

miR-17/20 Controls Prolyl Hydroxylase 2 (PHD2)/Hypoxia-Inducible Factor 1 (HIF1) to Regulate Pulmonary Artery Smooth Muscle Cell Proliferation

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Background—Previously we found that smooth muscle cell (SMC)-specific knockout of miR-17~92 attenuates hypoxia-induced pulmonary hypertension. However, the mechanism underlying miR-17~92-mediated pulmonary artery SMC (PASMC) proliferation remains unclear. We sought to investigate whether miR-17~92 regulates hypoxia-inducible factor (HIF) activity and PASMC proliferation via prolyl hydroxylases (PHDs).

Methods and Results—We show that hypoxic sm-17~92^{-/-} mice have decreased hematocrit, red blood cell counts, and hemoglobin contents. The sm-17~92^{-/-} mouse lungs express decreased mRNA levels of HIF targets and increased levels of PHD2. miR-17~92 inhibitors suppress hypoxia-induced levels of HIF1 α , VEGF, Glut1, HK2, and PDK1 but not HIF2 α in vitro in PASMC. Overexpression of miR-17 in PASMC represses PHD2 expression, whereas miR-17/20a inhibitors induce PHD2 expression. The 3'-UTR of PHD2 contains a functional miR-17/20a seed sequence. Silencing of PHD2 induces HIF1 α and PCNA protein levels, whereas overexpression of PHD2 decreases HIF1 α and cell proliferation. SMC-specific knockout of PHD2 enhances hypoxia-induced vascular remodeling and exacerbates established pulmonary hypertension in mice. PHD2 activator R59949 reverses vessel remodeling in existing hypertensive mice. PHDs are dysregulated in PASMC isolated from pulmonary arterial hypertension patients.

Conclusions—Our results suggest that PHD2 is a direct target of miR-17/20a and that miR-17~92 contributes to PASMC proliferation and polycythemia by suppression of PHD2 and induction of HIF1 α . (*J Am Heart Assoc.* 2016;5:e004510 doi: 10.1161/JAHA.116.004510)

Key Words: hypoxia • hypoxia-inducible factor 1 • miR-17~92 • prolyl hydroxylase 2 • pulmonary artery smooth muscle cell • pulmonary hypertension • pulmonary hypertension • smooth muscle cell

Pulmonary arterial hypertension (PAH) is a complex disease with multiple etiologic factors.^{1,2} Recent studies indicate that microRNAs (miRNAs) are key contributors to the pathogenesis of PAH.³ Previously, we have shown that the microRNA-17~92 cluster (miR-17~92) is a key factor in the development of pulmonary hypertension (PH).⁴ The miR-

17~92 cluster locates at the human chromosome 13 and encodes 6 mature miRNAs (mi-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a) organized in a polycistronic cluster.^{5,6} We have found that human pulmonary artery smooth muscle cells (hPASMC) isolated from idiopathic PAH (IPAH) and associated PAH (APAH) patients expressed lower levels of the miR-17~92 cluster than normal hPASMC.⁴ In both normal hPASMC and mouse PASMC (mPASMC), short-term hypoxia induces miR-17~92, whereas prolonged exposure to hypoxia decreases miR-17~92 levels.⁴ We also reported that SMC-specific knockout of miR-17~92 attenuates hypoxia-induced PH in mice.⁴ These results clearly indicate the clinical relevance of dysregulation of miR-17~92 in PAH. Furthermore, we show that miR-17~92 can induce both PASMC proliferation and differentiation.⁴ Although we have established that miR-17~92 mediates PASMC differentiation by directly targeting PDLIM5 to regulate the TGF- β /Smad2/3 pathway,⁴ the mechanism by which miR-17~92 contributes to PASMC proliferation remains unknown.

Hypoxia is a commonly used stimulus to induce experimental PH in rodents.⁷ During hypoxia, hypoxia-inducible factor

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Accompanying Table S1 and Figures S1 through S7 are available at <http://jaha.ahajournals.org/content/5/12/e004510/DC1/embed/inline-supplementary-material-1.pdf>

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(HIF) is stabilized and activated, and it acts as a master transcription factor that regulates cellular adaptation to hypoxia.^{8,9} HIF is a dimer of the short-lived HIF α and stable HIF β subunits. The stability of HIF1/2 α is regulated posttranslationally by prolyl hydroxylase protein domain proteins (PHDs) and von Hippel-Lindau protein (VHL), which was first identified as a tumor suppressor because of VHL gene mutations in tumors.^{10,11} VHL is a component of a ubiquitin protein ligase (E3) for proteasomal degradation of HIF1/2 α .^{12,13} Under normal conditions, PHDs catalyze the hydroxylation of 2 HIF α prolines (Pro-402 and Pro-564 on HIF1 α). Hydroxylated HIF α is recognized by VHL for proteasome degradation. Hypoxia decreases PHD activity and HIF α hydroxylation, resulting in HIF α stabilization and the transcription of numerous target genes. Another enzyme, FIH (factor inhibiting HIF-1), catalyzes the hydroxylation of HIF1 α at an asparagine residue within the C-terminal transactivation domain, blocking the binding of the transcriptional coactivators CBP and p300.^{14,15} Previous reports suggest that HIF1 α and HIF2 α are essential for the development of hypoxia-induced pulmonary hypertension (PH).¹⁶⁻¹⁸ However, it is not known whether miR-17~92 mediates the hypoxia response and contributes to the development of PH via the HIF pathway.

In this study we found that SMC-specific knockout of miR-17~92 in mice attenuates chronic hypoxia-induced HIF activation. We found that prolyl hydroxylase 2 (PHD2) is inhibited by miR-17~92 and that the 3'-UTR of PHD2 contains a miR-17/20a seed sequence, indicating that miR-17/20a can directly target PHD2 to regulate HIF and hypoxia-induced PH. SMC-specific knockout of PHD2 in mice enhances chronic hypoxia-induced HIF activation and PH. Our study provides the first evidence that a novel pathway of miR-17~92/PHD2/HIF1 is critical for the development of PH.

Methods

Cell Culture

Normal human pulmonary artery smooth muscle cells (hPASMC) were purchased from Lonza (Walkersville, MD). hPASMC from explanted lungs of normal donors and patients with idiopathic PAH (IPAH) or PAH associated with other diseases (APAH) were provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI) as we described previously.⁴ APAH samples include patients with collagen vascular disease/connective tissue disease and congenital systemic-to-pulmonary shunts. hPASMC were maintained in SmGM-2 medium (Lonza) containing 5% fetal bovine serum (FBS), growth factors, and 1% penicillin-streptomycin. hPASMC at passages 5 to 7 were used for the study.

For transfection, hPASMC were plated in 60-mm dishes to reach ~60% to 70% confluence and transfected with 100 pmol

miRVana™ miRNA inhibitors, miRNA mimics, or siRNAs (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen, Grand Island, NY), following the manufacturer's protocol. Six hours after transfection in opti-MEM (Invitrogen), cells were incubated with complete medium for 2 days. Inhibitor negative control 1, mimic negative control 1, and siRNA negative control (siNeg) (Ambion) were used as respective controls.

For overexpression of PHD1 and 2, hPASMC were plated in 100-mm dishes and transfected with 10 μ g pEGFP-N1-PHD1 and pEGFP-N1-PHD2 plasmids (Addgene, Cambridge, MA) using an Amaxa® Nucleofector® II device and an Amaxa® Basic Nucleofector® Kit for Primary Smooth Muscle Cells (Lonza).

For hypoxia exposure, hPASMC were exposed to 1% or 3% hypoxia in an INVIVO₂300 hypoxia chamber (Ruskin, Sanford, ME) for 6 hours on the second day of transfection.

All cells were maintained in a humidified incubator with a constant supply of 5% CO₂ at 37°C.

5-Bromo-2'-Deoxyuridine (BrdU) Proliferation Assay, Cell Viability Assay, and LDH Assay

Cell proliferation activity was determined using BrdU Proliferation Assay (Calbiochem, San Diego, CA) and CellTiter 96® Aqueous One Solution Cell Viability Assay (Promega, Madison, WI), according to the manufacturer's instructions. Cell death was determined using the cytotoxicity detection kit (LDH) (Roche, Indianapolis, IN), according to the manufacturer's instructions. Briefly, hPASMC were plated at 5000 cells/well into 96-well plates. Cells were transfected with siRNAs or plasmids using Lipofectamine 2000 on the next day. Twenty-four hours after transfection, cells transfected with plasmids for overexpression of PHDs were exposed to either 1% hypoxia in an INVIVO₂300 hypoxia chamber (Ruskin) or normoxia for 24 hours. Proliferation assays and LDH assays were performed 48 hours after transfection.

Isolation of Mouse Pulmonary Artery Smooth Muscle Cells

mPASMC were isolated from mouse lungs as we have previously described.⁴ The purity of mPASMC was validated with the coimmunofluorescence staining of SMA, surfactant protein C (type II airway epithelial cell marker), and DAPI (Figure S1).

