Identifying microRNAs targeting Wnt/β-catenin pathway in end-stage idiopathic pulmonary arterial hypertension

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Abstract

MicroRNAs (miRNAs) play important roles in the pathogenesis of pulmonary arterial hypertension (PAH). However, the pathways targeted by miRNAs in PAH have not been systematically investigated. We aim to identify dysregulated miRNAs for patients with idiopathic PAH (IPAH).

miRNA profiling was performed on lung tissue total RNA from eight IPAH patients and eight control subjects. Real-time quantitative RT-PCR (qRT-PCR) was used for validation of miRNA and mRNA expression levels in 14 IPAH patients and 14 control subjects.

Pathway enrichment analysis showed Wnt/β-catenin signaling is among the top PAH related pathways enriched in target genes of dysregulated miRNAs. We confirmed the significant increased expression levels of 5 miRNAs (*let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p* and

miR-656) targeting major PAH-related pathways. Moreover, qRT-PCR validation of Wnt/β-catenin pathway activation indicated multiple genes including receptors (*FZD4, FZD5*), core molecule (*CTNNB1*), and downstream targets (*CCND1, VEGFA* and *AXIN2*) were significantly upregulated. The expression level of *miR-199b-5p* was positively correlated with patients' hemodynamics (PVR: p=0.038) and pulmonary vascular remodeling (muscularization: p=0.021). We confirmed overexpression of *miR-199b-5p* in hypoxic pulmonary arterial endothelial cells that negatively regulates *GSK3B* expression.

In summary, miRNAs influence the pathogenesis of PAH by regulating major PAH related pathways including Wnt/β-catenin in end-stage IPAH.

Keywords

microRNA; Wnt/β-catenin; idiopathic pulmonary arterial hypertension; microarray

INTRODUCTION

Idiopathic pulmonary arterial hypertension (idiopathic PAH, IPAH) is a debilitating disease characterized by elevated pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR). Although the incidence of IPAH is low [1], it is often life-threatening, causing right heart failure. The main pathological changes of IPAH are pulmonary vascular remodeling located in but not limited to small pulmonary arteries, including intima and media thickening, small artery muscularization, plexiform lesion, perivascular inflammation and *in situ* thrombosis [2–4]. The biological behaviors of IPAH are similar to those of cancer. Traits of cancer, such as monoclonal cell proliferation [5], apoptosis resistance [6], Warburg effect [7] and angiogenesis [8] are found in IPAH.

Wnt signaling pathways consist of Wnt/β-catenin pathway (the canonical Wnt pathway), the noncanonical planar cell polarity pathway (Wnt/PCP pathway) and the noncanonical Wnt/ calcium pathway. Wnt pathways have been identified to be critical in embryonic development, cell proliferation, and carcinogenesis. Recent studies have revealed Wnt also plays a role in the pathogenesis of PAH. In the context of human lung tissue, both the Wnt/β-catenin [9] and Wnt/PCP pathways [10] are activated in pulmonary vasculature from patients with PAH. Thus, the Wnt signaling pathways have emerged as attractive treatment targets in PAH given their role in the preservation of pulmonary vascular homeostasis and the recent development of Wnt-specific compounds and biological therapies capable of modulating Wnt pathway activity.

microRNAs (miRNAs or miRs), a type of non-coding RNA, are key regulators in gene post-transcriptional regulation [11]. Previous studies have uncovered dysregulation of miRNAs in both tissue and plasma that are correlated with PAH severity and prognosis [12,13]. Systematic approaches combining transcriptomic data with the powerful predictive methods of network biology are warranted to identify essential pathways in the control of PAH and ultimately novel therapeutic interventions.

We hypothesized that an unbiased approach using miRNA profiling followed by pathway enrichment analysis would help identify dysregulated miRNAs and major pathways involved in the pathogenesis of PAH. Furthermore, these miRNAs would be correlated with hemodynamic and pathologic severity of the disease.

METHODS

Patient enrolment and tissue sample preparation

A total of 22 IPAH patients and 22 control subjects were enrolled in this study. An initial miRNA expression profiling was performed with the total RNA isolated from lung tissue of eight IPAH patients and eight age-matched controls (Screening cohort). In miRNA expression validation, the cohort of 14 IPAH patients and 14 control subjects were included (Validation cohort), and the entire cohort was used for gene expression validation. The IPAH tissue samples were obtained from explanted lungs of patients undergoing lung transplantation, and control tissue samples were from the lungs of failed organ donors (FD). All subjects were enrolled in the Pulmonary Hypertension Breakthrough Initiative (PHBI), and all lung tissue was collected according to the PHBI standardized tissue-processing protocol [4]. The diagnosis of IPAH was based on standard criteria with confirmation by right heart catheterization (RHC) and exclusion of other forms of pulmonary hypertension as per the most recent guidelines of diagnosis [14,15]. Lung tissue collection was approved by each Institutional Review Board at all lung transplant sites. Written informed consent from all subjects or their legal guardians was obtained upon enrolment.

miRNA microarray screening

Isolated RNA samples were hybridized to a Human miRNA Microarray V3 (G4872A, Agilent Technologies) platform according to the manufacturer's instructions in the JHMI Deep Sequencing and Microarray Core Facility (*see* the Supplementary Methods). The data from this study have been deposited in the National Centre for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE67597.

miRNA target gene exploration and pathway analysis

To identify biological functions and canonical pathways in which the significantly dysregulated miRNAs are involved, Ingenuity® Pathway Analysis (IPA) (QIAGEN, http://www.ingenuity.com/products/ipa) was applied. Utilizing the "microRNA Target Filter" tool in IPA, target genes experimentally observed and predicted with high confidence were obtained. Pathway enrichment analysis of these validated and putative target genes was performed by IPA's "Core Analysis" tool.

Selection of miRNAs targeting major PAH pathways for technical validation

A pathway-based target gene-to-miRNA searching approach was adopted to select the miRNAs for technical validation. First, miRSystem (http://mirsystem.cgm.ntu.edu.tw/), an online integrated miRNA target gene prediction tool was employed to find the Wnt-specific miRNAs targeting the 73 Wnt/β-catenin pathway genes identified by IPA (Table S1). Eleven dysregulated miRNAs identified by array analysis, were found to be targeting the 73 Wnt/β-

catenin pathway genes. Next, search of the literature indicated 7 out of these 11 Wnt-targeting miRNAs (*let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, miR-199b-5p, miR-205-5p* and *miR-656*) were linked to at least one of the following 3 major PAH-related pathways: hypoxia, inflammation or TGF-β signaling. Thus, these 7 miRNAs were selected for further miRNA qRT-PCR validation (Table S2, *see* the Supplementary Methods).

