by targeting the Notch pathway.³⁴ In non-small cell lung cancer (NSCLC), miR-223 regulates sensitivity toward erlotinib through the Notch pathway.³⁵ Although the Notch pathway plays an important role in both pulmonary fibrosis and the development of lung cancer, the present study is the first to provide evidence that the Notch signaling pathway regulates PMEC activity, which may have an impact on lung diseases such as pulmonary hypertension. Of course, the detailed molecular mechanisms underlying this regulation remain to be studied.

Notch1 is a cytoplasmic receptor and a member of the Notch protein family. Previous studies have indicated the high expression of Notch1 in endothelial cells, suggesting its critical regulatory functions in endothelial cell activities.³⁶ Endothelial Notch1 also plays a critical regulatory role in pulmonary hypertension.³⁷ Interestingly, our present study found that miR-375-3p affected the activities of PMECs by targeting Notch1. Under hypoxia, the expression level of miR-375-3p decreased, whereas the expression level of Notch1 increased. Our results further confirmed that miR-375-3p directly acts on the non-coding region of

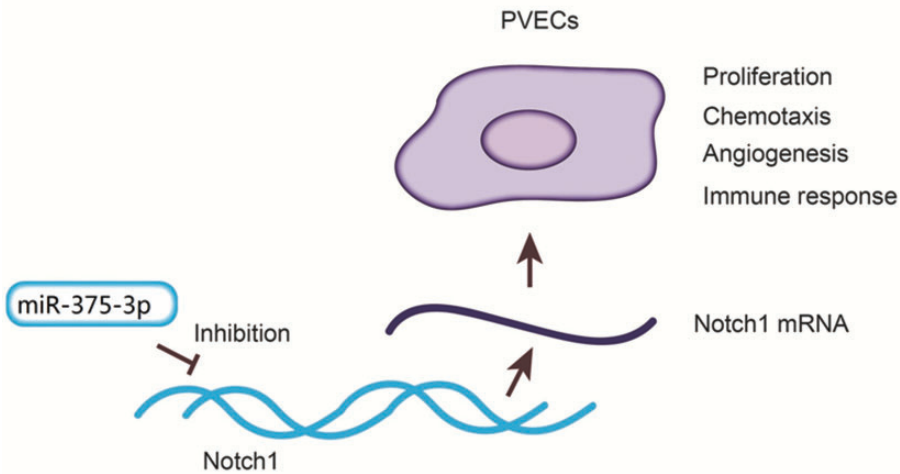


Figure 7. Schematic of the role of miR-375-3p in the regulation of PVECs. MiR-375-3p transcriptionally inhibits Notch1, thereby affecting PVEC activities.

Notch1, thus inhibiting the expression of Notch1 (Figure 7). Here, the inhibition of Notch1 signaling was shown to affect several key functions of PVECs, including cell proliferation, chemotaxis, angiogenesis, and inflammatory response. The interaction between Notch1 and miR-375-3p may, therefore, participate in the progression of various lung diseases. The Protein Atlas protein database (<https://www.proteinatlas.org/ENSG00000148400-NOTCH1/tissue>) indicates high expression of Notch1 in lung tissues, suggesting that Notch1 may indeed play a critical role in cell function in lung tissues, such as the regulation of cell proliferation and migration.³⁴ Through the screening of chip, we found a miRNA, miR-375-3p, had the potential to bind the promoter region of *Notch1* and regulate its expression.

Conclusions

In this study, we demonstrated that under hypoxia, the expression levels of miR-375-3p and Notch1 in PVECs were significantly down-regulated and up-regulated, respectively. Our results confirmed that miR-

375-3p directly targets Notch1 in PVECs, by inhibiting the transcription of *Notch1*. We further found that miR-375-3p regulates the proliferation, chemotaxis, angiogenesis, and inflammatory response of PVECs. Our findings suggest that miR-375-3p plays a key role in the regulation of PVEC function and indicate the potential influence of miR-375-3p on the development of lung diseases.


Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by grants from the National Key Research and Development Program (grant numbers 2016YFC0802806 and 2018YFC1504404-04) and the Great Program of Science Foundation of Tianjin (grant number 15ZXLCSY00040).

