miR-4632 mediates PDGF-BB-induced proliferation and antiapoptosis of human pulmonary artery smooth muscle cells via targeting cJUN

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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, tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), angiotensin II(ANG II), 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 the PDGF receptor (PDGFR) and the downstream signals (13, 35). It has been shown that the blockage of PDGF signaling pathway by the PDGFR inhibitor imatinib could reverse vascular remodeling and the symptoms of PAH (1, 11, 29). However, the underlying molecular mechanisms of how PDGF mediates these processes are far from being fully elucidated.

MicroRNAs (miRNAs) are a class of evolutionarily conserved small noncoding RNAs that negatively regulate gene expression through interacting mainly with the 3′-untranslated region (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, accumulating research suggests that miRNAs are indeed involved in the modulation of PASMC 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 a mediator of PDGF-stimulated hyperproliferative state 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 SMC proliferation (41). By contrast, Kim et al. (19) found that miR-365, inhibited by PDGF-BB, ANG II, and serum, displayed an antiproliferative effect in vascular SMC partially via targeting cyclin D1. Likewise, miR-638, which is decreased upon PDGF treatment, suppresses the vascular SMC proliferation and migration through targeting NOR1 (22).

Our previous work has profiled the genome-wide miRNA expression in PDGF-BB-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 it is a critical node in mediating the function of multiple PAH causative signaling pathways. In addition, we found that miR-4632 could regulate the proliferation and apoptosis of HPASMCs, which is at least partially through its targeting of the versatile transcriptional factor cJUN. Moreover, the PDGF-BB-induced decrease of miR-4632 expression was proposed to associate with deacetylation by the activation of PDGFR/phosphatidylinositol 3'-kinase (PI3K)/histone deacetylase (HDAC) 4 signaling. Finally, we showed that the miR-4632 level decreased in the serum of PAH patients.

MATERIALS AND METHODS

Cell culture and treatments. HPASMCs were purchased from ScienCell (San Diego, CA) and cultured in complete SMC medium containing 2% fetal bovine serum (FBS), 1% SMC growth supplement, and 1% penicillin/streptomycin. Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Growth factors and cytokines including PDGF-BB, TNF-α, angiotensin II (ANG II), transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), PDGF-AA, and β3-fibroblast growth factor (β3-FGF) were obtained from R&D Systems (Minneapolis, MN). For the treatment of growth factors, HPASMCs were starved in serum free of SMC growth factors, HPASMCs were then treated with cytokines including PDGF-BB, TNF-α, angiotensin II (ANG II), transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), PDGF-AA, and β3-fibroblast growth factor (β3-FGF) for 24 h. 

miRNA or siRNA oligonucleotide transfection. The 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 Ribobio (Guangzhou, China). Two siRNA of cJUN were also synthesized by Ribobio, and their sequences are as follows: for si-cJUN 1#: sense, 5'-GGCATGACGCUUAAACAGAAAdTdT-3', antisense, 5'-GUUUAAGCUGUGCCdTdT-3'. Cell transfections were performed using K2 lentivirus was generated by using a Lenti-X HT Packaging system in HEK293T cells according to the manufacturer's procedure (ClonTech, Mountain View, CA). To monitor the transduction efficiency, the open reading frame of EGFP with stop codon was inserted between the CMV promoter and pri-miR-4632. Plasmids were used for miR-4632 was used as a 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 HPASMC phenotype, proliferation, apoptosis, and migration. The phenotype, proliferation, and apoptosis of HPASMCs were indicated by their respective markers, namely the α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA), and cleaved-Ca3 (cleaved-caspase3), using immunoblotting assays as described in Western blotting. Cell proliferation was also determined by 5-ethyl-2'-deoxyuridine (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 Fisher Scientific, Wilmington, DE). The miRNA expression was performed on Step-One plus real-time PCR System (Applied Biosystems) using the S-Poly (T) real-time PCR method (16), and sn;44 was used as a reference gene. The primer sequences for miRNA determination were as follows: miR-4632 RT primer: 5'-GTCGAGGGTGCCGAGGTCGAGTTGGCTAAGTCAAGTTCGAG-3' and forward primer: 5'-GTCGCCCGCTCTCCTGCTG-3'; sn;44 RT primer: 5'-GTCGAGGGTGCCGAGGTCGAGGCGACCCACTGGGCAATTTTTTTTTTTAGTTCAG-3' and forward primer 5'-CATGAGGTTCTAATTAGCCTCA-3'. For mRNA expression analysis, 1 μg total RNA was 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 follows: for β-actin: 5'-AGAGATGGCCAGGCGTCTGCTT-3' (forward) and 5'-ATTTGCGGTTGACGAGG-3' (reverse); cJUN: 5'-CTTGCGTCTTAGGGCTTTC-3' (forward) and 5'-TGGCGCAATGTCCAACCAAA-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 blotting. Total proteins were extracted using ice-cold RIPA buffer supplemented with a protease inhibitor cocktail. The protein determination was performed by the BCA assay kit protocol (Thermo Scientific). Equal amounts of proteins (20 μg) were electrophoresed and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). 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), cleaved-caspase3 and PCNA (Sanying, Wuhan, China), β-actin, and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, respectively. After three 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). To construct the mutualistic 3'-UTR report vector, the region that base paired with miR-4632 seeding sequences was mutated by site-directed mutagenesis. The primer sequences were as follows: cJUN 3'-UTR WT forward primer with restriction site of EcoRI: 5'-CGGATATCAGTGGGCTGAGATGTTGAGCCTTTCC-3' and reverse primer with restriction site of XhoI: 5'-CCGCTGCTGAAATCTGTGCCAAGCCCTGC-3'. The 3'-UTR mutation forward primer: 5'-GGGGACGCGCCGACGAGCT-3' and reverse primer: 5'-CCCTCCTCGGGGGTCCGCGAACCCTCC-3'. 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 48 h 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 3,000 g
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 24 h of PDGF-BB treatment. It turned out that all of them were consistently altered upon PDGF-BB (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, the miR-4632 level decreased markedly after 24 h of hypoxia or TNF-α treatment, while the other three miRNAs were less altered, as compared with control (Fig. 1, C and D). Moreover, miR-4632 level decreased gradually from 2 to 48 h (Fig. 1E), showing a time-dependent manner upon PDGF-BB treatment. A similar expression pattern of miR-4632 was also found in response to 5% FBS stimulation (Fig. 1F). In addition, a dose-dependent inhibition of the miR-4632 level by PDGF-BB was observed (Fig. 1G). In addition, PDGF-BB, several growth factors that are known to trigger the proliferation of SMCs 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 with control. Taken together, these results indicated that miR-4632 level was negatively regulated in the proliferating HPASMCs.