LNA-ISH for *miR-199b-5p* and *miR-656*

We employed techniques for miRNA localization to reveal the spatial distribution of the dysregulated miRNAs. Locked nucleic acid (LNA) - *in situ* hybridization was performed on formalin-fixed paraffin embedded (FFPE) tissue samples according to the manufacturer's protocol (*see* the Supplementary Methods).

miRNA validated target gene search and network construction

First, experimentally validated gene targets of these miRNAs (*let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, miR-199b-5p* and *miR-656*) were obtained from a publicly available database: miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/mirnatargetpub.html). Then, genes in all of the 4 major PAH-related pathways (hypoxia, inflammation, TGF-β and Wnt) were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/gene/). Synonyms of each pathway were used for search. The overlapping genes of the above two lists were then subjected to network construction. Direct interactions between network constituents based on published literature were obtained using the "My Pathway" tool in IPA. Genes with multiple interactions with others in the network, so-called "gene hubs," were validated by qRT-PCR.

Gene expression analysis

Expression levels of microRNA target genes were detected by TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7300 Real-Time PCR System (*see* the Supplementary Methods).

Hypoxia treatment of cells and transfection of oligonucleotides for *miR-199b-5p*

Primary human pulmonary arterial endothelial cells (HPAECs) were purchased and propagated in EGM-2 cell culture media (Lonza). Experiments were performed at passages 4–9. HPAECs were exposed for 24 or 48 hours either to standard non-hypoxic cell-culture conditions (21% O_2, 5% CO_2, with N_2 balance at 37°C) or to hypoxia (4% O_2, 5% CO_2, with N_2 balance at 37°C), in a modular hypoxia chamber.

miR-199b-5p mimic (to mimic the mature form of miR-199b-5p), *miR-199b-5p* inhibitor (hairpin loop oligonucleotides that carry specific antisense sequences) and negative control molecules were obtained from Dharmacon (USA) and transfected into HPAECs, using Geneporter B reagent (GeneTherapy Systems), at a final concentration of 60 nM for the mimic and 100 nM for the inhibitor, according to the manufacturer's protocol. Transfected cells were allowed to recover in normal growth media for 24 hours prior to hypoxia treatment (24 hours).

Statistical analyses

For microarray analysis, the filtering criteria of fold change (FC, increase or decrease) 1.5 and p-value <0.05 were employed. An unpaired t-test or Mann-Whitney U test was applied to the comparisons of relative miRNA or mRNA expression levels between control subjects and IPAH patients, and a p-value of less than 0.05 was considered significant.

The correlations between – Ct values of miRNAs, genes and hemodynamics or histopathological parameters in IPAH patients were assessed by Pearson correlation. The hemodynamics at both diagnosis and the last RHC before transplantation were analyzed. Mean right atrial pressure (mRAP), mean PAP (mPAP), cardiac output (CO) and PVR were available as hemodynamic measurements. Pulmonary vascular pathological parameters, including scores for the pulmonary artery (PA) fractional thicknesses (Intima, Media and Adventitia), muscularization, Intima thickening (INTTHICK), Medial thickening (MEDTHICK) and Proliferation (PROLIF) were assessed [4]. Receiver operating characteristic (ROC) curve assessment of cut-off was employed to evaluate the diagnostic value of miRNAs. Calculations were performed with SPSS 19.0 (IBM, Armonk, NY).

An overview of our study design and work flow is summarized in Figure S1.

RESULTS

Patient characteristics

We have listed the demographic features, hemodynamic parameters (at diagnosis), six-minute walk distance (6MWD), WHO functional classification (WHO FC) and initial treatment of the screening cohort, validation cohort and entire cohort in Table 1. The entire cohort was composed predominantly of females (N=15/22, 68%) with a mean age of 46±12.4 years. Upon presentation, most patients were categorized as WHO FC III and IV (N=17/18, 94.4%) and had documented mean 6MWD of 347±81 m. All patients underwent RHC that showed an average mRAP of 15.0±6.0 mmHg, a median mPAP of 52 (49, 64) mmHg, mean CO of 4.36±1.51 L/min and median PVR of 11.41 (8.02, 15.63) Wood Units. Of note, there were no carriers of either *BMPR2* or *SMAD9* genetic mutation.

Identifying PAH related pathways

Of 2,006 miRNAs screened, 21 were differentially expressed in the lung of IPAH patients compared to controls including 13 downregulated and 8 upregulated (Figure S2).

To identify the top pathways enriched in target genes of differentially expressed miRNAs in IPAH, we retrieved 4,664 gene targets of the 21 dysregulated miRNAs. These genes were either validated by previous experiments or predicted *in silico* with high confidence. Pathway enrichment analysis of these target genes revealed that 'Molecular Mechanisms of Cancer', 'HGF', 'PTEN', 'TGF-β', 'Wnt/β-catenin' and 'Cardiac Hypertrophy' signaling were the top canonical pathways enriched in these target genes (Table S1 and Figure S3). Given the emerging role of Wnt/β-catenin signaling in the pathogenesis of PAH and other cardiovascular diseases [16], and the potential of recently developed Wnt-specific

compounds and biological therapies capable of modulating Wnt pathway activity [17], we have focused on the Wnt/β-catenin pathway in the following validations.

qRT-PCR validation confirmed the dysregulation of five miRNAs

By target gene-to-miRNA prediction from miRSystem, we found 11 dysregulated miRNAs to be targeting the 73 Wnt/β-catenin pathway genes (6 out of 11 were shown in Table S3), and 7 of them (*let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, miR-199b-5p, miR-205-5p* and *miR-656*) were also linked to at least one of the 3 additional major PAH-related pathways: hypoxia, inflammation or TGF-β signaling (Table S4). Significant upregulation of 5 of these miRNAs (*let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p* and *miR-656*) was confirmed by qRT-PCR validation (Figure 1). *miR-199b-5p* also showed a trend toward significance in both the validation cohort (p=0.056) and the entire cohort (p=0.046).

Expression levels of miRNAs were correlated with hemodynamics and histopathological parameters

The expression level of *miR-199b-5p* was significantly correlated with both mPAP (r=0.444, p=0.038) and PVR (r=0.522, p=0.038), a key determinant of PAH prognosis [18] as well as muscularization (r=0.540, p=0.021). Expression level of *miR-199b-5p* also had a borderline correlation with adventitia fractional thickness (r=0.462, p=0.054) (Figure 2). Expression level of *miR-656* had significant positive correlations with intima thickening, medial thickening, and proliferation (Figure S4) despite that the abnormal scores for these 3 parameters were observed only in very few patients. In addition, we found expression levels of *miR-199a-3p, miR-26b-5p* and *miR-27b-3p* were correlated with RAP at diagnosis; expression levels of *miR-656* were correlated with RAP before transplantation (Figure S5).

The strong positive correlation between overexpression of *miR-199b-5p* and *miR-656* and various features of vascular remodeling prompted us to pursue an in-depth investigation of their tissue distribution. We found intriguing specificity and intensity of expression of both miRNAs in and surrounding small and medium-sized vessels. *miR-199b-5p* displayed expression in the pulmonary vascular endothelium and adventitia; and *miR-656* showed detectable but very low-abundance expression (Figure 3).