ORCID iD

Shike Hou  <https://orcid.org/0000-0002-8325-0712>

References

1. Chen PI, Cao A, Miyagawa K, et al. Amphetamines promote mitochondrial dysfunction and DNA damage in pulmonary hypertension. *JCI Insight* 2017; 2: e90427.
2. Wojciak-Stothard B and Leiper J. Rho GTPases and hypoxia in pulmonary microvascular endothelial cells. *Methods Enzymol* 2008; 439: 267–283.
3. Siow RC, Sato H, Leake DS, et al. Induction of antioxidant stress proteins in vascular endothelial and smooth muscle cells: Protective action of vitamin C against atherogenic lipoproteins. *Free Radic Res* 1999; 31: 309–318.
4. Iannuzzi GL, D'Alto M, Formisano R, et al. Biomarkers in clinical management of pulmonary hypertension: Has the emperor no clothes? A call for action. *Biomark Med* 2019; 13: 235–238.
5. Badagliacca R, Papa S, Poscia R, et al. The added value of cardiopulmonary exercise testing in the follow-up of pulmonary arterial hypertension. *J Heart Lung Transplant* 2019; 38: 306–314.
6. Brandt M, Giokoglu E, Garlapati V, et al. Pulmonary arterial hypertension and endothelial dysfunction is linked to NADPH oxidase-derived superoxide formation in venous thrombosis and pulmonary embolism in mice. *Oxid Med Cell Longev* 2018; 2018: 1860513.
7. Tang H, Hao Q, Fitzgerald T, et al. Pyk2/CAK β tyrosine kinase activity-mediated angiogenesis of pulmonary microvascular endothelial cells. *J Biol Chem* 2002; 277: 5441–5447.
8. Doganci S, Yildirim V, Yesildal F, et al. Comparison of angiogenic and proliferative effects of three commonly used agents for pulmonary artery hypertension (sildenafil, iloprost, bosentan): Is angiogenesis always beneficial? *Eur Rev Med Pharmacol Sci* 2015; 19: 1900–1906.
9. Hu H, Zhang GW, Tian G, et al. miRNA profiling reveals the upregulation of osteogenesis-associated miRNAs in ovariectomy osteoporosis mice. *Clin Exp Obstet Gynecol* 2018; 45: 817–822.
10. Ors-Kumoglu G, Gulce-Iz S and Biray-Avci C. Therapeutic microRNAs in human cancer. *Cytotechnology* 2019; 71: 411–425.
11. Iswariya GT, Paital B, Padma PR, et al. MicroRNAs: Epigenetic players in cancer and aging. *Front Biosci (Schol Ed)* 2019; 11: 29–55.
12. Avnit-Sagi T, Kantorovich L, Kredon-Russo S, et al. The promoter of the pri-miR-375 gene directs expression selectively to the endocrine pancreas. *PLoS One* 2009; 4: e5033.
13. Li X. MiR-375, a microRNA related to diabetes. *Gene* 2014; 533: 1–4.
14. Hong M, He J and Li S. SNW1 regulates Notch signaling in neuroblastoma through interacting with RBPJ. *Biochem Biophys Res Commun* 2019; 509: 869–876.
15. Ma SC, Li Q, Peng JY, et al. Claudin-5 regulates blood-brain barrier permeability by modifying brain microvascular endothelial cell proliferation, migration, and adhesion to prevent lung cancer metastasis. *CNS Neurosci Ther* 2017; 23: 947–960.
16. Saltarella I, Frassanito MA, Lamanuzzi A, et al. Homotypic and heterotypic activation of the Notch pathway in multiple myeloma-enhanced angiogenesis: A novel therapeutic target? *Neoplasia* 2019; 21: 93–105.
17. Gao Y, Yin Y, Xing X, et al. Arsenic-induced anti-angiogenesis via miR-425-5p-regulated CCM3. *Toxicol Lett* 2016; 254: 22–31.
18. Yang W, Hanley FL, Chan FP, et al. Computational simulation of postoperative pulmonary flow distribution in Alagille patients with peripheral pulmonary artery stenosis. *Congenit Heart Dis* 2018; 13: 241–250.
19. Palmiero G, Melillo E, Ferro A, et al. Significant functional mitral regurgitation affects left atrial function in heart failure patients: Haemodynamic correlations and prognostic implications. *Eur Heart J Cardiovasc Imaging* 2019; 20: 1012–1019.
20. Xiao R, Zhu L, Su Y, et al. Monocrotaline pyrrole induces pulmonary endothelial damage through binding to and release from erythrocytes in lung during venous blood reoxygenation. *Am J Physiol Lung Cell Mol Physiol* 2019; 316: L798–L809.
21. Tao W, Sun W, Zhu H, et al. MiR-205-5p suppresses pulmonary vascular smooth muscle cell proliferation by targeting

