MiR-4632 mediates PDGFBB-induced proliferation and anti-apoptosis of human pulmonary artery smooth muscle cells via targeting cJUN

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Running title: miR-4632 regulates cell proliferation and apoptosis

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Abstract

MicroRNAs (miRNAs) can regulate proliferative status of pulmonary artery smooth muscle cells (PASMCs), which is a core factor modulating pulmonary vascular remodeling diseases, such as atherosclerosis and pulmonary arterial hypertension (PAH). Our previous work has shown that miR-4632, a rarely reported miRNA, is significantly downregulated in Platelet-Derived Growth Factor (PDGF)BB-stimulated human pulmonary artery smooth muscle cells (HPASMCs), yet its cell function and the underlying molecular mechanisms remain to be elucidated. Here, we find that miR-4632 is highly expressed in HPASMCs and its expression significantly decreased in response to different stimuli. Functional studies revealed that miR-4632 inhibited proliferation and promoted apoptosis of HPASMCs, but had no effects on cell contraction and migration. Furthermore, the cJUN was identified as a direct target gene of miR-4632, while knockdown of cJUN was necessary for miR-4632-mediated HPASMCs proliferation and apoptosis. In addition, the downregulation of miR-4632 by PDGFBB was found to associate with histone deacetylation through the activation of PDGFR/PI3K/HDAC4 signaling. Finally, the expression of miR-4632 was reduced in serum of patients with PAH. Overall, our results suggest that miR-4632 plays an important role in regulating HPASMCs proliferation and apoptosis by suppression of cJUN, providing a novel therapeutic miRNA candidate for the treatment of pulmonary vascular remodeling diseases. It also implies that serum miR-4632 has the potential to serve as a circulating biomarker for PAH diagnosis.

Keywords: HPASMC, PDGFBB, miRNA, proliferation, cJUN
INTRODUCTION

Pulmonary arterial hypertension (PAH) is a currently incurable cardiopulmonary disease, featured by pulmonary vascular remodeling and vasoconstriction, resulting in a progressive increase in pulmonary artery pressure and vascular resistance (10, 24, 25). Albeit multiple cellular mechanisms have been implicated, the abnormal proliferation, migration and resistance to apoptosis of pulmonary arterial smooth muscle cells (PASMCs) are accepted as key pathological processes in the occurrence and development of PAH (25, 30). A number of studies indicate that the dysregulation of PASMCs can be triggered by numerous environmental and extracellular factors, such as hypoxia, tumour necrosis factor-alpha (TNF-α), platelet-derived growth factor (PDGF), angiotensin (Ang) 2 and endothelin (ET)-1 (12, 26). Among them, PDGF which is known as a potent mitogen and chemoattractant for smooth muscle cells (SMCs), contributes essentially to the progression of PAH. PDGF can regulate various cellular functions and gene expression through the activation of PDGF receptor (PDGFR) and the downstream signals (13, 35). It has been shown that the blockage of PDGF signaling pathway by PDGFR inhibitor imatinib could reverse vascular remodeling and the symptom of PAH (1, 11, 29). However, the underlying molecular mechanisms of how PDGF mediates these processes are far to be fully elucidated.

MicroRNAs (miRNAs) are a class of evolutionarily conserved small non-coding RNAs that negatively modulate gene expression through interacting mainly with the 3’-UTR of mRNA transcripts. It has long been recognized that miRNAs play notable regulatory role in a wide range of biological activities and disease genesis. Particularly, accumulative research suggest that miRNAs are indeed involved in the modulation of PASMCs function and PAH progression (2, 5, 43). In addition, recent studies suggest that PDGF-regulated miRNAs play crucial roles in controlling cell dysfunction, and hence serve as mediator of PDGF-stimulated hyperproliferative states of SMCs. For instance, miR-221 expression induced by PDGF is required for
the regulation of cell contraction and proliferation by suppressing p27Kipl in both vascular SMCs and pancreatic cancer cells (6, 34). PDGF-induced miR-136 through the ERK1/2 pathway promotes the vascular SMCs proliferation (41). By contrast, Kim et al. found that miR-365, inhibited by PDGFBB, Ang2 and serum, displayed an anti-proliferative effect in vascular SMCs partially via targeting cyclin D1 (19). Likewise, miR-638 which is decreased upon PDGF treatment, suppresses the vascular SMCs proliferation and migration through targeting NOR1 (22).