Diagnostic value of validated miRNAs for IPAH

Expression levels of *let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, miR-199b-5p* and *miR-656* were associated with the diagnosis of IPAH on ROC analysis. Subjects with higher tissue levels of these six miRNAs are more likely to be IPAH patients. Area under curve (AUC) and cut-off values from ROC analyses of these miRNAs are shown in Table 2 and Figure S6.

Activation of Wnt/ β-catenin pathway in end-stage IPAH lung tissue

Expression levels of Wnt ligand inhibitor (*WIF1*), major ligands (*WNT5A, WNT7A* and *WNT16*), receptors (*FZD4, FZD5* and *FZD10*), components of the Wnt inhibition complex (*APC* and *GSK3B*), core gene β-catenin (*CTNNB1*), β-catenin inhibitors (*CTNNBIP1* and *HDAC1*) and downstream target genes (*CCND1, VEGFA* and *AXIN2*) were analyzed by

qRT-PCR. We found Wnt inhibitor (*WIF1*), receptors (*FZD4, FZD10*), core gene (*CTNNB1*) and its downstream targets (*CCND1, VEGFA* and *AXIN2*) were significantly upregulated in lung tissue from patients with IPAH (Figure 4 and Table S5).

Network construction and validation

Given the importance of these miRNAs at the crucial intersection of hypoxia, inflammation, TGF-β, and Wnt signaling in PAH, we evaluated the miRNA's broader effects using a network approach. We retrieved 439 experimentally validated gene targets of the 6 validated miRNAs (Table S6). A total number of 71 genes were 'common' genes involved in all 4 major PAH-related pathways (Figure S7). Among them, 39 were 'core' genes, not only involved in all 4 pathways (Figure S8), but also being targeted by all the qRT-PCR verified miRNAs, except for *miR-656* (Figure S9). We further constructed a network consisting of these genes and their direct interactions using IPA. In this network, an edge between two nodes means these two genes have experimentally observed direct interactions in human (Figure 5A). We also constructed 5 separate networks for each of the 5 miRNAs, *let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p*, and *miR-199b-5p* (Figure S10). Gene expression levels of the 12 out of 39 core genes were selected for validation by qRT-PCR and we observed significant upregulation of genes *CDKN1A, ESR1* and *HIF1A* (Figure 5B). Interestingly, expression levels of *ESR1*, which encodes an estrogen receptor alpha, were significantly higher in male IPAH patients than in female patients. However, we did not observe this difference between genders among the controls (Figure S11).

Overexpression of *miR-199b-5p* by hypoxia and negative regulation of *GSK3B* in HPAECs

We observed overexpression of *miR-199b-5p* in the lung of IPAH patients, which was correlated with features of vascular remodeling. Given that it was localized to the endothelium, as suggested by the *in situ* hybridization, we further validated *miR-199b-5p* expression in HPAECs. Expression of mature *miR-199b-5p* was increased 2.29 fold after exposure of the cells to 4% O_2 for 48 hours (p=0.037; Figure 6A), consistent with its substantial contribution to the dysregulated pathophenotype observed in PAH. Through target prediction analyses using miRSystem, *GSK3B*, which encodes the multifunctional kinase GSK3-β, a protein involved in signal transduction from the Wnt/β-catenin pathway, was identified as a putative target gene of miR-199b-5p (Table S3). To determine whether this potential binding can translate into efficient *GSK3B* regulation in HPAECs, we transfected oligonucleotides regulating *miR-199b-5p* expression to test this hypothesis. Interestingly, we found hypoxic HPAECs displayed decreased levels of *GSK3B* (fold change=0.74, p=0.01; Figure 6B, left), as assessed by RT-PCR in HPAECs. Moreover, forced expression of *miR-199b-5p* by mimic displayed a significant downregulation of *GSK3B* expression (fold change=0.66, p=0.024; middle). In contrast, the *miR-199b-5p* inhibitor upregulated *GSK3B* expression (fold change=1.57, p=0.032; right). Together, these data demonstrate the antagonistic function of *GSK3B* on β-catenin signaling is reduced by *miR-199b-5p*, potentially constituting a pathological feed forward mechanism, affecting activation status of downstream positive effectors of β-catenin.

DISCUSSION

This is the first study utilizing miRNA profiling as an unbiased approach to identify dysregulated miRNAs in lung tissue of IPAH patients, providing specific epigenetic information regarding the pathophysiology of PAH.

miRNAs are small non-coding RNAs which regulate gene expression by binding to the 3'-untranslated region (UTR) of gene transcripts and causing mRNA degradation or inhibition of translation [19]. Current miRNA studies in PAH revealed dysregulation of several miRNA families in PAH, including *miR-17/92* cluster [20], *miR-21* [21–23], *miR-130/301* cluster [24,25], *miR-143/145* cluster [26–28], *miR-204* [13,29] and *miR-424/503* cluster [30,31]. Recent reports found that *miR-223* was down-regulated in human PAH lungs, in both the right heart and lungs from rodent models of PH. Downregulation of *miR-223* triggers PARP-1 and insulin-like growth factor 1 receptor (IGF-1R) overexpression, subsequent pathologic DNA damage repair, and increased proliferation [32, 33]. The relationship between miRNAs and target genes *in vivo* is a complex network. Thus far, very few studies looked into the systematic interactions between miRNA and gene signaling pathways. In the present study, we performed pathway enrichment analysis utilizing unbiased miRNA microarray data, and have identified top canonical pathways highly relevant to the pathogenesis of PAH, including the Wnt/β-catenin signaling. Further validation by qRT-PCR confirmed activation of the Wnt/β-catenin pathway in IPAH.

The Wnt/β-catenin pathway is highly conserved among species, regulating stem cell pluripotency and cell fate decisions during development [34]. Wnt/β-catenin pathway activation triggers displacement of GSK-3β from a regulatory APC/Axin/GSK-3β-complex and inhibits degradation of β-catenin. Translocation of β-catenin into the nucleus activates transcription of downstream targets, *e.g.*, cyclin D1, VEGF and survivin. Wnt/β-catenin signaling is essential in vascular development and remodeling [35]. Previous studies have discovered that Wnt pathways, both canonical and noncanonical, are operative in PAH [9,10,36,37]. We observed increased expression of cell membrane receptors (*FZD4, FZD10*), core gene (*CTNNB1*) and downstream targets (*CCND1, VEGFA* and *AXIN2*) in lung tissue from patients with IPAH. These findings argue for the existence of a coordinated regulatory cascade in which the selective upregulation of these miRNAs activates β-catenin via membrane receptors and modulators (*e.g.*, suppression of the GSK-3β inhibition complex), and the induction of downstream positive effectors (*e.g., CCND1* and *VEGFA*), leading to vascular remodeling and poor outcome of PAH.