Quantitative Real-Time Reverse Transcription PCR

Total RNA was isolated using a miRNeasy Mini Kit (Qiagen, Valencia, CA) and treated with an RNase-free DNase set (Qiagen). After quantification with a Nanodrop 2000

spectrophotometer (ThermoScientific, Rockford, IL), total RNA was reverse transcribed using high-capacity reverse transcription kits (Applied Biosystems, Foster City, CA). Ribosomal protein L19 (RPL19) was used as internal control. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a StepOnePlus or ViiA 7 Real-Time PCR System (Applied Biosystems). Primer sequences are provided in Table S1.

Western Blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS) 3 times and lysed in mRIPA buffer (50 mmol/L Tris pH 7.4, 1% NP-40, 0.25% deoxycholate, 150 mmol/L NaCl, and protease inhibitors). After incubation on ice for 30 minutes, the cell lysates were cleared by centrifugation at 13 000g for 10 minutes at 4°C, and protein concentrations of the supernatants were determined using Bio-Rad protein assay solution (Bio-Rad, Hercules, CA). Typically, 20 to 50 µg protein was then separated by SDS-polyacrylamide gel electrophoresis and transferred to BA85 nitrocellulose membrane (PROTRAN, Whatman, Dassel, Germany). Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific). The following primary antibodies were used in this study: HIF1α (Cat# 610959, BD Biosciences, San Jose, CA), HIF2α (Cat#NB100-122), PHD1 (Cat#NB100-310), PHD2 (Cat#NB100-137), PHD3 (Cat#NB100-303) (Novus Biologicals, Littleton, CO), α-tubulin (Cat#T5168), α-smooth muscle actin (SMA)(Cat#A5228), calponin (Cat#C2687) (Sigma-Aldrich, St. Louis, MO), smooth muscle protein 22-α (SM22α) (Cat#ab10135, Abcam, Cambridge, MA), myocardin (Cat#MAB4028, R&D Systems), and proliferating cell nuclear antigen (PCNA) (Cat#10205-2-AP, Proteintech Group, Chicago, IL). Antimouse (Cat#172-1011), antirabbit (Cat#172-1034), and antigoat (Cat#172-1019) IgG-HRP conjugates were purchased from Bio-Rad. The gray density of the protein bands was quantified with ImageJ software.

PHD2 3'-UTR Luciferase Reporter Assay

To construct the luciferase-PHD2 3'-UTR (Wt-luc) reporter plasmid, a 272-bp 3'-UTR of human PHD2 gene containing the predicted miR-17/20a binding site was amplified from human genomic DNA and inserted downstream of the luciferase reporter gene in the pGL3-promoter vector (Promega) through the XbaI endonuclease restriction site. We mutated the predicted miR-17/20a binding site on the Wt-luc reporter plasmid to generate the Mut-luc reporter using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutated sequences are highlighted in Table S1. All constructs were confirmed by DNA sequencing.

hPASMC were plated in 60-mm dishes and cotransfected with 2 µg of either Wt-luc or Mut-luc reporter plasmid, 1 µg Renilla reporter plasmid, and 100 pmol of either miRNA mimics or inhibitors using Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after transfection, the cells were lysed, and the luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) on a GloMax[®]-96 Microplate luminometer (Promega). Relative luciferase activities were calculated by comparing the firefly/renilla luciferase ratio.

miR-17~92 Knockout Mice and smmhc-PHD2 Knockout Mice

We generated a strain of smooth muscle cell (SMC)-specific miR-17~92 knockout (sm-17~92^{-/-}) mice as previously described.⁴ We also created a strain of inducible SMC-specific PHD2 knockout mice by crossbreeding PHD2^{fl/fl} mice (obtained from the Jackson Laboratory) with smmhc-CreER^{T2} mice.¹⁹

To study the role of PHD2 in hypoxia-induced PH, 4-hydroxytamoxifen (4-OHT), which activates Cre recombinase in SMMHC-positive cells, was given by intraperitoneal (IP) injection for 5 consecutive days to achieve knockout of PHD2. Mice injected with corn oil were used as controls. Then, the mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 4 weeks in a Biospherix A chamber (Biospherix, Lacona, NY), and the oxygen concentration (10%) was monitored with a Proox Model P110 oxygen controller (BioSpherix).

To study the role of PHD2 in established hypoxia-induced PH, 8- to 10-week-old smmhc-CreER^{T2}-PHD2^{fl/fl} mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 2 weeks in a Biospherix A-chamber (BioSpherix). Then, 4-OHT was administered for 5 consecutive days to induce the knockout of PHD2. We found that mPASMC isolated from wild-type mice (PHD2^{fl/fl}) and from smmhc-PHD2^{fl/fl} mice given corn oil IP contain similar amounts of PHD2 and HIF downstream genes, suggesting that these mice share the same PHD2 function. From our previous experience we did not find a difference in response to hypoxia between 4-OHT and corn oil-injected wild-type mice. Thus, we chose to inject mice with corn oil as controls. Indeed, we did not observe that 4-OHT itself affects the parameters we were measuring; therefore, PHD2^{fl/fl} and smmhc-PHD2^{fl/fl} mice injected with corn oil are good controls for smmhc-PHD2^{fl/fl} mice injected with 4-OHT in our system. These mice were then exposed to room air or 10% oxygen for another 2 weeks. The oxygen concentration (10%) was monitored with a ProOx Model P110 oxygen controller (BioSpherix).

To investigate the potential use of PHD2 activators in the treatment of PH, we employed the use of R59949 (Sigma-Aldrich, St. Louis, MO), which has been shown to activate PHD2 and inhibit HIF activation.²⁰ R59949 was dissolved in

DMSO and administered intraperitoneally in a volume of 100 μ L at the dose of 0.125 mg/25 g body weight (modified from Dominguez et al²¹). Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 2 weeks in a BioSpherix A chamber to induce PH. Mice were then weighed, and we injected R59949 daily, 5 days a week, for the next 2 weeks while the mice remained in normoxia or hypoxia.

Right-ventricular pressure (RVP) was measured and recorded with a 1.4F pressure transducer catheter (Millar Instruments, Houston, TX) and AcqKnowledge software (Biopac Systems Inc, Goleta, CA). The right-ventricular systolic pressure (RVSP) was used as a surrogate for pulmonary arterial pressure. Blood was drawn to determine the complete blood count (CBC), and hearts were excised and dissected to determine the RV/(LV+S) ratio (right ventricle/[left ventricle+septum]) as a parameter of RV hypertrophy. Lung tissues were fixed, embedded, and sectioned. Slides were stained with hematoxylin and eosin for morphometric analysis to quantify pulmonary arterial wall thickness.⁴ Arterial wall thickness was analyzed by measuring the areas bounded by the external and internal elastic laminae with AxioVision LE software (Zeiss, Vienna, Austria) as follows: (area of external wall–area of inner wall)/area of external wall. All animals were handled according to National Institutes of Health guidelines and the Institutional Animal Care and Use Committee-approved experimental protocols.

Statistical Analysis

All experiments were repeated at least 3 to 5 times independently (mostly 5 experiments), and, for experiments in mice, at least 5 mice were studied in each group except in experiments where we isolated mouse PASMCMC (at least 3 mice in each group), where we used pooled cells for repeating experiments. The Shapiro-Wilk normality test was carried out for the assessment of normality. Two-way ANOVA, 1-way ANOVA, and t tests were performed using GraphPad Prism 4 (GraphPad, San Diego, CA) when applicable. Bonferroni posttests were carried out after 2-way ANOVA and 1-way ANOVA and were only indicated where there was a statistical difference. Data are presented as mean \pm SEM. The significant difference values were set at 0.05 and 0.01.

Results

miR-17~92 Is Required for Hypoxia-Induced HIF Activation

We exposed SMC-specific miR-17~92 knockout mice (sm-17~92^{-/-}) and their wild-type littermates to normoxia or hypoxia (10% oxygen) for 3 weeks and found that knockout of

miR-17~92 in SMC attenuated the hypoxia-induced increase in right ventricular systolic pressure (RVSP) (Figure 1A) and pulmonary arterial wall thickness (Figure 1C), without changing in hypoxia-induced RV hypertrophy (RV/[LV+S] ratio) (Figure 1B), confirming that SMC-specific miR-17~92 is key to the development of hypoxia-induced PH.⁴ HIF is known to contribute to hypoxia-induced polycythemia and PH.^{16–18,22} To investigate the relationship between miR-17~92 and HIF, we measured hematocrits (HCT), red blood cell (RBC) counts, and hemoglobin (HGB) contents in these mice. We found that sm-17~92^{-/-} mice had an attenuated increase in HCT, RBC counts, and HGB contents under hypoxia, indicating a decreased HIF activity in sm-17~92^{-/-} mice (Figure 1D through 1F). We also found that the mRNA expression levels of HIF targets, such as VEGF, and PGK-1, were lower in the whole lung tissues of sm-17~92^{-/-} mice than in those of their wild-type littermates (Figure 1G). However, mRNA levels of HIF upstream regulators VHL, PHD1, and PHD3 remained unchanged, whereas PHD2 mRNA levels were higher in the whole lung tissues of sm-17~92^{-/-} mice than in those of their wild-type littermates (Figure 1G), further justifying a decreased HIF activity in sm-17~92^{-/-} mice. In normal hPASMCMC, miR-17~92 inhibitors decreased the stabilization of HIF1 α but not that of HIF2 α (Figure 1H) and attenuated hypoxia-induced increase in VEGF (Figure 1I).