Our previous work has profiled the genome-wide miRNA expression in PDGFBB-treated human pulmonary artery smooth muscle cells (HPASMCs), and identified miR-4632 as the most downregulated miRNA (27). In the present study, we further investigated the function and regulatory molecular mechanisms of miR-4632. Our results demonstrate that miR-4632 was abundantly expressed in HPASMCs and its expression was significantly repressed by various PAH related stimuli, suggesting its critical node in mediating the function of multiple PAH causative signaling pathways. Besides, we found that miR-4632 could regulate the proliferation and apoptosis of HPASMCs, which is at least partially through its targeting of a versatile transcriptional factor cJUN. Moreover, PDGFBB-induced decrease of miR-4632 expression was proposed to associate with deacetylation by the activation of PDGFR/PI3K/HDAC4 signaling. Finally, we showed that miR-4632 level decreased in serum of PAH patients.

MATERIALS AND METHODS

Cell culture and treatments

Human pulmonary arterial smooth muscle cells (HPASMCs) were purchased from Sciencell (San Diego, CA, USA) and cultured in complete smooth muscle cell medium (SMCM) containing 2% fetal bovine serum (FBS), 1% smooth muscle cell growth supplement (SMCGS) and 1% penicillin/streptomycin. Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection
(ATCC, Manassas, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Growth factors and cytokines including PDGFBB, TNF-α, angiotensin 2 (Ang2), transforming growth factor beta (TGF-β), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), PDGFAA and beta fibroblast growth factor (β–FGF) were obtained from R&D Systems (Minneapolis, MN, USA). For the treatment of growth factors, HPASMCs were starved in serum free of SMCM for 12h and then stimulated with each factor (12, 27).

**miRNA or siRNA oligonucleotide transfection**

Chemically synthesized mimic/inhibitor miR-4632 for overexpressing/suppressing the endogenous mature miR-4632, and the corresponding negative control (notarget or anti-miR-con) were provided by Ruibobio (Guangzhou, China). Two siRNA of cJUN were also synthesized by Ruibobio, and their sequences are as follow: for si-cJUN 1#: sense, 5’-GGCACAGCUUAAACAGAAAdTdT-3’, antisense, 5’-UUUCUGUUAAGCUGUGCCdTdT-3’; for si-cJUN 2#: sense, 5’-CGCAGCAGUUGCAAACAUUdTdT-3’, antisense, 5’-AAUGUUUGCAACUGCUGCGdTdT-3’. Cell transfections were performed using K2 transfection reagent (Biontex, Germany) according to the manufacturer’s instruction. The mimic/inhibitor miR-4632 and the cJUN siRNA were transfected in a final concentration of 20, 50 and 50nM, respectively.

**Lentivirus-based miR-4632 overexpression.**

The primary miR-4632 sequence was amplified by PCR from human genomic DNA with the forward primer: 5’-CCGCTCGAGGACGAGCAGGACTGCGGA-3’ and reverse primer: 5’-CCGGAATTCCAAGGACCTGAGCCCCAC-3’. Then, the PCR products were cloned into pLVX-Puro vector (Clontech, Mountain View, CA). To monitor the transduction efficiency, the open reading frame of EGFP with stop codon was inserted between CMV promoter and pri-miR-4632. pLVX/EGFP vector without
miR-4632 was used as miRNA negative control. High-titer lentivirus was generated by using a Lenti-X HT Packaging system in HEK293T cells according to the manufacturer’s procedure (Clontech). Lentiviral supernatants produced by the transfected packaging cells were used to infect HPASMCs. To ensure complete infection, cells were selected with puromycin.

**Determination of HPASMCs phenotype, proliferation, apoptosis and migration**

The phenotype, proliferation and apoptosis of HPASMCs were indicated by their respective marker, namely the α-SMA (smooth muscle actin), PCNA (proliferating cell nuclear antigen) and cleaved-Cas3 (cleaved-caspase3), using immunoblotting assays as described in next western blot section. Cell proliferation was also determined by EdU labeling using the EdU Assay Kit (Ribobio, Guangzhou, China) according to the manufacturer’s instruction. Cell migration was measured by transwell and wound healing assays as described before (27, 40).

**Quantification of miRNA and mRNA expression**

Total RNA was extracted with RNAiso Plus (Takara, Dalian, China) and quantified using the NanoDrop 2000c Spectrophotometer (Thermo FisherScientific, Wilmington, DE). The miRNA expression was performed on Step-One plus real-time PCR System (Applied Biosystems) using S-Poly (T) real-time PCR method (16), and sno44 was used as reference gene. The primer sequences for miRNA determination were:

- miR-4632 RT primer: 5’-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTT
  - forward primer: 5’-GTGCCGCCCTCTCGCTG

- sno44 RT primer: 5’-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTT
  - forward primer 5’-CATGAAGGTCTTAATTAGCTC

For mRNA expression analysis, 1μg total RNA were used for first-strand cDNA synthesis and the quantitative PCR experiments were performed using SYBR green-I Master PCR Mix with gene specific primers. The β-actin was used as an
internal control for normalization. Primers sequences are listed as follow: for β-actin:
5’-AGAGATGGCCACGGCTGCTT-3’ (forward) and 5’-ATTGCCTGGAGACGAT
GGAG-3’ (reverse); cJUN: 5’-CTGCGTCCTTAGGCTTCTCC-3’ (forward) and 5’-C
TCGCCAAGTCTGAA-3’ (reverse). All reactions were performed in duplicate
and the relative expression of miRNA and mRNA levels were calculated using the 2^ΔΔCt
method.