We validated and confirmed the dysregulation of 5 miRNAs: *let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p* and *miR-656*. The *let-7* miRNA family is the first known human miRNA and is highly conserved across species; its family members are induced by hypoxia and involved in angiogenesis [38]. *let-7f* was downregulated during the development of PAH in both rat chronic hypoxia and MCT models but *let-7a* was reduced only in MCT [39]. *miR-26b-5p* shares the same seed region with *miR-26a*. The expression level of *miR-26a* was decreased in both the MCT-PAH rat model and PAH patients' plasma, and was positively correlated with patients' 6MWD suggesting it could serve as a potential biomarker for PAH [40]. *miR-27b-3p* has the same seed region as *miR-27a*. We confirmed

upregulation of *miR-27b-3p*, which is consistent with previous findings from Courboulin *et al* done on PASMCs [13]. A subsequent study found that overexpression of *miR-27a* promoted human pulmonary artery endothelial cell proliferation, and *miR-27a* and PPARγ repressed each other in hypoxia-induced PH [41]. Upregulation of both *miR-199a-3p* and *miR-199b-5p* was first discovered in a murine RVH model [42].

Among the miRNAs we validated, tissue levels of *miR-199b-5p* had positive correlations with patients' hemodynamics. We further examined target in Wnt/β-catenin signaling with artificial reconstitution of *miR-199b-5p* expression changes. Notably, our data show for the first time that *miR-199b-5p* impairs Wnt/β-catenin signaling in HPAECs through negative regulation of *GSK3B*. However, further experiments are warranted to demonstrate the presence of a functional conserved *miR-199b-5p* seed region in the 3' UTR of *GSK3B*, and *miR-199b-5p* silences *GSK3B* expression via 3′UTR binding.

We further constructed gene networks for the 39 common core genes shared by the 4 major PAH-related pathways and targeted by the 5 relevant validated miRNAs *let-7a-5p, miR-26b-5p, miR-27b-3p, miR-199a-3p, miR-199b-5p*. Interestingly, we found only 3 genes were dysregulated in the qRT-PCR validation of network components (*HIF1A, ESR1* and *CDKN1A*). *HIF1A*, targeted by *miR-199b-5p*, encodes the alpha subunit of hypoxia-inducible factor-1 (HIF-1). Recent evidence shows HIF-1A in smooth muscle contributes to pulmonary vascular remodeling and PH in chronic hypoxia [43]. *ESR1* encodes estrogen receptor alpha which is essential for sexual development and involved in pathological processes of cancers. Rajkumar *et al* discovered upregulation of *ESR1* in patients with PAH [44]. Interestingly, we found the expression level of *ESR1* in male IPAH patients was significantly higher than that in female patients. *CDKN1A* encodes a cyclin-dependent kinase inhibitor. Recent study reveals *CDKN1A* is a direct target of *miR-130a*. Hypoxia has been seen to induce *miR-130a* expression and thus repress *CDKN1A* translation [45]. These findings suggest experimental validation at the post-transcriptional level is necessary to confirm the role(s) of target genes of dysregulated miRNAs.

Surprisingly, some known dysregulated genes in PAH didn't show significant change by qRT-PCR validation in our study, including: *TGFB1, PPARG, NOTCH1* and *TP53*. This may be due to posttranscriptional regulation and modification. Many microRNA exert an effect on translation, we only analyzed the mRNA levels of the genes in our study thus would not have seen protein-level dysregulations.

There remain limitations to our study. First, the microarray screening served solely as a discovery tool. This may explain in part the discrepancy observed between microarray and qRT-PCR validation for *miR-656*. Second, all tissue samples from IPAH patients were isolated post-transplant. Using lung explants, we cannot trace dynamic changes in lung miRNA profiles during PAH initiation and propagation. Third, lung tissue homogenate was used in this study for miRNA profiling and validations of miRNAs and their target genes. miRNA biosynthesis and function are highly regulated and this regulation may be cell type specific. Examination of miRNA levels in other relevant tissue (*e.g.*, right ventricle) and cell-types (PASMCs and lung fibroblasts) is warranted to investigate the consequences of miRNA expression changes that relate to PAH pathobiology. Finally, investigation of

circulating miRNAs is necessary to explore the clinical utility of these miRNAs as novel biomarkers for PAH because lung biopsy is invasive, and particularly risky for patients with PAH.

In summary, this is the first report utilizing miRNA profiling in lung tissue samples from patients with IPAH. We confirmed the dysregulation of 6 miRNAs targeting major PAH-related pathways including Wnt/β-catenin. The wide-ranging activation of Wnt/β-catenin pathway genes in our study substantiates the significance of this pathway in the pathogenesis of PAH and strongly supports further investigation of the Wnt/β-catenin pathway in PAH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Messages

- It is the first miRNA profiling study in lung tissue from end-stage idiopathic PAH.

- We identified dysregulated miRNAs and major pathways (*e.g.*, Wnt signaling) in IPAH.

- Levels of miRNA expression were correlated with hemodynamics and pathological changes.

- We observed aberrant expression of target genes in the Wnt/β-catenin pathway.