Increasing evidence indicates a metabolic abnormality with a shift to increased aerobic glycolysis during the pathogenesis of PAH.^{23,24} HIF1 α activation in PASMCMC is likely to induce a metabolic remodeling associated with increase glycolysis and suppressed mitochondrial glucose oxidation (GO).²⁵ Therefore, we measured HIF1 α downstream targets implicated in metabolism and subsequent proliferation and apoptosis resistance in PASMCMC, for example, glucose transporter 1 (Glut1), the glycolytic enzyme hexokinase II (HKII), and the inhibitor of mitochondrial GO, pyruvate dehydrogenase kinase 1 (PDK1). We also found that these HIF targets were downregulated in hPASMCMC treated with miR-17~92 inhibitors (Figure 1J through 1L). Taken together, these results suggest that miR-17~92 contributes to the activation of HIF1 α during hypoxia.

miR-17~92 Directly Suppresses PHD2 via its 3'-UTR

HIF consists of an α and a β subunit, and its activity is primarily regulated by the stabilization of α subunits under low-O₂ conditions.^{8,26} In normoxia, prolyl hydroxylases (PHDs) induce prolyl hydroxylation of HIF α , which is then targeted by von Hippel-Lindau protein (VHL) for degradation in proteasomes.¹⁷ Because HIF activity was reduced in sm-17~92^{-/-} mice (Figure 1), we speculated that miR-17~92 may suppress PHD or VHL to increase HIF activity. However, we found that

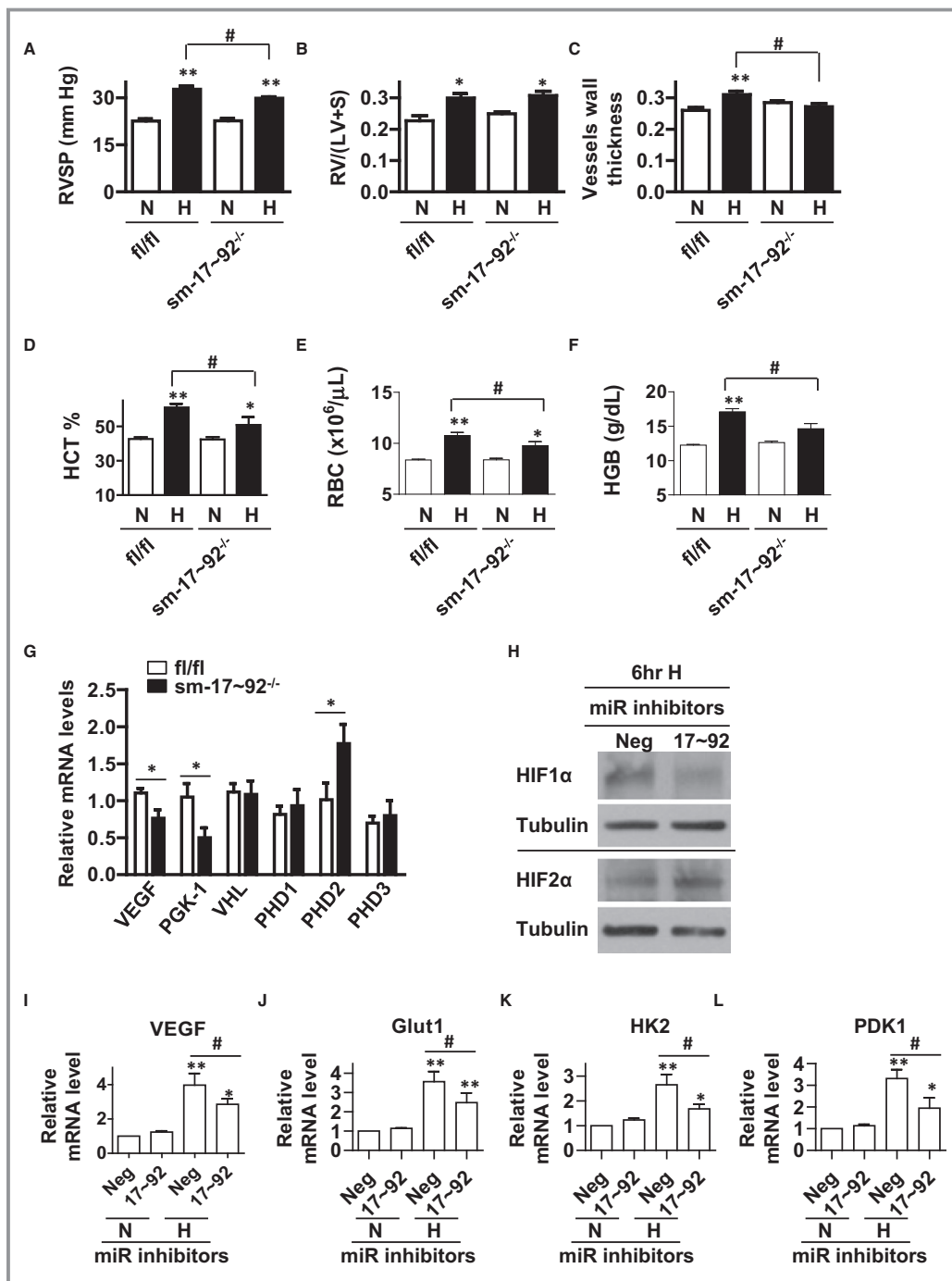


Figure 1. Loss of miR-17-92 decreases HIF activity in mice and PASM. A through F, miR-17-92^{fl/fl} (fl/fl) and sm-17-92^{-/-} mice were exposed to normoxia (N) and hypoxia (H) for 3 weeks. We calculated RVSP based on the measured RVP (A) and determined RV/(LV+S) ratio (B) and pulmonary arterial wall thickness (C). Mouse blood samples were collected, and hematocrit (HCT) (D), red blood cell (RBC) numbers (E), and hemoglobin (HGB) levels (F) were measured. n=5, 5, 8, 5. Data are presented as mean±SEM. *P<0.05; #P<0.05; **P<0.01. There were at least 5 mice in each group. G, mRNA levels of described genes in lung samples of miR-17-92^{fl/fl} (fl/fl) and sm-17-92^{-/-} mice: n=6 for each group. H through L, hPASM were treated with miR inhibitors or control miR (Neg) followed by exposure to normoxia (N) and hypoxia (H) for 6 hours. Protein levels of HIF1α and HIF2α were measured (H). mRNA levels of VEGF (I), Glut1 (J), HK2 (K), and PDK1 (L) were determined after 24 hours of exposure to hypoxia. n=8, 8, 4, 6. HIF indicates hypoxia-inducible factor; PASM, pulmonary artery smooth muscle cell; RVP, right-ventricular pressure; RVSP, right-ventricular systolic pressure. *P<0.05; #P<0.05; **P<0.05. Data are presented as mean±SEM.

the mRNA levels of PHD2, but not VHL, PHD1, or PHD3, were significantly higher in the whole lung tissues of *sm-17~92^{-/-}* mice than in their wild-type littermates (Figure 1G). This suggests that miR-17~92 may target PHD2 to control HIF activity in PASM. Among 6 mature miRNAs in the miR-17~92 cluster, miR-17 and miR-20a are identical except for 2 nonseed nucleotides and often share common targets.⁶ We searched in TargetScan and found that, among the 3 PHDs and VHL, only PHD2 and PHD3 contain a putative binding site for miR-17 and miR-20a in their 3'-UTRs (Figure 2A). Other members of miR-17~92 do not contain seed sequences for PHDs and VHL. Furthermore, overexpression of miR-17~92 inhibited PHD2 protein levels, had little effect on the PHD3 protein levels, but induced PHD1 protein levels in hPASM (Figure 2B). Consistently, loss of miR-17~92 increased PHD2

in mPASM (Figure 2C). These results suggest that PHD2 may be a direct target of the miR-17~92 cluster, particularly miR-17 and miR-20a.