**Western blot**

Total proteins were extracted using ice-cold RIPA buffer supplemented with a
protease inhibitor cocktail. The protein concentration was determined by BCA assay
protein kit (Thermo scientific). Equal amounts of proteins (20μg) were
electrophoresed and transferred to a polyvinylidene fluoride (PVDF) membrane
(Millipore, Billerica, MA, USA). The membranes were blocked for 2 h at room
temperature and incubated with antibodies against α-SMA, cJUN, total AKT,
phosphorylation AKT, total HDAC4, phosphorylation HDAC4 (CST, USA),
cleaved-caspase3 and PCNA (Sanying, Wuhan, China), β-actin and β-tubulin (Santa
Cruz, CA) overnight at 4°C, respectively. After 3×10 min washes, the membrane was
incubated for 1 h at room temperature with horseradish peroxidase-conjugated
secondary antibody. The protein bands were visualized using the SuperSignal
chemiluminescent detection module (Pierce).

**Luciferase reporter assay**

The 3’-UTR of cJUN was amplified from human genomic DNA and cloned into
pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). To construct
mutational 3’-UTR report vector, the region that base-paired with miR-4632 seeding
sequences were mutated by site-directed mutagenesis. The primer sequences were,
cJUN 3’-UTR WT forward primer with restriction site of EcoR1: 5’-CGGAATTCA
AGTGTCGAGAAGCTAAAGCC-3’ and reverse primer with restriction site of Xho1: 5’-CCGCTCGAGATATGCGCAATACTTCCT-3’; cJUN-1 3’-UTR mutation forward primer: 5’-GGGGAGCCCGCCGAGGAGGTTTGTGA-3’ and reverse primer: 5’-CCCTCCTCGGCGGGCTCCCCAACCCTCCCCCG’.

All the constructs were confirmed by DNA sequencing. For luciferase activity measurement, HEK293 cells were seeded in 48-well plates and co-transfected with luciferase reporter plasmid together with either mimic miR-4632 or mimic negative control (Notarget). The cells were lysed for luciferase activity measurement using a Lumat LB9508 luminometer (Berthold, Bad Wildbad, Germany) after 48h of transfection. The firefly luciferase activity expressed in the same vector was used as an internal control for normalization.

Human serum sample collection

The collection of human serum samples and their clinical information have been detailed in our previous work (27). In brief, two batches of serum were collected from healthy participants and patients with PAH in the Fuwai Hospital (Beijing, China) and Sun Yat-Sen Cardiovascular Hospital (Shenzhen, China), respectively. The blood samples were centrifuged at 3000g for 10 min at 4°C and stored at -80°C until use. The study was approved by the ethics committee of the two Hospitals. All subjects who participated in the study provided written informed consent.

Statistical analysis

Statistical analyses were carried out using the SPSS package for Windows. All data are presented as mean values ± standard deviation (SD) of three experiments. When only two groups were compared, the statistical differences were evaluated with the double-sided Student’s t-test. Significant differences between groups were analyzed.
by one-way analysis of variance (ANOVA), taking $p$ value less than 0.05 (* or
$\#<0.05$, ** or $\#\#<0.01$, ***$<0.001$) as a significant difference.

RESULTS

miR-4632 expression is decreased in proliferating HPASMCs

The expression patterns of 15 miRNAs, as we identified previously (27), were further
analyzed in HPASMCs after 0, 6, 12 and 24h of PDGFBB treatment. It turned out that
all of them were consistently altered upon PDGFBB (Fig. 1A). Among them, four
miRNAs including miR-4497, miR-4632, miR-1181 and miR-1469 were noted as the
most highly expressed in HPASMCs (Fig. 1B). Interestingly, miR-4632 level
decreased markedly after 24 h of hypoxia or TNF-$\alpha$ treatment, while the other three
miRNAs were less altered, as compared with control (Fig. 1C and D). Moreover,
miR-4632 level decreased gradually from 2 to 48h (Fig. 1E), showing a
time-dependent manner upon PDGFBB treatment. Similar expression pattern of
miR-4632 was also found in response to 5% FBS stimulation (Fig. 1F). In addition, a
dose-dependent inhibition of miR-4632 level by PDGFBB was observed (Fig. 1G).
Besides PDGFBB, several growth factors that are known to trigger the proliferation of
smooth muscle cells were used to treat HPASMCs. As shown in Fig. 1H, most of the
applied growth factors (with the exception of ET-1) significantly inhibited miR-4632
as compared to control. Taken together, these results indicated that miR-4632 level
was negatively regulated in the proliferating HPASMCs.