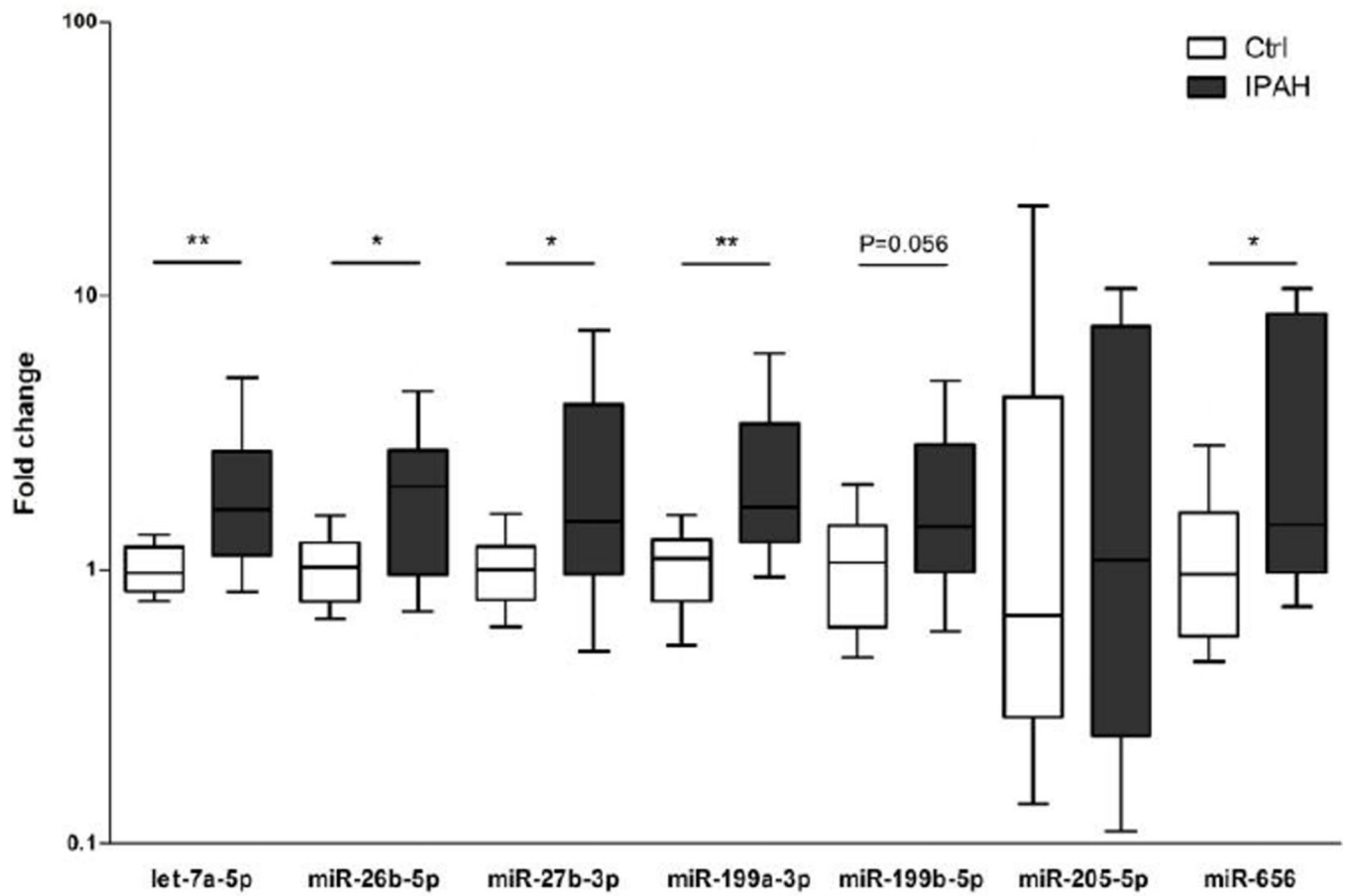- miRNAs influence the pathogenesis of PAH by regulating major PAH related pathways.