In order to address whether miR-17 or miR-20a is the main regulator of PHD2 in this miRNA cluster, we transfected hPASM individually with miR-17 or miR-20a mimics and measured the expression levels of PHD1, PHD2, and PHD3 proteins. As shown in Figure 2D through 2F, overexpression of miR-17 and miR-20a did not change the protein levels of PHD1 and PHD3, whereas overexpression of miR-17 but not miR-20a was sufficient to inhibit PHD2 protein levels. Inhibition of PHD2 by miR-17 alone was moderate (Figure 2E). miR-20a mimic transfection had a trend to reduce PHD2 levels without significance (Figure 2E). Given the similarities between miR-17 and miR-20a seed sequences, we examined

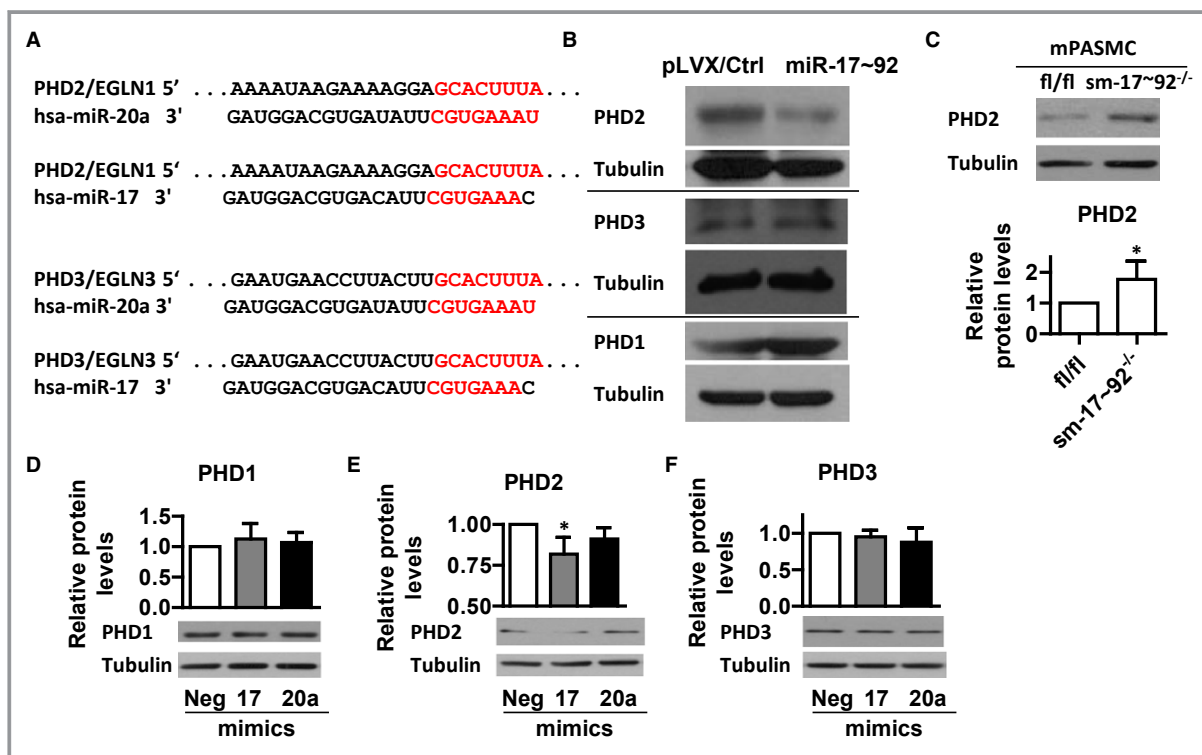


Figure 2. miR-17~92 directly targets PHD2. A, The putative binding site of miR-17 and miR-20a in the 3'-UTR of PHD2 and PHD3. B, The expression levels of PHD1, PHD2, and PHD3 proteins were determined in cell lysates of normal hPASM infected with control lentiviruses (pLVX/Ctrl) or lentiviruses encoding the whole cluster of miR-17~92 (miR-17~92). Tubulin was used as the internal control. C, We determined the protein levels of PHD2 in freshly isolated mPASM from miR-17~92^{fl/fl} and *sm-17~92^{-/-}* mice. In each strain mPASM from at least 3 mice were subjected to Western blotting. Representative blots are shown in the top panel and quantification in the bottom panel. D through F, Normal hPASM were treated with miR-17 and miR-20a mimics as well as negative control (Neg) and harvested 48 hours after transfection to determine the levels of PHD1 (D), PHD2 (E), and PHD3 (F). The amount of tubulin was used as loading control. n=3. G through I, Normal hPASM were treated with miR-17 and miR-20a inhibitors or negative control (Neg) and harvested 48 hours after transfection to determine the levels of PHD1 (G), PHD2 (H), and PHD3 (I). The amount of tubulin was used as loading control. n=5. J, Diagram of the wild-type (Wt-Luc) and mutated (Mut-Luc) 3'-UTR of PHD2 luciferase reporter constructs. Open triangle represents the binding site of miR-17/20a, and the solid triangle represents the mutated miR-17/20a binding site. K through N, Normal hPASM were cotransfected with the Wt-Luc reporter (K and L) or Mut-Luc (M and N) and inhibitors (K and M) or mimics (L and N) of miR-17 and miR-20a. n=3 for C through I, 9 for K and L, 5 for M, 4 for N. hPASM indicates human pulmonary artery smooth muscle cell; mPASM, mouse pulmonary artery smooth muscle cell; PHD, prolyl hydroxylase. **P*<0.05; ***P*<0.01. Data are presented as mean±SEM.

the combined effect of miR-17/miR-20a on PHD protein levels. We found that a mixture of miR-17/20a inhibitors induced protein levels of PHD2 (Figure 2H) but not PHD1 (Figure 2G) or PHD3 (Figure 2I). To determine whether miR-17/20a directly binds to the 3'-UTR of PHD2, we constructed a luciferase reporter containing a 272-bp 3'-UTR of PHD2/EGLN1 (3'-UTR full length 2660 bp, miR-17/20 site 562-569) into pGL3-promoter vector downstream of the luciferase gene (Figure 2J, Wt-Luc). A mutant construct was also generated with a mutation of the putative miR-17/20a site (Figure 2J, Mut-Luc). The miR-17/20a inhibitors and mimics increased or suppressed the luciferase reporter activity of Wt-Luc (Figure 2K through 2N), respectively, whereas miR-17/20a inhibitors and mimics did not change the luciferase reporter activity of the mutated construct Mut-Luc (Figure 2K through 2N). These results indicate that miR-17/20a directly targets and suppresses PHD2 in PASC in normal conditions. In previous reports,^{27,28} hypoxia is known to induce PHD2. To address whether miR-17/20a is able to inhibit PHD2 in hypoxic condition, we transfected hPASC with miR-17/20a mimics and exposed them to both normoxia and hypoxia (1% O₂). We found that in spite of hypoxia-mediated induction of PHD2 mRNA levels, miR-17/20a mimics inhibited the PHD2 mRNA levels in both normoxic and hypoxic hPASC

(Figure S2A). The miR-17/20a mimics did not alter PHD1 mRNA levels in either condition, but increased hypoxia-induced PHD3 mRNA levels (Figure S2B and S2C). More importantly, we found that in the hypoxic condition, miR-17/20a mimics or inhibitors repressed or induced PHD2 protein levels, respectively (Figure S2D and S2E). These results indicate that the direct targeting of PHD2 by miR-17/20a is independent of oxygen levels and further confirm that PHD3 is not a direct target of miR-17/20a. Together with the results in Figure 1 that miR-17~92 regulates HIF1 activity and the fact that PHD2 controls HIF1 activity, we speculate that miR-17~92 mainly regulates HIF1 activity via miR-17/20a-mediated suppression of PHD2.