MiR-4632 regulates HPASMCs proliferation and apoptosis

The above findings prompted us to ask whether alteration of miR-4632 level is
involved in HPASMCs function. To this end, transfection of miR-4632 inhibitor was
firstly performed, leading to almost 80% reduction of miR-4632 level in HPASMCs
(Fig. 2A). Accordingly, as compared with anti-miR-control, suppression of miR-4632
resulted in increased PCNA and decreased cleaved-Cas3 protein levels (Fig. 2B), both of which are known as indicators of cell proliferation and apoptosis respectively. On the contrary, miR-4632 expression raised over 80-fold in HPASMCs transfected with miR-4632 mimic (Fig. 2D). Consequently, the expression of PCNA decreased, while cleaved-Cas3 increased, as compared to the control (Fig. 2E). However, the protein level of α-SMA and the migration of HPASMCs were not significantly affected irrespective of miR-4632 expression (Fig. 2B, C, E and F). To further validate the function of miR-4632, lentiviral infection for overexpressing miR-4632 in HPASMCs was performed, leading to 10-fold increase in the expression of miR-4632 (Fig. 2G). Similar to the results of miR-4632 mimic transfection, lentivirus-based miR-4632 overexpression resulted in significant decrease of PCNA and increase of cleaved-Cas3, but unaltered α-SMA and migratory rate in HPASMCs (Fig. 2H and I).

In addition, the function of miR-4632 was also investigated in PDGFBB-stimulated HPASMCs. As shown in Fig. 3A, PDGFBB induced PCNA protein expression was in turn suppressed by the transfection of miR-4632 mimic. By contrast, the cleaved-Cas3 protein level was enhanced in miR-4632 mimic transfected HPASMCs both with and without PDGFBB treatment (Fig. 3A and B). The effect of miR-4632 on HPASMCs proliferation was also determined by EdU labeling assay. Similarly, PDGFBB induced increase of EdU incorporation was significantly inhibited by overexpressing miR-4632 (Fig. 3C and D). Collectively, these data demonstrated that miR-4632 plays an important role in the regulation of the HPASMCs proliferation and apoptosis, and can at least partially recover the PDGF induced HPASMC dysfunction (i.e., hyperproliferation and anti-apoptosis).

cJUN is a direct target of miR-4632 in HPASMCs

To explore the mechanism of miR-4632 involved in HPASMCs function, the Targetscan database (http://www.targetscan.org/) was used to predict the possible
targets of miR-4632. By KEGG pathway and gene ontology analysis, the candidate
targets of miR-4632 were found highly enriched in cancer-related pathways and might
be involved in signal transduction, cell proliferation and migration. Via a further
search on literatures, three potential target genes including FGF2, cJUN and CCND1
(cyclin D1) were found as tightly correlated to PDGF signaling and SMC dysfunction.
Moreover, preliminary data indicated that the expression of cJUN rather than FGF2
and CCND1 were reduced in HPASMCs transfected with miR-4632 mimic (data not
shown), thus cJUN was chosen for further verification. As shown in Fig.4A,
overexpression of miR-4632 caused significant downregulation of cJUN at both
mRNA and protein level; while the inhibition of miR-4632 resulted in an opposite
effect (Fig. 4B). Moreover, PDGFBB-stimulated HPASMCs showed a significant
increase in the expression of cJUN (Fig. 4C). To further determine whether miR-4632
could interact with its putative binding site in the 3’-UTR sequence of cJUN (Fig. 4D),
the luciferase reporter assays were performed. As compared to notarget control, the
luciferase activity was significantly decreased by the cotransfection of miR-4632
mimic with cJUN reporter vector (Fig. 4D). By contrast, mutation of miR-4632
binding site resulted in a complete loss of the inhibitory effect of miR-4632 on the
luciferase activity (Fig. 4D). Thus, these observations implied that miR-4632 binds to
the 3’-UTR of cJUN and regulates its expression in HPASMCs.

Subsequently, we questioned whether cJUN mediated the effects of miR-4632 on
proliferation and apoptosis of HPASMCs. To find out this, we firstly investigated the
effects of cJUN knockdown on HPASMCs function. As shown in Fig.5A and B, the
expression of cJUN at both mRNA and protein levels were significantly suppressed
by the transfection of two cJUN siRNAs. As a consequence, the PCNA level was
decreased while cleaved-Cas3 level was increased (Fig. 5C). In addition, the effects of
anti-miR-4632 on PCNA and cleaved-Cas3 expression were partially restored by
knockdown of cJUN in HPASMCs (Fig. 5D). Overall, these results suggested that
miR-4632 regulates HPASMCs proliferation and apoptosis at least partially through regulating the cJUN.