Figure 1.
Validation of selected miRNAs in lung tissue samples of IPAH patients and controls by qRT-PCR. Expression levels of *miR-656, let-7a-5p, miR-26b-5p, miR-27b-3p* and *miR-199b-5p* were significantly increased in IPAH patients (N=14) compared with the controls (N=14). In all experiments, miRNA levels were normalized to U6 snRNA. Data are expressed as median and $25^{th}/75^{th}$ (boxes) and $10^{th}/90^{th}$ percentiles (whiskers). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
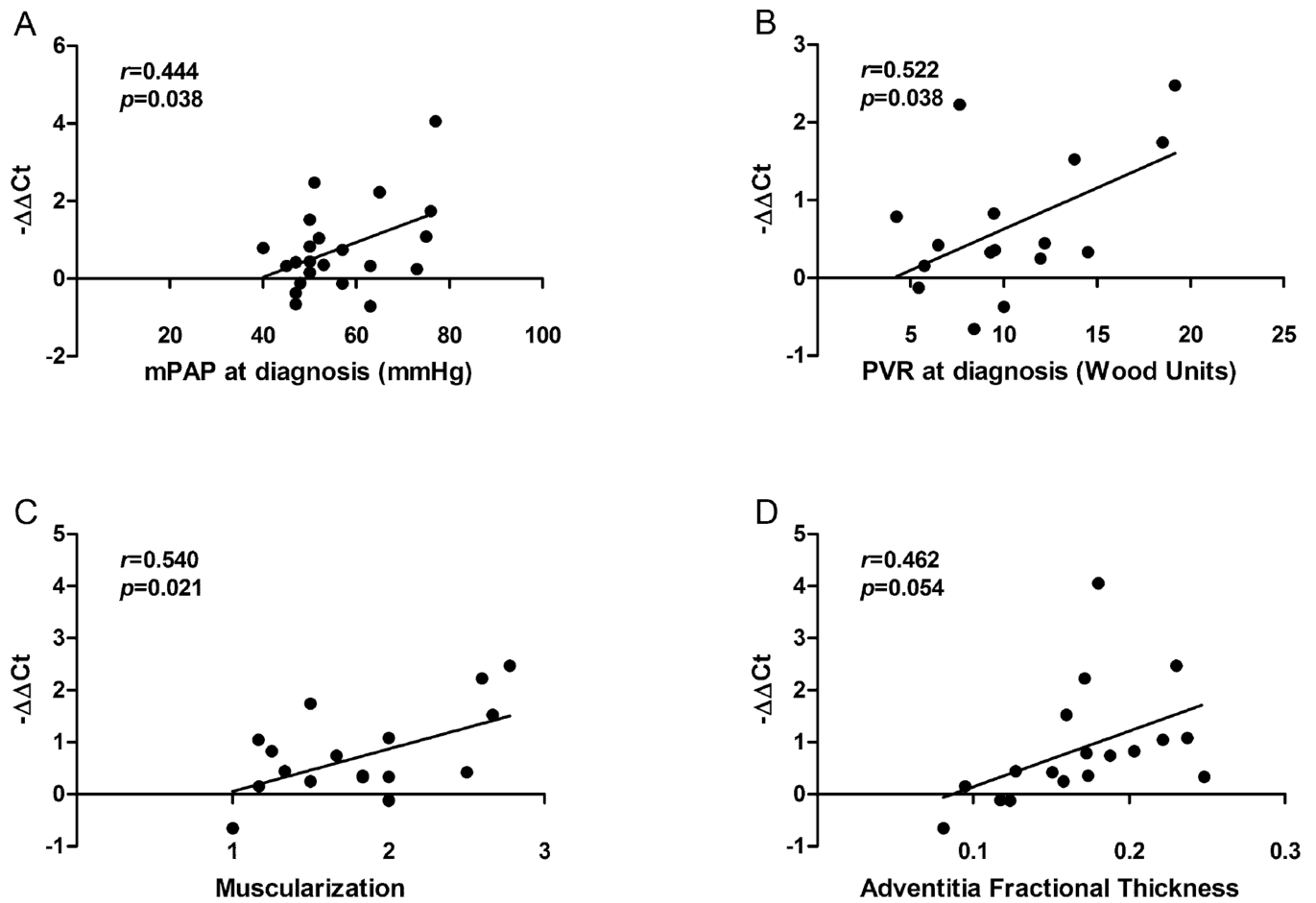
Figure 2.
Pearson correlation between expression levels of miR-199b-5p and (A) mean pulmonary artery pressure (mPAP) at diagnosis (N=22, r=0.444, p=0.038), (B) pulmonary vascular resistance (PVR) at diagnosis (N=16, r=0.522, p=0.038), (C) muscularization (N=18, r=0.540, p=0.021), and (D) adventitia fractional thickness (N=18, r=0.462, p=0.054).
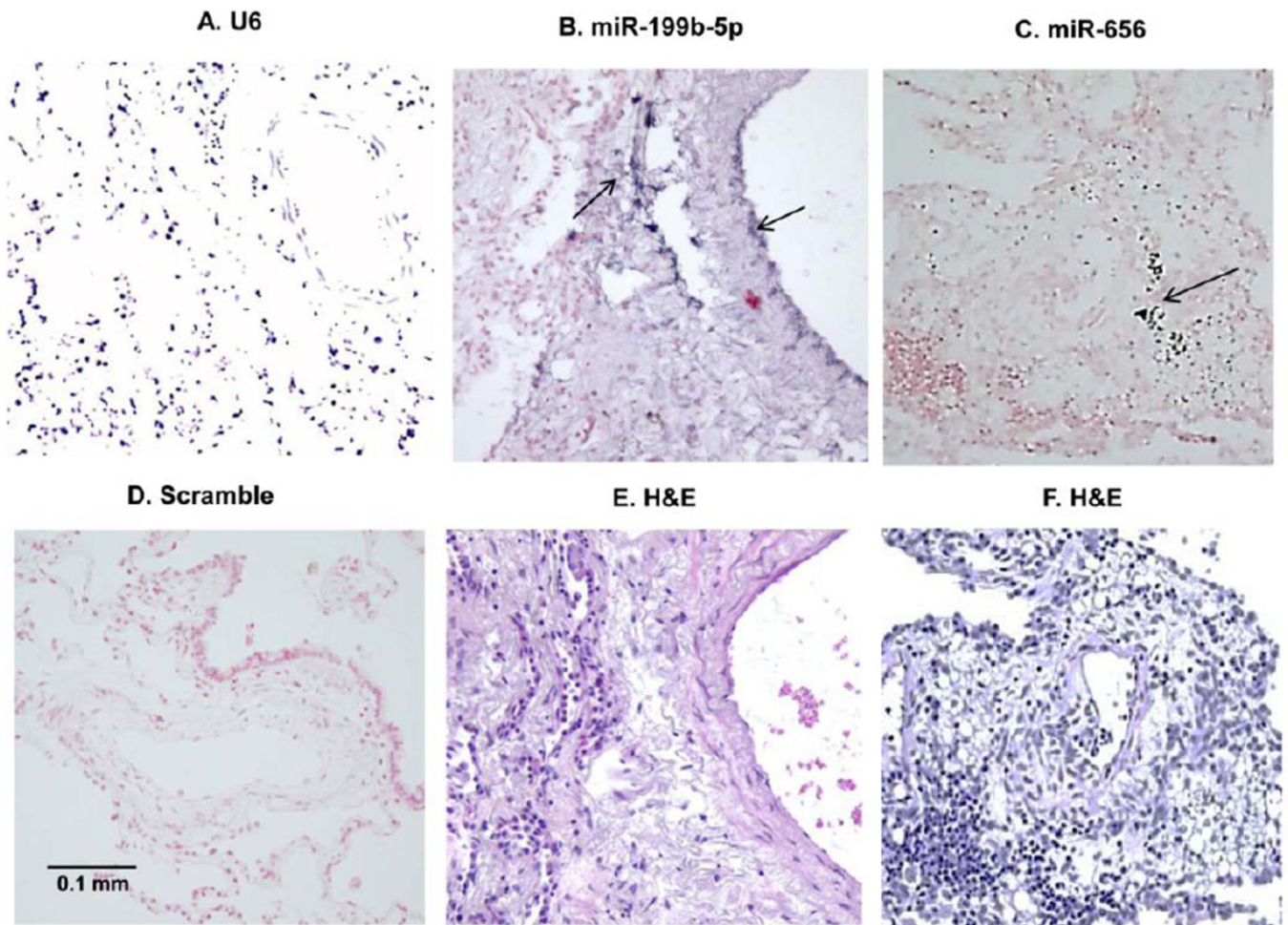
Figure 3.
In situ hybridization of U6 snRNA (A, positive control, to determine ISH sensitivity, control subject was used), *miR-199b-5p* (B) and *miR-656* (C) with DIG-labeled LNA probe (LNA-ISH) in human IPAH lung. The scrambled (negative control, D) and matching H&E staining for *miR-199b-5p* (E) and *miR-656* (F) are also shown. For LNA-ISH, positive staining is dark blue (arrows). Magnification: 20×, using Olympus BX 51 Microscope. Scale bar = 0.1 mm.
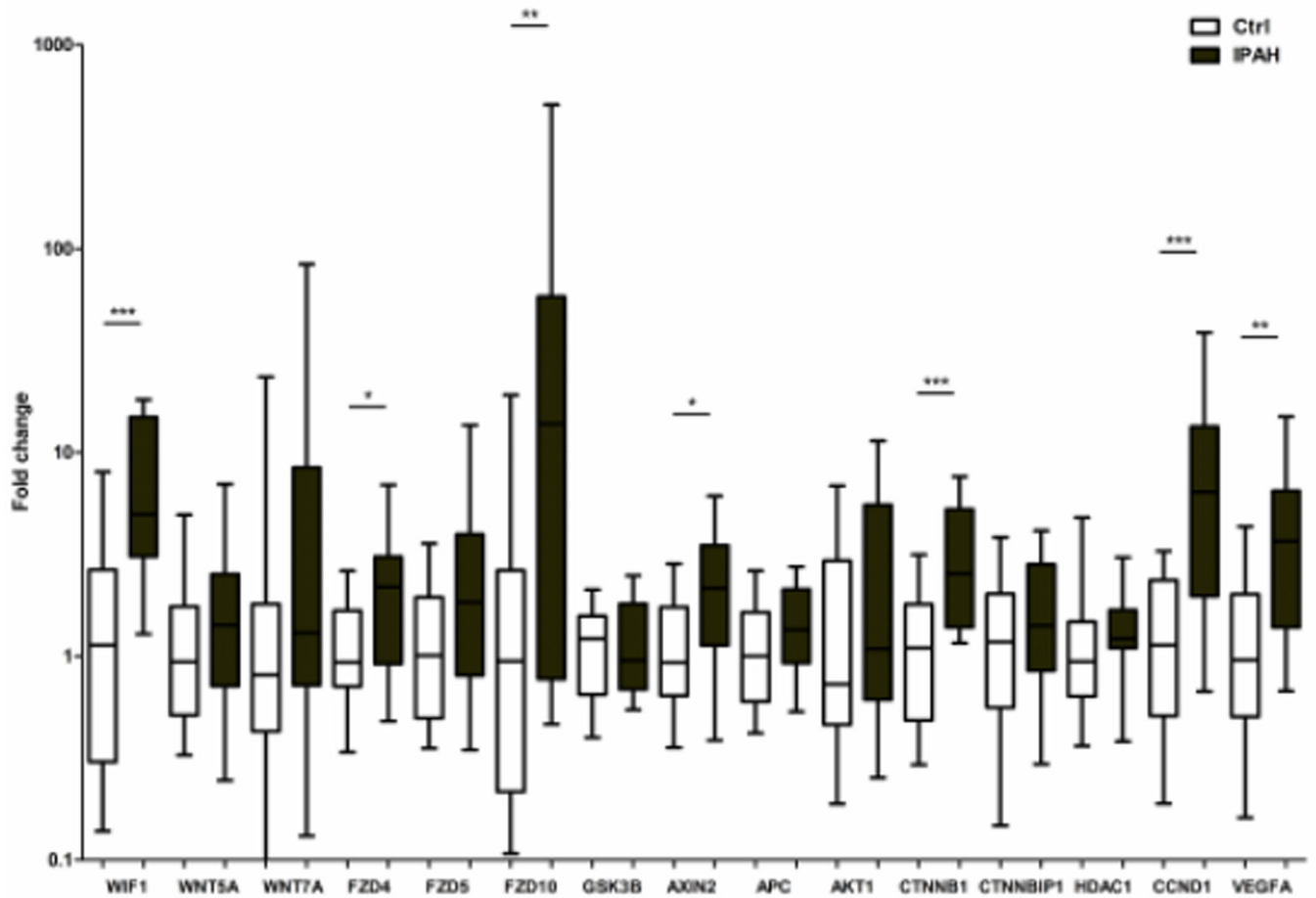
Figure 4.
Evaluation of mRNA expression levels of major Wnt/ β-catenin pathway genes by qRT-PCR.