- MICAL2-mediated Erk1/2 signaling. *Microvasc Res* 2019; 124: 43–50.
22. Ma M, Dai J, Tang H, et al. MicroRNA-23a-3p inhibits mucosal melanoma growth and progression through targeting adenylate cyclase 1 and attenuating cAMP and MAPK pathways. *Theranostics* 2019; 9: 945–960.
 23. Yang Y, Zhao Z, Xie CW, et al. Dual-targeting liposome modified by glutamic hexapeptide and folic acid for bone metastatic breast cancer. *Chem Phys Lipids* 2020; 228: 104882.
 24. Zhao Z, Zhao Y, Xie CW, et al. Dual-active targeting liposomes drug delivery system for bone metastatic breast cancer: Synthesis and biological evaluation. *Chem Phys Lipids* 2019; 223: 104785.
 25. Zhao Z, Chen CQ, Xie CW, et al. Design, synthesis and evaluation of liposomes modified with dendritic aspartic acid for bone-specific targeting. *Chem Phys Lipids* 2020; 226: 104832.
 26. Xu H, Jiang J, Zhang J, et al. MicroRNA-375 inhibits esophageal squamous cell carcinoma proliferation through direct targeting of SP1. *Exp Ther Med* 2019; 17: 1509–1516.
 27. Liu L, Shen W, Zhu Z, et al. Combined inhibition of EGFR and c-ABL suppresses the growth of fulvestrant-resistant breast cancer cells through miR-375-autophagy axis. *Biochem Biophys Res Commun* 2018; 498: 559–565.
 28. Garikipati VNS, Verma SK, Jolardarashi D, et al. Therapeutic inhibition of miR-375 attenuates post-myocardial infarction inflammatory response and left ventricular dysfunction via PDK-1-AKT signalling axis. *Cardiovasc Res* 2017; 113: 938–949.
 29. Liu G, Huang K, Jie Z, et al. CircFAT1 sponges miR-375 to promote the expression of Yes-associated protein 1 in osteosarcoma cells. *Mol Cancer* 2018; 17: 170.
 30. Li GF, Cheng YY, Li BJ, et al. MiR-375 inhibits the proliferation and invasion of glioblastoma by regulating Wnt5a. *Neoplasma* 2019; 66: 350–356.
 31. Shah JS, Gard GB, Yang J, et al. Combining serum microRNA and CA-125 as prognostic indicators of preoperative surgical outcome in women with high-grade serous ovarian cancer. *Gynecol Oncol* 2018; 148: 181–188.
 32. Zhou XY and Shu XM. TRIP13 promotes proliferation and invasion of epithelial ovarian cancer cells through Notch signaling pathway. *Eur Rev Med Pharmacol Sci* 2019; 23: 522–529.
 33. Long YJ, Liu XP, Chen SS, et al. MiR-34a is involved in CSE-induced apoptosis of human pulmonary microvascular endothelial cells by targeting Notch-1 receptor protein. *Respir Res* 2018; 19: 21.
 34. Zhao S, Xiao X, Sun S, et al. MicroRNA-30d/JAG1 axis modulates pulmonary fibrosis through Notch signaling pathway. *Pathol Res Pract* 2018; 214: 1315–1323.
 35. Zhao FY, Han J, Chen XW, et al. MiR-223 enhances the sensitivity of non-small cell lung cancer cells to erlotinib by targeting the insulin-like growth factor-1 receptor. *Int J Mol Med* 2016; 38: 183–191.
 36. Jiao J, Tian W, Qiu P, et al. Induced pluripotent stem cells with *NOTCH1* gene mutation show impaired differentiation into smooth muscle and endothelial cells: Implications for bicuspid aortic valve-related aortopathy. *J Thorac Cardiovasc Surg* 2018; 156: 515–522.
 37. Babicheva A and Yuan JX. Endothelial Notch1 in pulmonary hypertension. *Circ Res* 2019; 124: 176–179.