The downregulation of miR-4632 is associated with PDGFR/PI3K/HDAC4 signaling

To investigate the possible signaling in modulation of miR-4632 expression, several inhibitors of PDGF pathway were used. Firstly, the regulation of miR-4632 by PDGFBB was verified by using PDGFR inhibitor imatinib. It was found that the higher concentration of imatinib applied, the more restoration of miR-4632 level displayed in PDGFBB stimulated HPASMCs (Fig. 6A). Then, we tested additional 6 inhibitors of PDGF downstream signals, including pictilisib, enzastaurin, SP600125, saracatinib, SCH772984 and SH-4-54. Among them, only the pictilisib, which prevents the PI3K signaling, markedly recovered the expression of miR-4632 to control level (Fig. 6B), showing a concentration dependent manner (Fig. 6C). The activation of PI3K signals and its inhibition by pictilisib was indicated by the changes of AKT phosphorylation status (Fig. 6D).

We have previously showed that PDGFBB inhibited miR-328 expression in an epigenetic way (27), thus we hypothesized similar mechanism might exist in this work. As shown in Fig. 6E, HPASMCs pretreated with trichostatin A (TSA) but not 5-aza-2’-deoxycytidine (5’-aza-dC), of which are known as inhibitor of HDACs and DNMTs respectively, significantly prevented the PDGFBB-stimulated reduction of miR-4632. The inhibition of miR-4632 by PDGFBB was dose-dependently restored upon TSA application (Fig. 6F). Moreover, pictilisib significantly inhibited PDGFBB-stimulated HDAC4 phosphorylation (Fig. 6G), while the protein level of HDAC4 was found upregulated upon PDGF treatment (Fig. 6H). Taken together, these data suggested that the decrease of miR-4632 by PDGFBB is likely related to deacetylation through PDGFR/PI3K/HDAC4 pathway.
miR-4632 expression is reduced in serum of patients with PAH

To correlate the physiological significance of miR-4632, the changes of miR-4632 levels were measured in serum from two batches of healthy donors and patients with PAH. Most of the patients are coupled with ventricular septal defect (VSD) or atrial septal defects (ASD) (27). The first batch of serum was collected from newborns consisting of 20 healthy donors and 49 patients. Compared with healthy control, miR-4632 level decreased significantly in serum of PAH newborns (Fig. 7A). Likewise, the expression of miR-4632 was also reduced in serum samples of 26 PAH adult patients as compared to 24 healthy controls (Fig. 7B). Thus, these findings indicated a potential value of miR-4632 as a circulating biomarker in PAH diagnosis.

DISCUSSION

Despite promising progresses have been achieved in discovering the function of miRNA in the dysregulated HPASMCs and etiology of PAH, the cellular role and underlying molecular mechanisms are highly variable among distinct miRNAs (3, 5, 28). In the present study, we demonstrated that PDGF ligand is likely to activate deacetylation through PDGFR/PI3K/HDACs pathway, leading to the downregulation of miR-4632; while the inhibition of miR-4632 resulted in the upregulation of its target gene cJUN, which contributes to the increased proliferation and decreased apoptosis dysfunctions of HPASMCs (Fig. 8).

In this study, we showed that the 15 differentially expressed miRNAs, as we identified previously (27), were consistently influenced by PDGFBB (Fig. 1A). Of them, four miRNAs namely miR-4497, miR-4632, miR-1181 and miR-1469 were observed as the most highly expressed in HPASMCs (Fig. 1B). However, they are rarely studied except for miR-1181, which has been implicated in pancreatic cancer (15). The reasons might be that these miRNAs are lately identified and poorly conserved among different species. It has been shown that many miRNAs could be regulated by hypoxia and TNF-α, two important contributors of PAH (3, 26, 33, 40).
In this study, we found miR-4632 rather than other three miRNAs was significantly downregulated in HPASMCs exposed to both hypoxia and TNF-α (Fig. 1C and D), indicating the downregulation of miR-4632 indeed occurred in dysregulated HPASMCs. Moreover, our results further showed that miR-4632 expression was inhibited by the treatment of PDGFBB at different time points and concentrations, FBS and several growth factors (Fig. 1 E-H). Since all the factors are tightly related to the proliferation and dysfunction of HPASMCs (23, 30), it implies that miR-4632 is sensitive to all the tested environmental and extracellular HPASMC-proliferation stimuli, and it may serve as a central node mediating the effect of these stimuli on HPASMC. Based on these findings, miR-4632 was used for further study in the subsequent work, in attempt to explore its function and the underlying molecular mechanisms.