Both miR-106a/363 and miR-106b/25 are paralogues of the miR-17~92 cluster, and individual miRNAs in these 2 clusters share similar or identical seed sequences with the members of the miR-17~92 cluster,²⁹ suggesting a potential involvement of these 2 clusters in the regulation of PHD2. Because hypoxia is known to regulate miR-17~92 in a biphasic manner,⁴ we investigated the expression levels of individual miRNAs of miR-106a/363 and miR-106b/25 in response to hypoxia. We exposed hPASC to hypoxia for 6 and 24 hours and measured the expression levels of 7 miRNAs in miR-106a/363 and miR-106b/25. As shown in

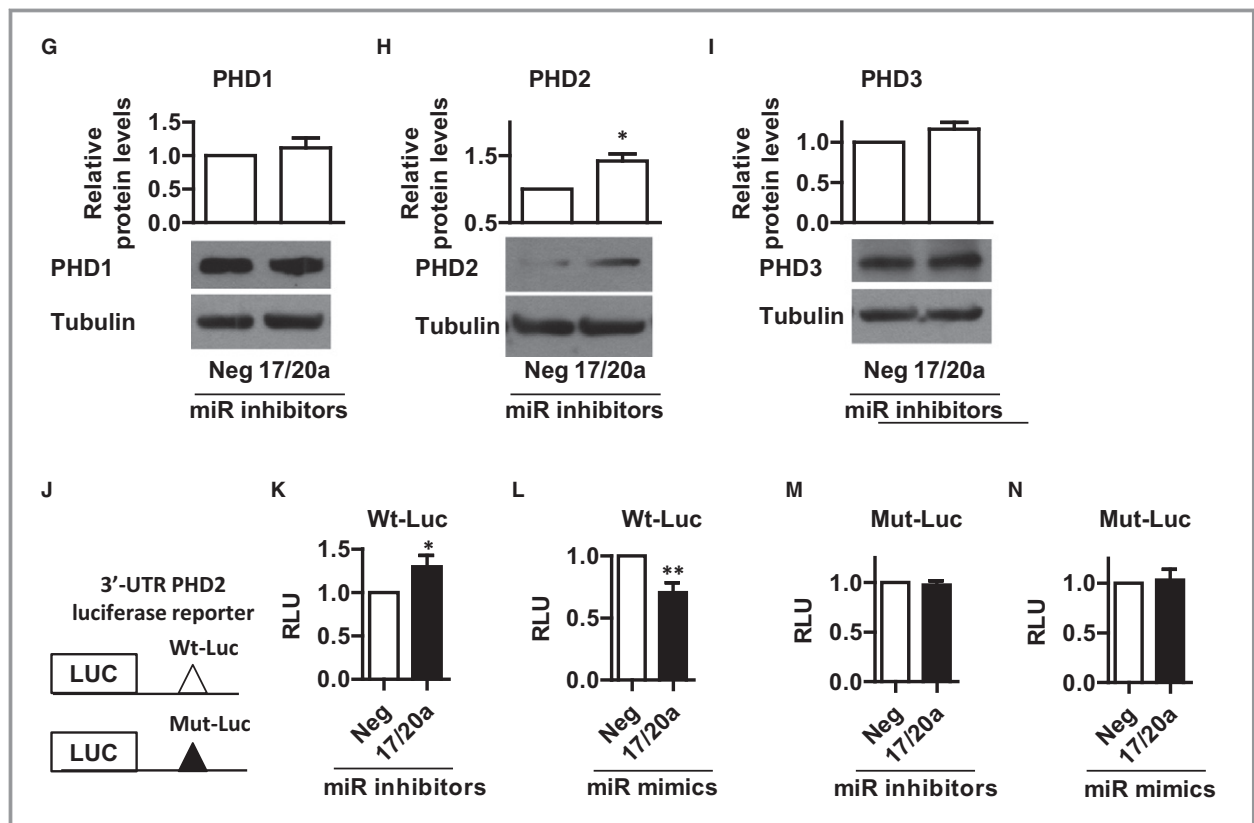


Figure 2. Continued

Figure S3, both short-term and prolonged hypoxic exposure did not appear to affect expression of these miRNAs except for a transient downregulation of miR-363-3p after 6 hours of exposure to hypoxia. MiR-363-3p belongs to the miR-92 family and does not contain a seed sequence that can bind to the 3'-UTR of PHD2. Therefore, these results demonstrate the specificity of the regulation of PHD2 by miR-17/20a during hypoxia.

Loss of PHD2 Induces HIF1 Activity in PASC

To examine the consequences of regulation of PHDs on HIF activity in hPASC, we suppressed expression of the PHDs individually or all together using siRNAs. As shown in Figure S4A, siPHDs specifically and efficiently inhibited their cognate PHD protein expression. Importantly, we found that suppression of PHD2 was most efficient in inducing HIF1 α but not HIF2 α , whereas suppression of PHD1 and PHD3 induced HIF2 α (Figure S4A). None of the PHD siRNAs affected VHL mRNA levels (Figure S4B). These results suggest an isoform-specific PHD2/HIF1 α axis in PASC. Furthermore, we found that VEGF, a known downstream target of HIF, was upregulated by suppression of PHDs, especially suppression of PHD2 (Figure S4C). We also found that suppression of PHD2 and PHD1, especially suppression of PHD2, induced expression of HIF1 downstream targets Glut1, HK2, and PDK1, which are implicated in the metabolic shift in response to hypoxia and PAH (Figure S4D through S4F).

Loss of PHD2 Induces PASC Proliferation

We then investigated the roles of PHDs in the PASC phenotype. We found that suppression of any PHD induced the expression levels of PCNA, a proliferation cell marker, but not the levels of SMC markers SMA, calponin, SM22 α , or myocardin (Figure S5A through S5F), suggesting that suppression of PHDs induces PASC proliferation but not differentiation. We further performed a viability assay, a BrdU incorporation assay, and an LDH assay (a measurement of cell death). We found that although suppression of PHDs ubiquitously induced PASC viability to a similar extent (Figure S5G), suppression of PHDs induced PASC proliferation in varying degrees, with the suppression of PHD1 exerting the highest induction of cell proliferation (Figure S5H). However, suppression of PHD2 had little effect on LDH activity, whereas suppression of PHD1 and PHD3 decreased LDH activity (Figure S5I). These results suggest that there are isoform-specific functions of PHDs: PHD2 promotes PASC viability through induction of cell proliferation, whereas PHD1 and PHD3 promote PASC viability via both increased cell proliferation and decreased cell death. Suppression of PHD1 increased BrdU incorporation but not PCNA expression

(Figure S5B and S5H), suggesting that loss-of-PHD1-mediated DNA replication is PCNA independent.^{30,31} This observation again supports the above-mentioned isoform-specific functions of PHDs.

Previously we have shown that miR-17~92 can induce PASC proliferation and SMC contractile protein expression.⁴ Knowing that miR-17/20a directly targets and suppresses PHD2, we examined whether the miR-17~92-induced PASC proliferation is PHD2 dependent. We simultaneously transfected hPASC with miR-17~92 inhibitors and siRNA against PHDs and exposed these cell to normoxia or hypoxia (1% O₂). We found that in both normoxic and hypoxic conditions, silencing of PHD2 restored or even enhanced hPASC proliferation that was inhibited by miR-17~92 inhibitors (Figure S6A and S6B). Silencing of PHD1 rescued hPASC proliferation in normoxia but not hypoxia, whereas silencing of PHD3 had little effect on miR-17~92-inhibitors-mediated inhibition of PASC proliferation. More importantly, only silencing of PHD2 restored the hypoxia-mediated induction of PASC proliferation (Figure S6C). These results indicate that PHD2 is essential for miR-17~92-mediated PASC phenotype.

Because suppression of PHDs induces HIF activity (Figure S4), we next investigated whether induction of HIF activity alone is sufficient to induce PASC proliferation. We used CoCl₂ and DMOG, which are known to induce HIF under normoxic conditions, as we have published previously,^{32,33} and found that activation of HIF alone was sufficient to induce PASC proliferation in normoxia (Figure S5J). Another approach to stabilize HIF α is to saturate pVHL binding by an exogenous peptide containing HIF1 α -ODDD-wt (amino acids from 531 to 575). HIF1-ODDD-mut, which has a mutation of proline 564 to alanine, has no significant effect on stabilization of endogenous HIF α .³² We used adenovirus encoding HIF-ODDD-wt to infect hPASC at MOI=1:100 according to previous reports. The HIF-ODDD-mut was used as a negative control. We found that overexpression of HIF-ODDD-wt had a higher BrdU incorporation rate than HIF-ODDD-mut (Figure S5K). Together these results suggest that loss of PHD2 leads to HIF-dependent PASC proliferation.

Overexpression of PHD2 Inhibits HIF Activity

In order to establish the biological consequences of the gain of function of PHD2 in PASC, we overexpressed PHD1 and PHD2 with the transfection of pEGFP-N1-PHD1 and pEGFP-N1-PHD2 plasmids, respectively (Figure 3A). We found that overexpression of either PHD1 or PHD2 slightly decreased HIF1 α but not HIF2 α in normoxia (Figure 3A); however, the mRNA levels of VEGF and endothelin-1 (ENDO-1) were unchanged (Figure 3B and 3C). In addition, overexpression of PHDs did not affect VHL mRNA levels (Figure 3D). Overexpression of PHD2 also decreased expression levels of PCNA and induced SMA levels