MiR-4632 regulates HPASMC proliferation and apoptosis. The above findings prompted us to ask whether alteration of the miR-4632 level is involved in HPASMC function. To this end, transfection of the miR-4632 inhibitor was first performed, leading to an 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 increased over 80-fold in HPASMCs transfected with the miR-4632 mimic (Fig. 2D). Consequently, the expression of PCNA decreased, while cleaved-Cas3 increased, as compared with 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. 2, B, C, E, and F). To further validate the function of miR-4632, lentiviral infection for overexpressing miR-4632 in HPASMCs was performed, leading to a 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 a significant decrease of PCNA and an increase of cleaved-Cas3 but unaltered α-SMA and migratory rate in HPASMCs (Fig. 2, H and I).

In addition, the function of miR-4632 was also investigated in PDGF-BB-stimulated HPASMCs. As shown in Fig. 3A, PDGF-BB-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 PDGF-BB treatment (Fig. 3, A and B). The effect of miR-4632 on HPASMC proliferation was also determined by EdU labeling assay. Similarly, the PDGF-BB-induced increase of EdU incorporation was significantly inhibited by overexpressing miR-4632 (Fig. 3, C and D). Collectively, these data demonstrated that miR-4632 plays an important role in the regulation of HPASMC proliferation and apoptosis and can at least partially recover the PDGF induced HPASMC dysfunction (i.e., hyperproliferation and antiapoptosis).

cJUN is a direct target of miR-4632 in HPASMCs. To explore the mechanism of miR-4632 involved in HPASMC 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 in the literature, three potential target genes including FGFR2, 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 FGFR2 and CCND1 was reduced in HPASMCs transfected with the 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, PDGF-BB-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), luciferase reporter assays were performed. As compared with nontarget control, the luciferase activity was significantly decreased by the cotransfection of miR-4632 mimic with the cJUN reporter vector (Fig. 4D). By contrast, mutation of the miR-4632 binding site resulted in a complete loss of the inhibitory effect of miR-4632 on 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 first investigated the effects of cJUN knockdown on HPASMC function. As shown in Fig. 5, A and B, the expression of cJUN at both mRNA and protein levels was significantly suppressed by the transfection of two cJUN siRNAs. As a consequence, the PCNA level was decreased while the 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 HPASMC proliferation and apoptosis at least partially through regulating the cJUN.

The downregulation of miR-4632 is associated with PDGFR/P13K/HDAC4 signaling. To investigate the possible signaling in modulation of miR-4632 expression, several inhibitors of PDGFR pathway were used. First, the regulation of miR-4632 by PDGFR inhibitor imatinib. It was found that the higher concentration of
imatinib applied, the more restoration of the miR-4632 level displayed in PDGF-BB-stimulated HPASMCs (Fig. 6A). Then, we tested additional six 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 were indicated by the changes of Akt phosphorylation status (Fig. 6D).

We have previously shown that PDGF-BB inhibited miR-328 expression in an epigenetic way (27); thus we hypothesized that a similar mechanism might exist in this work. As shown in Fig. 6E, HPASMCs pretreated with trichostatin A
miR-4632, through the PDGFR/PI3K/HDAC4 pathway. Taken together, these data suggested that the decrease of HDAC4 was found upregulated upon PDGF treatment (Fig. 6F), while the protein level of PDGF-BB-stimulated reduction of PCNA, Caspase-3 (Casp3) (Fig. 2), and the subsequent changes of α-SMA, β-Tubulin was used as an internal control. Transwell assays were carried out for the determination of cell migration (C, F, and I). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with control; ns, not significant.