Recently, growing studies have reported that PDGF-mediated aberrant expression of miRNAs essentially involved in PASMCs dysfunction and vascular disease (23, 27). For example, the downregulation of miR-204 by PDGF account for the proliferative and antiapoptotic function of PAH-PASMCs and is correlated with the PAH severity (3). Similarly, miR-638 that is inhibited by PDGFBB regulates the human VSMCs proliferation and migration (23). By contrast, PDGF induced upregulation of miR-210 is critical for modulation of VSMCs phenotype (32). In this work, we found that knockdown of miR-4632 in HPASMCs showed increased PCNA and decreased cleaved-Cas3 level, while overexpression of miR-4632 by both miRNA mimic transfection and lentivirus infection had the opposite effects (Fig. 2). These data suggest that miR-4632 could regulate the proliferation and apoptosis of HPASMCs. However, the contraction and migration of HPASMCs were not affected regardless of miR-4632 level, as indicated by the expression of smooth muscle cell-specific phenotype marker α-SMA and transwell assay (Fig. 2). Moreover, it was speculated that miR-4632 might play an important role in the regulation of PDGFBB-associated HPASMCs proliferation (19, 22, 27). As expectation, our
subsequent results revealed that overexpressing miR-4632 significantly suppressed the PDGF-modulated PCNA and cleaved-Cas3 expression and Edu incorporation (Fig.3), indicating that miR-4632 also plays a regulatory role in PDGFBB-stimulated HPASMCs. Nevertheless, it would be interesting to investigate the function of miR-4632 in HPASMCs stimulated by other growth factors in future.

It is well accepted that miRNAs negatively regulate their target genes at transcript level, thus we subsequently aimed to identify the target of miR-4632. By searching in the online prediction tools and computational analysis, we found a binding site of miR-4632 in the 3'-UTR of the cJUN (Fig. 4D). Recent studies showed that miRNAs such as miR-139 or miR-200 targeting the cJUN could mediate the dysfunction of gastric cancer cells or inflammatory response of microglia (14, 42). However, they would not be the miRNAs regulating cJUN in HPASMC, as our previous miRNA profiling found their unchanged expression in PDGFBB-treated HPASMC. In this study, we demonstrated that miR-4632 directly interacted with the 3’UTR of cJUN, and the modulation of miR-4632 level inversely regulated the cJUN expression in HPASMCs (Fig. 4A and B), indicating that cJUN was a potential target of miR-4632. However, it should be notified that cJUN may not be the target gene of miR-4632 in other species, since miR-4632 is a poorly conserved miRNA.

Known as a major component of the activatorprotein-1 (AP-1) transcription factor complex, the cJUN has been intensively studied for its important role in the regulation of cell proliferation, differentiation, apoptosis and cellular migration (17, 32). Activation of cJUN is also involved in the formation and progression of vascular remodeling diseases (20, 31, 32). For instance, Khachigian et al. reported that cJUN expression was induced in animal arterial injury models and in human atherosclerotic lesions, while the knockdown of cJUN abrogated SMCs repair following scraping injury in vitro and intimal thickening in injured rat carotid arteries in vivo (18). Likewise, knockdown of cJUN suppresses vascular permeability and transendothelial emigration of leukocytes in murine models of vascular deseases (9). In this work, we
also found that the cJUN expression was upregulated by PDGFBB (Fig. 4C) (39), while knockdown of cJUN in HPASMCs resulted in decreased PCNA and increased cleaved-Cas3 expression (Fig. 5A-C). These data were tightly coordinated with the finding that miR-4632 decreased in response to PDGFBB and its effects on HPASMCs proliferation and apoptosis (Fig. 2). Furthermore, our results showed the proliferative and antiapoptotic effects of anti-miR-4632 were partially restored by the suppression of cJUN in HPASMCs (Fig. 5D). Taken together, these results suggest that miR-4632 directly targets cJUN in HPASMCs, of which the proliferation and apoptosis is regulated through this pathway.