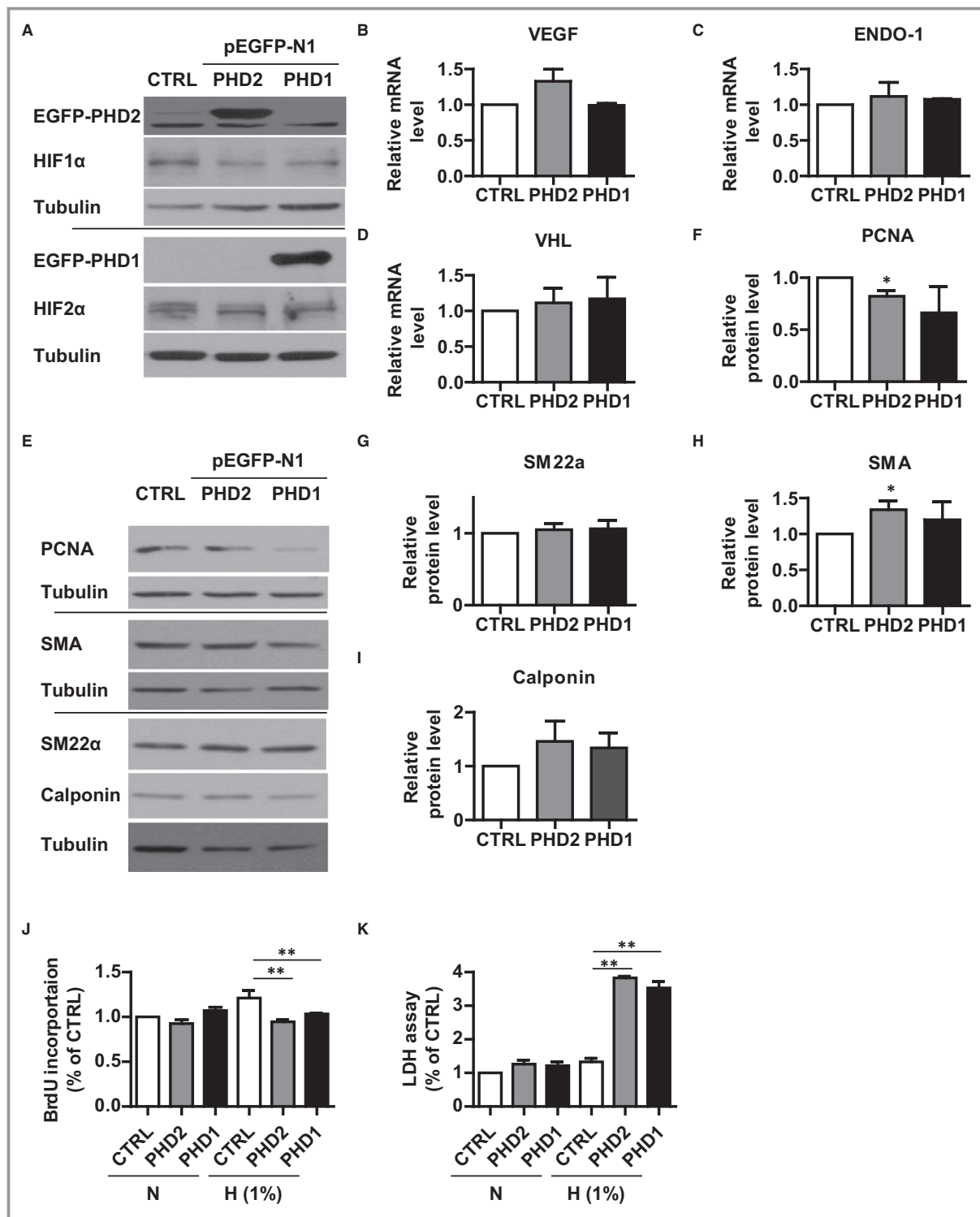


Figure 3. Overexpression of PHD2 inhibits HIF activity and cell proliferation in PASMC. A through J, hPASMC were transfected with a control plasmid, pEGFP-N1-PHD2, or pEGFP-N1-PHD1. Exogenous PHD2 or PHD1 and endogenous HIF1 α and HIF2 α levels were detected by Western blotting (A). mRNA levels of VEGF (B), ENDO-1 (C), and VHL (D) were determined by qRT-PCR. n=6. Protein levels of PCNA, SMA, SM22 α , and calponin were measured by Western blotting (E). The quantification of these proteins is shown (F through I). n=4. J and K, hPASMC transfected with a control plasmid, pEGFP-N1-PHD2, or pEGFP-N1-PHD1 were exposed to normoxia or hypoxia (1% oxygen) and were subsequently subjected to the BrdU incorporation assay (J) and LDH assay (K). n=6. Data are presented as mean \pm SEM. HIF indicates hypoxia-inducible factor; PASMC, pulmonary artery smooth muscle cell; PHD, prolyl hydroxylase. * P <0.05; ** P <0.01.

without affecting expression levels of SM22 α and calponin (Figure 3E through 3I). On the other hand, overexpression of PHD1 did not alter expression levels of PCNA, SMA, SM22 α , or calponin (Figure 3E through 3I). Although overexpression of PHD1 or PHD2 had no effect on BrdU incorporation and LDH release under normoxic conditions, it decreased BrdU incorporation and increased LDH release under hypoxic conditions (Figure 3J and 3K). These results suggest that overexpression of PHD2 inhibits PASMC proliferation and enhances cell death via inhibition of HIF activity.

SMC-Specific Knockout of PHD2 Enhances Hypoxia-Induced Pulmonary Vessel Remodeling

To investigate the biological function of PHD2 *in vivo*, we crossed SMMHC-CreER^{T2} mice in which Cre expression is induced by tamoxifen¹⁹ with STOCK EglN1tm1Kael/J mice (STOCK EglN1tm1Kael/J mice possess loxP sites on either side of exons 2 and 3 of the PHD2/EglN1 gene, PHD2^{fl/fl}) to generate inducible SMC-specific PHD2 knockout mice (smmhc-PHD2^{fl/fl}). The genotypes of these mice were confirmed as shown in Figure 4A. To confirm the deletion of PHD2 in SMC, we isolated mPASC from wild-type mice (PHD2^{fl/fl}) and *smmhc-PHD2^{fl/fl}* mice given corn oil IP or 4-OHT IP for 5 consecutive days. For *smmhc-PHD2^{fl/fl}* mice given 4-OHT, we isolated mPASC immediately (0W) or 1 week (1W) after the final injection of 4-OHT. Deletion of PHD2 in mPASC was validated in the freshly isolated mPASC from these mice (Figure 4B). Deletion of PHD2 can induce HIF activity, leading to the upregulation of HIF targets.^{8,26} To further confirm the deletion of PHD2 and activation of HIF in mPASC, we measured the mRNA levels of VEGF, PGK-1, and VHL^{8,26,27,32,34} and of CXCL12 (not a HIF1 target). We found that deletion of PHD2 induced mRNA levels of VEGF, PGK-1, and VHL immediately after the final injection of 4-OHT (Figure 4C). Deletion of PHD2 did not alter CXCL12 mRNA levels (Figure 4C). We also found that deletion of PHD2 in PASMC induced mRNA levels of Glut1, HK2, and PDK1 (Figure 4C). These results suggest that PHD2 is effectively deleted in PASMC of *smmhc-PHD2^{fl/fl}* mice and that HIF is activated.

To determine the effects of SMC-specific knockout of PHD2 in hypoxia-induced PH, we treated *smmhc-PHD2^{fl/fl}* mice with 4-OHT for 5 consecutive days to delete PHD2 in SMMHC-positive cells. PHD2^{fl/fl} mice were given corn oil at the same frequency and volume as controls. Mice were then exposed to hypoxia (10% oxygen) or normoxia for 4 weeks, and RVSP, RV hypertrophy, and pulmonary arterial wall thickness were measured. As shown in Figure 4D, mice with SMC-specific PHD2 knockout had a slightly higher (not statistically significant) increase in RVSP than the control mice in both normoxia and hypoxia. Loss of PHD2 in SMC resulted in a decrease in hypoxia-induced RV hypertrophy

(Figure 4E) but significantly increased pulmonary arterial remodeling in hypoxia (Figure 4F and 4G). These results suggest that PHD2 is predominantly an inhibitor of hypoxia-induced pulmonary arterial remodeling.

SMC-Specific Knockout of PHD2 Exacerbates Elevation of RVSP in Existing Hypertension in Mice

To further address the role of PHD2 in hypoxia-induced PH, we exposed mice to hypoxia (10% oxygen) for 14 days to induce PH, then gave *smmhc-PHD2^{fl/fl}* mice 4-OHT or corn oil IP for 5 consecutive days to delete PHD2 in SMMHC-positive cells while they were in hypoxia or normoxia and then maintained the mice in hypoxia or normoxia for 16 days after the last injection of 4-OHT. We measured RVSP, RV hypertrophy, and pulmonary arterial wall thickness in these mice. We found that deletion of SMC-specific PHD2 after the establishment of hypoxia-induced PH caused a further significant increase in hypoxia-induced RVSP (Figure 5A) and slightly increased hypoxia-induced RV hypertrophy (though not significant) (Figure 5B). Deletion of PHD2 did not augment hypoxia-induced arterial remodeling (Figure 5C and 5D). These results confirm that PHD2 is an inhibitor of hypoxia-induced PH.