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 were coupled with ventricular septal defect or atrial septal defects (27). The first batch of serum was collected from newborns consisting of 20 healthy donors and 49 patients. Compared with healthy control, the 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 with 24 healthy controls (Fig. 7B). Thus these findings indicated a potential value of miR-4632 as a circulating biomarker in PAH diagnosis.

DISCUSSION

Despite the promising progress that has 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 the PDGF ligand is likely to activate deacetylation through the PDGFR/P13K/HDAC4 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 PDGF-BB (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. 1, C 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 PDGF-BB 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 (22, 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 HPASMCs. Based on these findings, miR-4632 was used for further study in the subsequent work, in an attempt to explore its function and the underlying molecular mechanisms.

Recently, growing studies have reported that PDGF-mediated aberrant expression of miRNAs is essentially involved in PASMC dysfunction and vascular disease (23, 27). For example, the downregulation of miR-204 by PDGF accounts for the proliferative and antiapoptotic function of PAH-PASMCs and is correlated with the PAH severity (3). Similarly, miR-638 that is inhibited by PDGF-BB regulates the human vascular SMC (VSMC) proliferation and migration (23). By contrast, PDGF-induced upregulation of miR-210 is critical for modulation of VSMC 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 the miR-4632 level, as indicated by the expression of the SMC-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 PDGF-BB-associated HPASMC 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 PDGF-BB-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 the 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' untranslated region (UTR) of cJUN (Fig. 4D). Recent studies showed that miRNAs such as miR-139 or miR-200 targeting 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 HPASMCs, as our previous miRNA profiling found their unchanged expression in PDGF-BB-treated HPASMCs. 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 activator protein-1 transcription factor complex, 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. (18) reported that cJUN expression was induced in animal arterial injury models and in human atherosclerosis. 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. 4, A 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.
sclerotic lesions, while the knockdown of cJUN abrogated SMC repair following scraping injury in vitro and intimal thickening in injured rat carotid arteries in vivo. Likewise, knockdown of cJUN suppresses vascular permeability and transendothelial emigration of leukocytes in murine models of vascular diseases (9). In this work, we also found that the cJUN expression was upregulated by PDGF-BB (Fig. 4C) (39), while its activity was attenuated by the blockage of PI3K and signal transducer and activators of transcription pathway (13, 35). In the present work, we found that PDGF-BB-induced downregulation of miR-4632 was dose dependently restored by inhibitors of PDGFR (i.e., imatinib) and PI3K (i.e., pictilisib) signals (Fig. 6, A–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 concentrations of TSA (Fig. 6, E 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. Furthermore, we showed that PDGF-BB increased the expression and activity of HDAC4, while its activity was attenuated by the blockage of PI3K signaling (Fig. 6, G and H). Hence, the downregulation of miR-4632 might be due to histone deacetylation modulated via the PDGFR/PI3K/HDAC4 pathway. This may provide an alternative explanation for a previous report revealing that HDAC4 mediates PDGF-BB-induced vascular SMC proliferation and migration through p38-activated heat shock protein 27 signals (36) by suggesting that HDAC4 might regulate miR-4632 expression to exert its effect on SMC proliferation.

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 and B) and protein level of PCNA and cleaved-Cas3 (C and D) were measured. The si-cJUN 2# and anti-miR-4632 were cotransfected in HPASMCs, and the protein expression of PCNA and cleaved-Cas3 was assayed by immunoblotting. Results are shown as means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with si-control.
Fig. 6. The downregulation of miR-4632 is associated with deacetylation through the PDGF receptor phosphatidylinositol 3'-kinase (PI3K)/histone deacetylase (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 PDGF-BB. After 12-h treatment, the miR-4632 expression was examined with different concentrations of imatinib (A), or with different inhibitors (B), or with different concentrations 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 trichostatin A (TSA; 100 nm) for 48 h and then subjected to PDGF-BB, and the expression of miR-4632 was measured (E). The change of miR-4632 level in response to different concentration of TSA is shown (F). Effects of pictilisib on phosphorylation of HDAC4 (G) and PDGF on HDAC4 expression (H) are shown. Results are shown as means ± SD of 3 independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with control without PDGF-BB treatment (PDGF-BB -).
Nevertheless, more in-depth work should be taken in HPASMCs to elucidate the exact underlying regulatory mechanism regulating miR-4632 in the 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 the blood and such circulating miRNAs are highly stable in both plasma and serum (21). Our results showed that the 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. 1, C–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 PDGF-BB-induced downregulation of miR-4632 is likely to associate with deacetylation through PDGFR/PI3K/HDAC4 signaling and miR-4632 regulates HPASMC 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 the PDGF signaling pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z. Q. and D. G. conceived and designed research; Z. Q. and J. C. performed experiments; Z. Q., Y. L., and J. C. analyzed data; Z. Q. prepared figures; Z. Q. and D. G. approved final version of manuscript.

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