PDGF isoforms exert their cellular effects by binding and activating PDGFR, which subsequently trigger the signal transduction through downstream signaling molecules, such as tyrosine kinases of the Src family, phospholipase C-\(\gamma\) (PLC-\(\gamma\)), phosphatidylinositol 3\(^{'\prime}\)-kinase (PI3K) and signal transducer and activators of transcription (STATs) pathway (13, 35). In the present work, we found that PDGFBB-induced downregulation of miR-4632 was dose-dependently restored by inhibitors of PDGFR (i.e., imatinib) and PI3K (i.e., pictilisib) signals (Fig. 6A-C), suggesting the involvement of PDGFR/PI3K signaling in the regulation of miR-4632. Meanwhile, we also observed similar recovery of miR-4632 expression by different concentration of TSA (Fig. 6E and F), which has been known to inhibit HDAC class I and II activity and acts as an inducer of histone hyperacetylation (37, 38), implying the suppression of miR-4632 could be related to reduced histone acetylation. Further, we showed that PDGFBB increased the expression and activity of HDAC4, while its activity was attenuated by the blockage of PI3K signaling (Fig. 6G and H). Hence, the downregulation of miR-4632 might be due to histone deacetylation modulated via PDGFR/PI3K/HDAC4 pathway. This may provide an alternative explanation for previous report revealing that HDAC4 mediates PDGFBB-induced vascular SMCs proliferation and migration through p38-activated heat shock protein 27 signals (36), by suggesting HDAC4 might regulate miR-4632 expression to exert its effect on SMC
proliferation. Nevertheless, more in depth work should be taken in HPASMCs to elucidate the exact underlying regulatory mechanism regulating miR-4632 in PDGF signaling pathway.

There is now accumulating evidence that circulating miRNAs could be indicative and serve as diagnostic biomarkers for various diseases (4, 21). Extracellular miRNAs are included in lipid or lipoprotein complexes, which can circulate in blood and such circulating miRNAs are highly stable in both plasma and serum (21). Our results showed that miR-4632 level was significantly decreased in serum of both newborn and adult patients with PAH (Fig.7), indicating a potential value of circulating miR-4632 as a biomarker in PAH diagnose. Meanwhile, the decrease of serum miR-4632 was also coordinated with the observation that miR-4632 expression was downregulated in HPASMCs responding to different PAH-associated factors (Fig. 1C-H). Recent studies indicated that human VSMCs could secrete miRNAs and their expression was affected by the treatment of atherogenic lipoproteins (7, 8). Nevertheless, whether the decrease of serum miR-4632 was due to its reduction in HPASMCs; and the physiological significance of miR-4632 deserve to be explored with extended experiments in a larger number of clinical specimens.

CONCLUSION

In summary, our results demonstrate that PDGFBB-induced downregulation of miR-4632 is likely to associate with deacetylation through PDGFR/PI3K/HDAC4 signalings, and miR-4632 regulates HPASMCs proliferation and apoptosis by targeting cJUN, suggesting a novel miRNA to serve as potential therapeutic target for preventing and treating pulmonary vascular remodeling diseases (e.g. PAH) in PDGF signaling pathway.

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DISCLOSURES

All authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Z.Q. and D.G. conceived and designed the research; Z.Q., Y.L., and J.C. performed experiments; Z.Q., Y.L. and J.C. analyzed results of experiments; Z.Q. and Y.L. prepared figures and drafted the manuscript; Z.Q., Y.L., X.L. and D.G. edited and revised manuscript; all authors approved final version of manuscript.

Reference


Figure legends

**Fig.1 miR-4632 expression is decreased in proliferating HPASMCs.** Human pulmonary artery smooth muscle cells (HPASMCs) were treated with PDGFBB (20ng/mL) for 0, 6, 12 and 24h, and the expression of 15 miRNAs were analyzed by quantitative real-time PCR (A). Relative expression of these miRNAs were calculated using $2^{-\Delta\Delta Ct}$ method in control group (B). The expression of four highly expressed miRNAs including miR-4497, miR-4632, miR-1181 and miR-1469 were investigated in HPASMCs exposed to hypoxia (3% O$_2$) and TNF-α (100 ng/mL) (C and D). The miR4632 level was further measured in HPASMCs treated by PDGFBB and 5%FBS at different time and concentration (E, F and G). Growth factors including Ang2 (100 ng/mL), TGF-β (20 ng/mL), IGF (20 ng/mL), VEGF (20 ng/mL), ET-1 (25 ng/mL ), PDGFAA (20 ng/mL) and β-FGF (20 ng/mL) were used to treat HPASMCs, and the miR-4632 level was determined (H). Data are expressed as means ± SD with at least three independent experiments. *p < 0.05, ** p <0.01 and ***p <0.001 compared to control without treatment.