Activation of PHD2 by R59949 Reverses Vessel Remodeling in Existing Hypoxia-Induced PH *In Vivo*

To explore the potential for PHD2 as a novel therapeutic target for the treatment of PAH, we tested whether R59949, a PHD2 activator that inhibits HIF activation,²⁰ reverses existing PH in hypoxic mice. We exposed C57BL/6 mice to hypoxia or normoxia for 2 weeks to induce PH, followed by injection of R59949 or DMSO once daily for another 2 weeks. After exposure, we measured the RV/(LV+S) ratio, arterial wall thickness, and RVSP. Although R59949 was unable to reverse the elevated RVSP and RV hypertrophy in hypoxic mice (Figure 6A and 6B), it did decrease hypoxia-induced arterial wall thickening (Figure 6C and 6D) in hypoxic mice. R59949 did not appear to alter RVSP, RV/(LV+S), or wall thickness in normoxic mice (Figure 6). This result suggests that activation of PHD2 may partially reverse existing PH.

Dysregulation of PHD in Lungs of PAH Patients

To address the clinical relevance of PHDs in PAH, we obtained hPASC samples from normal donors, patients with idiopathic PAH (IPAH), and patients with PAH associated with other diseases (APAH). We compared the protein levels of PHD1 through 3 among control, IPAH, and APAH PASMC. We found that PHD1 levels were significantly lower in APAH

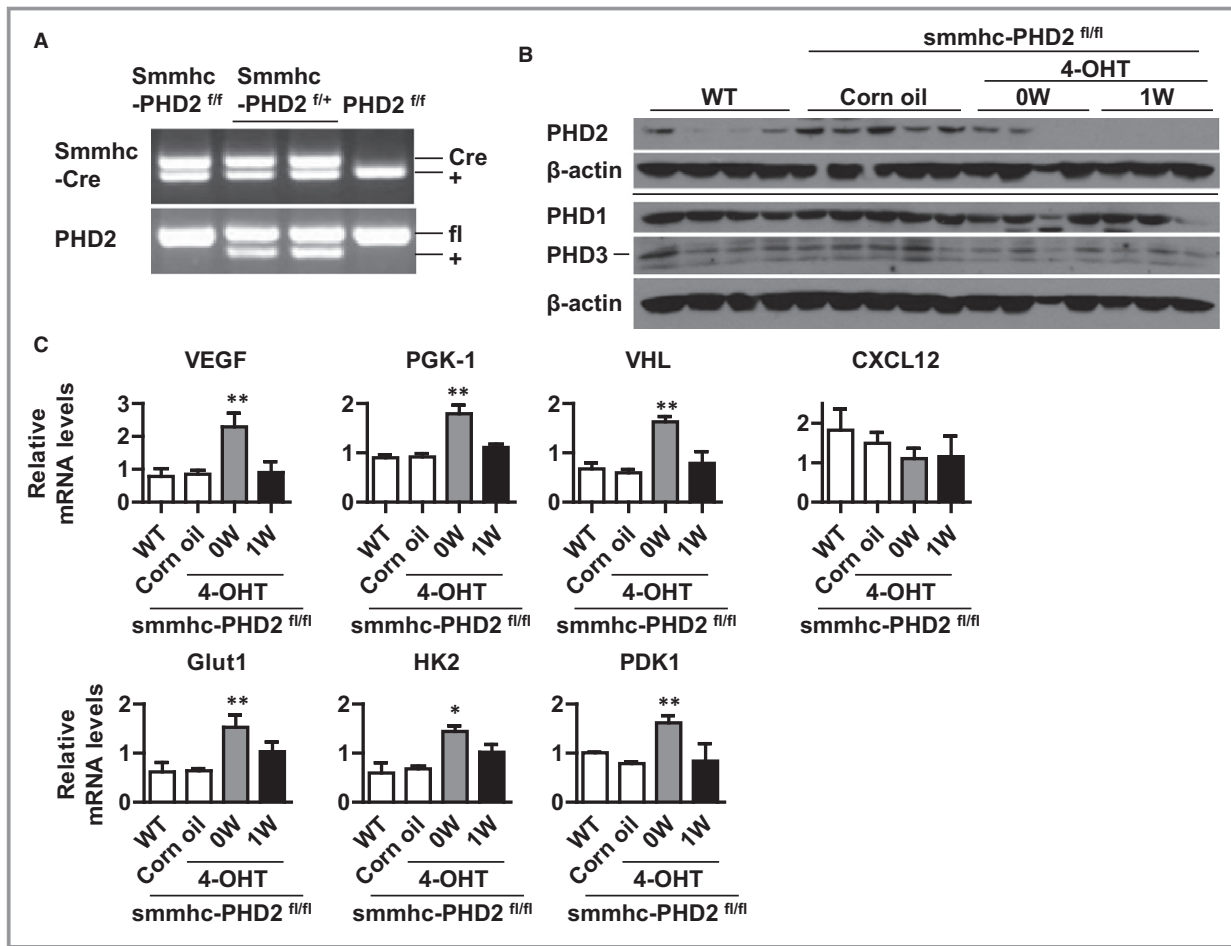


Figure 4. SMC-specific knockout of PHD2 enhances hypoxia-induced pulmonary arterial remodeling. A, Genotyping of smmhc-PHD2 mice. B and C, smmhc-PHD2^{fl/fl} mice were given 4-hydroxytamoxifen (4-OHT) by intraperitoneal injection for 5 consecutive days followed by 2 days of recovery. Mice were used for isolation of mouse PASM (mPASM) immediately (0W) or after another 1 week (1W) of maintenance. PHD2^{fl/fl} mice (WT) and smmhc-PHD2^{fl/fl} mice given corn oil (corn oil) were used to isolate control mPASM. The protein levels of PHD1, PHD2, and PHD3 were detected by Western blotting in which β -actin was used as control for equal loading (B). The mRNA levels of VEGF, PGK-1, VHL, CXCL12, Glut1, HK2, and PDK1 in these freshly isolated mPASM were measured by qRT-PCR with RPL19 as internal control (C). D through G, smmhc-PHD2^{fl/fl} mice were given 4-OHT by intraperitoneal injection for 5 consecutive days to achieve knockout of PHD2. PHD2^{fl/fl} mice injected with corn oil were used as controls. After 2 days of recovery, mice were exposed to normoxia (N) or hypoxia (10% oxygen, H) for 4 weeks. These mice were subjected to measurement of RVSP (D), right ventricular hypertrophy (E), and pulmonary arterial wall thickness (F and G). Representative image of pulmonary arteries are shown in (G). The scale bar indicates 50 μ m of length. n=5 for each group. Data are presented as mean \pm SEM. PASM indicates pulmonary artery smooth muscle cell; PHD, prolyl hydroxylase; RVSP, right-ventricular systolic pressure; SMC, smooth muscle cell. * P <0.05; ** P <0.01; ### P <0.01.

PASM (Figure S7A through S7D). In addition, PHD1 mRNA levels were decreased in IPAH and APAH PASM; however, PHD2 mRNA levels were higher in APAH PASM (Figure S7E and S7F). PHD3 mRNA appeared to be no different between normal and PAH PASM (Figure S7G). Thus, there is a dysregulation of PHD signaling in PAH.

Discussion

Mounting evidence demonstrates the significance of HIF and HIF α E3 ligase VHL in the development of PH^{16-18,35-37};

however, less is known whether PHDs, upstream regulators of HIF α , participate in PH. Our results suggest that miR-17/20a directly targets and suppresses PHD2 to induce HIF1 α in PASM. In PASM, suppression of PHD2 activates HIF1 and cell proliferation, but overexpression of PHD2 inhibits HIF1 and hypoxia-induced cell proliferation. Accordingly, loss of SMC-specific PHD2 enhances hypoxia-induced PH and exacerbates existing PH, suggesting that PHD2 is a negative regulator of PH.