Expression levels of *WIF1, FZD4, FZD10, AXIN2, CTNNB1, CCND1*, and *VEGFA* were
up-regulated in IPAH patients (N=22) compared with the controls (N=22). In all
experiments, level of miRNAs was normalized to GAPDH. Data are expressed as median
and 25th/75th (boxes) and 10th/90th percentiles (whiskers). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
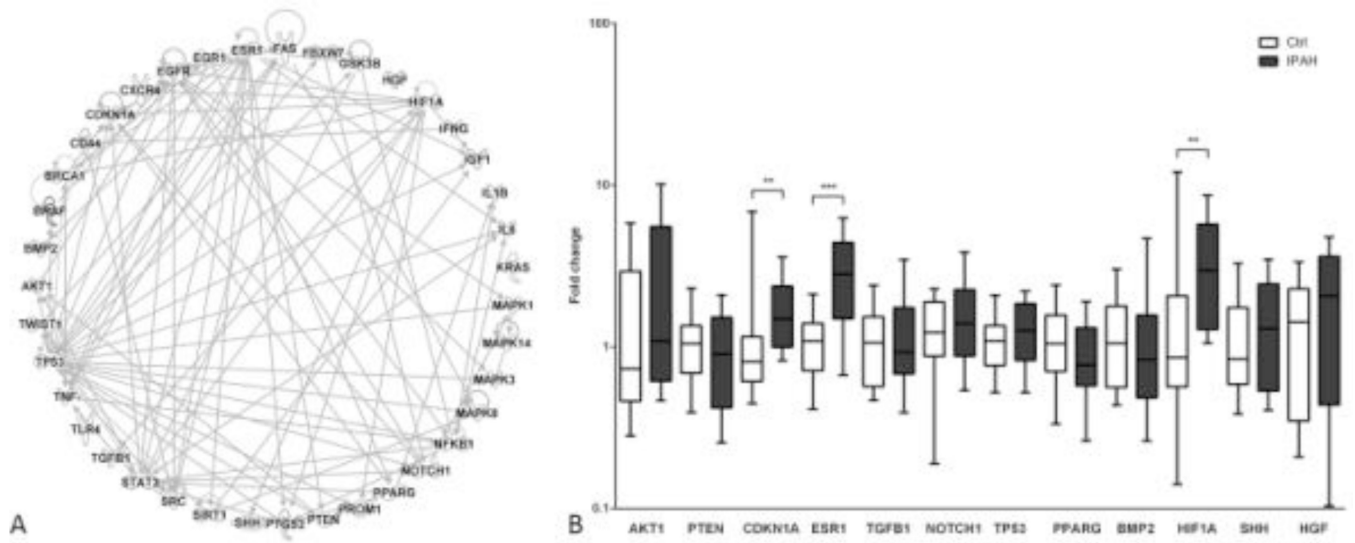
Figure 5.
Network analysis of 39 validated target genes for the 6 miRNAs and the qRT-PCR validation of selected network constituents (gene hubs). (A) Gene-gene interaction network showing experimentally observed direct interactions between gene targets. (B) Expression levels of selected genes of the network. *CDKN1A, ESR1* and *HIF1A* were upregulated in IPAH patients compared to the controls. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
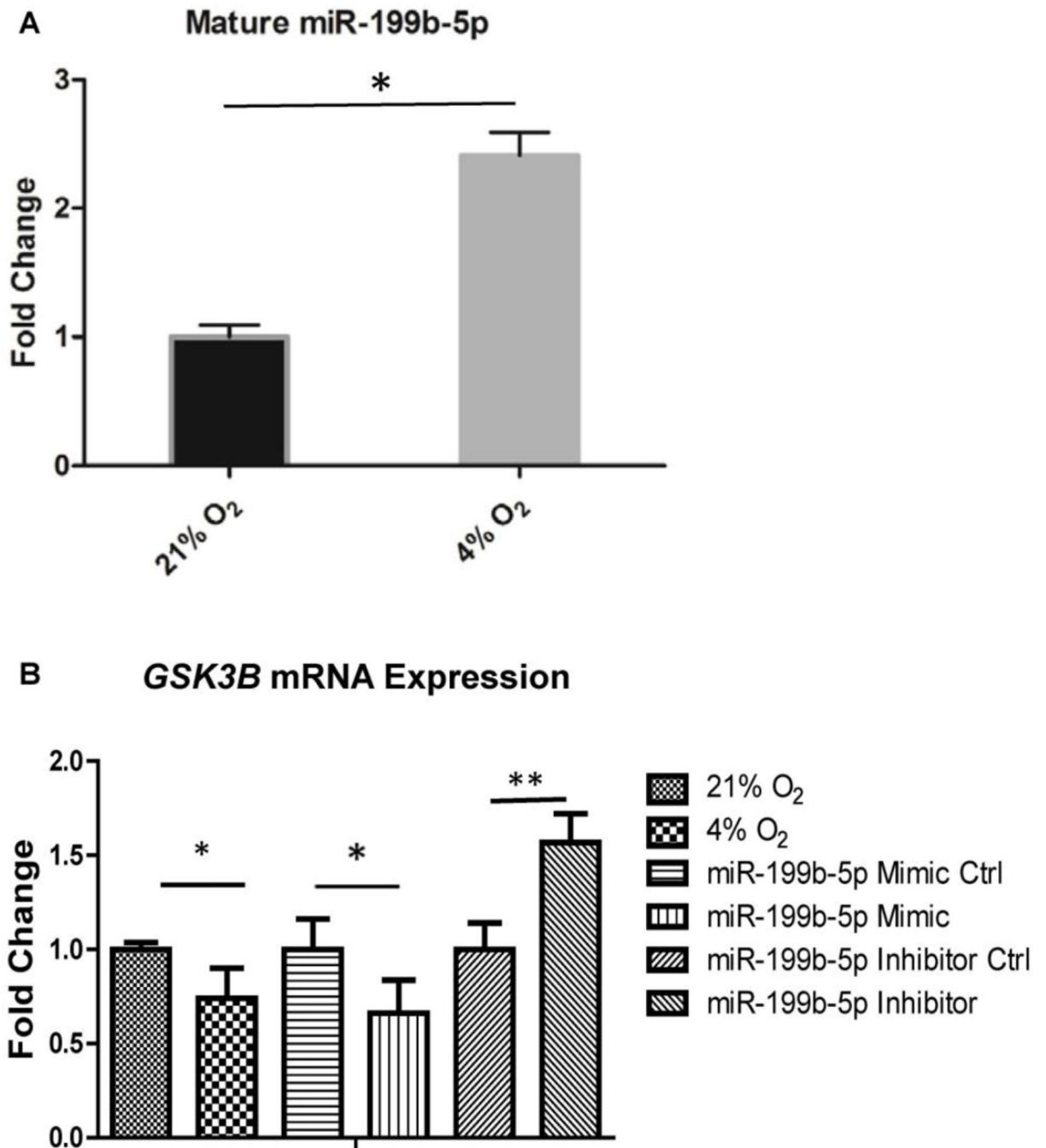
Figure 6.
Assessing the regulation of miR-199b-5p expression and effects on *GSK3B* in cultured HPAECs. (A) miR-199b-5p is upregulated by hypoxia, as measured by reverse transcription polymerase chain reaction (RT-PCR), in human pulmonary arterial endothelial cells (HPAECs). (B) Left, *GSK3B* expression is decreased in HPAECs after chronic exposure to 4% O_2 for 48 hours. Right, inhibition of miR-199b-5p expression by miR-199b-5p inhibitor upregulates *GSK3B* expression. In contrast, forced expression of miR-199b-5p reciprocally downregulated *GSK3B* expression (middle panel). Conditions utilized as control in each