**Fig.2 miR-4632 inhibits HPASMCs proliferation and induces apoptosis.** HPASMCs were cultured in complete SMCM. Following subculture, HPASMCs were transfected with 20 nM miRNA mimics, 50 nM miRNA inhibitor (anti-miR-4632) or their respect control. Forty-eight hours after transfection, the cells were collected for the analysis of miR-4632 expression (A & D), protein level of α-SMA, PCNA and cleaved capase-3 (Cas3) (B & E). Overexpression of miR-4632 was also generated by lentivirus infection with HPASMCs (G), and the subsequent changes of α-SMA, PCNA and Cas3 expression were measured (H). The β-tubulin was used as an internal control. Transwell assays were carried out for the determination of cell migration (C, F & I). ns, no significant, * p < 0.05, ** p <0.01 and ***p <0.001 compared to control.
Fig.3 miR-4632 mediates the function of PDGFBB-stimulated HPASMCs. After the transfection of miR-4632 mimic, HPASMCs were starved in serum free of SMCM for 12h, and treated with PDGFBB for 48h. Then the immunobloting for PNCA and cleaved-Cas3 (G) and EdU incorporation assays (H) were performed. Data are presented as means ± SD with three independent experiments; * p < 0.05 and ** p <0.01 compared to mimic NC without PDGFBB treatment (PDGFBB-), # p < 0.05 and ## p < 0.05 compared to notarget control with the PDGFBB treatment.

Fig.4 cJUN is a direct target gene of miR-4632 in HPASMCs. The mRNA and protein level of cJUN in HPASMCs transfected with miR-4632 mimic (A) and miR-4632 inhibitor (anti-miR-4632) (B). After 48h of PDGFBB treatment, the expression of cJUN were measured at both mRNA and protein level (C). β-tubulin was used as an internal control. * p < 0.05 and ** p <0.01 compared to control or without PDGFBB treatment (PDGFBB-). The luciferase reporter assay was performed to assess the interaction between miR-4632 and 3’-UTR of cJUN, and the putative binding sites of MiR-4632 in human cJUN 3’-UTR was shown (D). cJUN WT: cJUN wild-type, cJUN Mut: mutated of miR-4632 binding sites. Results are shown as means ± SD of three independent experiments. * p <0.05 compared to cotransfection of notarget and cJUN WT vector.

Fig.5 MiR-4632 function is partially mediated by cJUN. Two siRNA of cJUN were synthesized and transfected in HPASMCs (50 nM), and their effects on cJUN expression (A & B) and protein level of PCNA and cleaved-Cas3 (C & D) were measured. The si-cJUN 2# and anti-miR-4632 were cotransfected in HPASMCs, and the protein expression of PCNA and cleaved-Cas3 were assayed by immunoblotting (F). Results are shown as means ± SD of three independent experiments. * p <0.05, ** p <0.01 and *** p < 0.001 compared to si-Control.
Fig.6 The downregulation of miR-4632 is associated with deacetylation through PDGFR/PI3K/HDAC pathways. PDGF signaling pathway inhibitors including imatinib, pictilisib, enzastaurin, sp600125, saracatinib, SCH772984 and SH-4-54 were used to pretreat HPASMCs, which was then stimulated by PDGFBB. After 12h treatment, the miR-4632 expression was examined with different concentration of imatinib (A), or with different inhibitors (B), or with different concentration of pictilisib (C). The activation of PI3K signal and its inhibition by pictilisib was indicated by the change in phosphorylation of AKT (D). HPASMCs were pretreated with 5’-Aza-d (5 µm) or TSA (100 nm) for 48h then subjected to PDGFBB, and the expression of miR-4632 was measured (E). The change of miR-4632 level in response to different concentration of TSA was shown (F). Effects of pictilisib on phosphorylation of HDAC4 (G) and PDGF on HDAC4 expression (H) were shown. Results are shown as means ± SD of three independent experiments. * p <0.05, ** p <0.01 and *** p < 0.001 compared to control without PDGFBB treatment (PDGFBB-).

Fig.7 MiR-4632 level is decreased in serum of PAH patients. Serum miR-4632 expression was determined in serum of healthy human donors and patients with PAH by qRT-PCR. Relative miR-4632 level in 20 healthy versus 49 PAH newborns (A), and a second group of 24 healthy versus 26 adult PAH patients (B) were investigated. **p<0.01 compared to healthy controls.

Fig.8 A schematic mechanism by which PDGFBB-induced downregulation of miR-4632 might be partially attributed to the increase of deacetylation through activating PDGFR/PI3K/HDACs pathways. PDGF: platelet derived growth factor; PDGFR: receptor of platelet derived growth factor. PI3K: phosphatidylinositol 3’-kinase. HDAC: histone deacetylases. PASMC: pulmonary artery smooth muscle cell.
Figure 1
A

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C

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**cJUN 3' UTR-WT** 5' GGGAGGGUUGGGGAGGAGG...3'

**hsa-miR-4632** 5' GAUCUCGUCGCUCUCCCGCCG...3'

**cJUN 3' UTR-Mut** 5' GGGAGGGUUGGGGAGCCCGCCGAGGAGG...3'
Figure 8