comparison are assigned a fold change of 1, with which other conditions are compared. Error bars reflect SEM. *, $p < 0.05$; **, $p < 0.01$; n 3 in each group.

Table 1

Subject demographics, histopathological parameters and clinical information.

Clinical Variable	Screening cohort		Validation cohort		Entire cohort	
	Control Group (N=8)	IPAH (N=8)	Control Group (N=14)	IPAH (N=14)	Control Group (N=22)	IPAH (N=22)
Age at transplant, year	43.9 (14.8)	43.8 (14.1)	46.7 (11.3)	47.3 (11.7)	45.7 (12.4)	46.0 (12.4)
Gender, n						
Female	2	4	7	11	9	15
Male	6	4	7	3	13	7
Race, n						
White	8	6	13	12	21	18
AA	0	2	1	2	1	4
mRAP, mmHg	NA	16 (6)	NA	14 (6)	NA	15 (6)
PCWP, mmHg	NA	11 (3)	NA	9 (3)	NA	10 (3)
mPAP, mmHg	NA	55 (51, 75)	NA	54 (10)	NA	52 (49, 64)
CO, L/min	NA	3.81 (1.26)	NA	4.67 (1.59)	NA	4.36 (1.51)
PVR, Wood Units	NA	12.08 (9.91, 17.02)	NA	10.96 (4.73)	NA	11.41 (8.02, 15.63)
6MWD, m	NA	307 (76)	NA	369 (76)	NA	347 (81)
WHO FC, I/II/III/IV*	NA	0/0/2/4*	NA	0/1/8/3*	NA	0/1/10/7*
Morphological data						
Intima fractional thickness	0.196 (0.080)	0.307 (0.109)	0.160 (0.070)	0.347 (0.138)	0.176 (0.074)	0.329 (0.124)
Media fractional thickness	0.198 (0.044)	0.241 (0.042)	0.204 (0.044)	0.235 (0.047)	0.201 (0.042)	0.238 (0.044)
Adventitia fractional thickness	0.178 (0.032)	0.167 (0.045)	0.152 (0.043)	0.170 (0.053)	0.164 (0.039)	0.169 (0.048)

Clinical Variable	Screening cohort		Validation cohort		Entire cohort	
	Control Group (N=8)	IPAH (N=8)	Control Group (N=14)	IPAH (N=14)	Control Group (N=22)	IPAH (N=22)
Muscularization	0.17 (0.00, 1.00)	1.83 (1.38, 2.00)	0.00 (0.00, 0.73)	1.75 (1.19, 2.58)	0.00 (0.00, 0.83)	1.83 (1.27, 2.00)
Targeted therapy, n						
Epoprostenol	NA	5	NA	2	NA	7
ERA	NA	4	NA	5	NA	9
PDE-5 inhibitor	NA	6	NA	4	NA	10
Prostacyclin analog	NA	1	NA	2	NA	3
Combined therapy	NA	7	NA	4	NA	11
CCB**	NA	0	NA	0	NA	0
Anticoagulated, n	NA	6	NA	11	NA	17

Data are presented as number, mean (standard deviation) or median (25th percentile, 75th percentile). AA=African American; mRAP=mean right atrial pressure; PCWP= pulmonary capillary wedge pressure; mPAP=mean pulmonary artery pressure; CO=cardiac output; PVR=pulmonary vascular resistance; 6MWD= six-minute walk distance; WHO FC=World Health Organization functional classification; ERA=endothelin receptor antagonist; PDE-5=phosphodiesterase type 5; CCB=calcium channel blocker; NA=not available.

*
WHO FC data is available for 18 IPAH patients

**
CCB for treatment of pulmonary arterial hypertension with positive acute vascular test

Table 2

Area under curve (AUC), significance, cutoff value, sensitivity and specificity of 6 dysregulated miRNAs from ROC analyses.

miRNA	AUC	95% CI	p-value	Cutoff value	Sensitivity	Specificity
let-7a-5p	0.841	0.720–0.962	0.0001	1.385	0.727	0.864
miR-26b-5p	0.736	0.579–0.892	0.0075	1.703	0.545	1.000
miR-27b-3p	0.833	0.706–0.959	0.0002	1.440	0.773	0.864
miR-199a-3p	0.859	0.752–0.966	0.0001	1.426	0.773	0.810
miR-199b-5p	0.706	0.551–0.860	0.0210	1.153	0.727	0.619
miR-656	0.797	0.662–0.931	0.001	1.301	0.789	0.727