We have previously shown that miR-17~92 can induce both PASM proliferation and differentiation; however, its direct

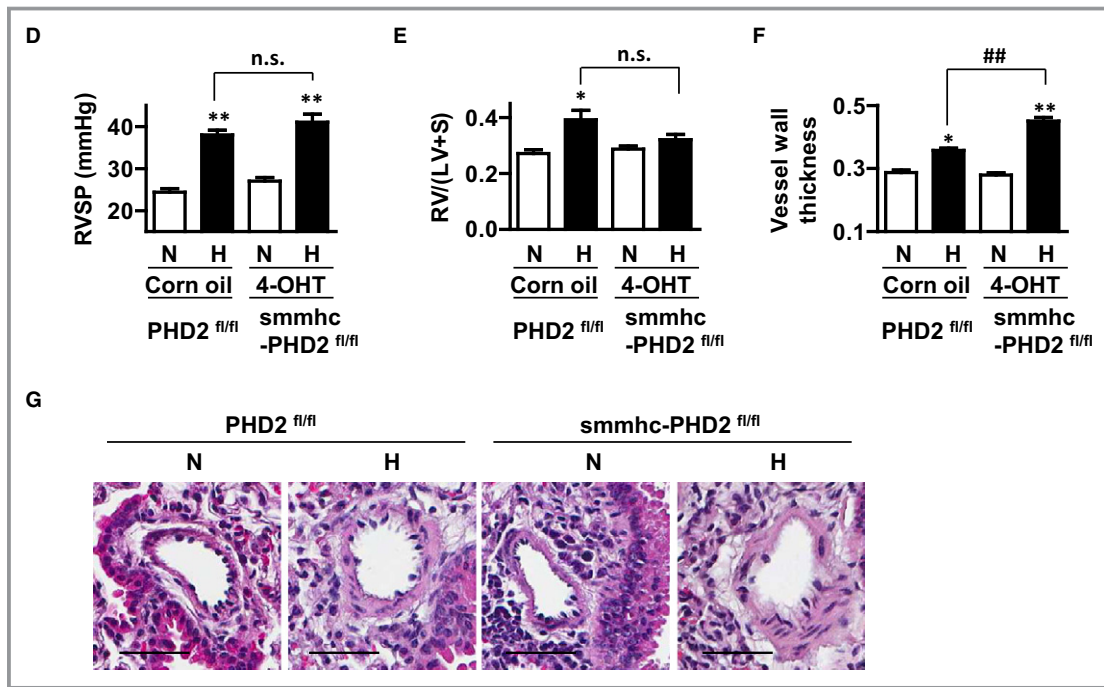


Figure 4. Continued

target PDLIM5 only modulates PSMC differentiation, suggesting that there are other mechanisms responsible for miR-17~92-induced cell proliferation.⁴ In this study, we have identified PHD2 as a novel target of miR-17/20a and, more importantly, shown that through the inhibition of PHD2, miR-17~92 induces HIF1 and cell proliferation. Thus, the dual function of miR-17~92 appears to be achieved by 2 distinct pathways: (1) by targeting PDLIM5, miR-17~92 induces TGF- β /Smad signaling and PSMC differentiation; (2) by targeting PHD2, miR-17/20a induces HIF1 and proliferation in PSMC (Figure 6E). It is important to point out that although miR-106a/363 and miR-106b/25, 2 paralogues of the miR-17~92 cluster, share similar or identical seed sequences with the members of the miR-17~92 cluster,²⁹ hypoxia has little effect on the expression of miR-106a/363 and miR-106b/25 clusters (Figure S3), which is different from the hypoxia-mediated biphasic regulation of miR-17~92.⁴ This suggests that miR-106a/363 and miR-106b/25 are regulated by different mechanisms from that of miR-17~92, and therefore, the regulation of PHD2 and PDLIM5 in pulmonary hypertension is specific to miR-17~92, particularly miR-17.

Our finding that miR-17~92 contributes to HIF activation (Figure 1) may have broad implications in several human diseases including cancer: miR-17~92 is known to be oncogenic,^{4,5,38} and HIF is implicated in cancer progression.^{15,39} Therefore, our study suggests that miR-17~92 may promote tumorigenesis via induction of HIF. Interestingly, previous reports suggest that miR-17~92 directly targets and inhibits HIF α .^{40,41} However, in lung cancer cells, miR-17~92-

mediated suppression of HIF1 α does not affect hypoxia-induced HIF1 α .⁴⁰ In macrophages, on the other hand, miR-17~92-mediated suppression of HIF α causes inhibition of HIF α in hypoxia.⁴¹ These results suggest a context-dependent regulation of HIF by miR-17~92 at multiple levels.

We show that SMC-specific knockout of miR-17~92 significantly decreases hypoxia-induced elevation in hematocrit, RBC, and HGB in mouse lungs (Figure 1D and 1F). This may appear to be surprising because erythropoietin (EPO), inducer of hematocrit, RBC, and HGB, is mainly produced in renal interstitial/fibroblast cells.⁴²⁻⁴⁴ A plausible explanation is the nonspecificity of sm22 α -Cre mice we used in our study to delete miR-17~92 in SMC. Sm22 α is also known as transgelin and is expressed in fibroblasts.⁴⁵ Therefore, crossing sm22 α -Cre with miR-17~92^{fl/fl} mice also deletes miR-17~92 in renal fibroblasts to induce PHD2 and inhibit HIF/EPO production in these mice when exposed to hypoxia. This may raise the question of whether the effects of the loss of miR-17~92 on pulmonary vessels and hypoxia-induced PH are due to the changes in circulating EPO. Interestingly, a recent study shows that there are increased levels of EPO in the plasma of PAH patients and that PAH serum or recombinant EPO promotes pulmonary artery endothelial cell network formation and smooth muscle cell proliferation in a cell culture system, respectively.⁴⁶ However, multiple studies suggest that EPO is beneficial in protecting against murine models of MCT-induced or hypoxia-induced PH in vivo.⁴⁷⁻⁴⁹ Further studies suggest that EPO and EPO receptor may protect mice from hypoxia-induced PH in vivo by the

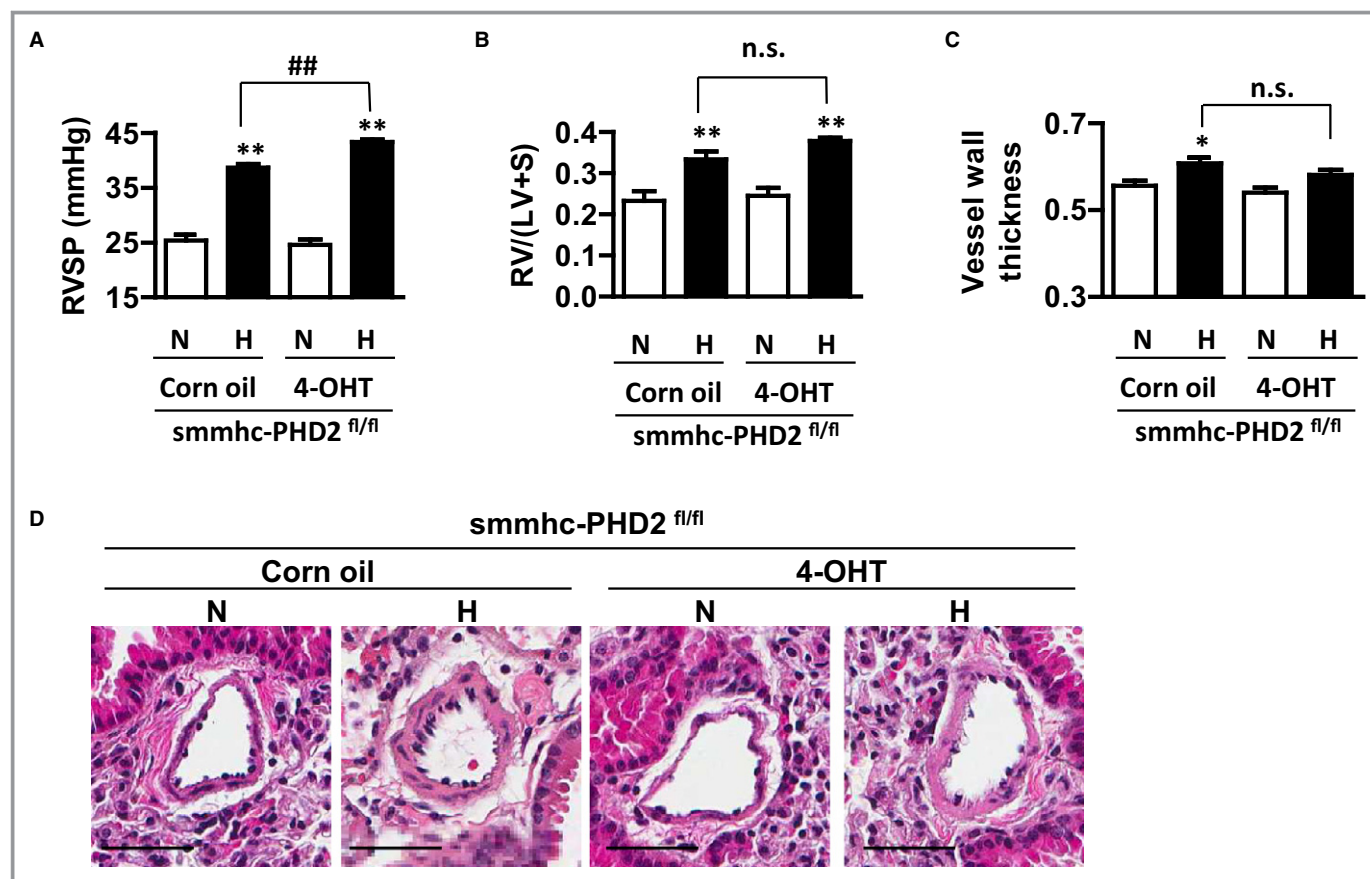


Figure 5. SMC-specific knockout of PHD2 exacerbates existing PH. Eight- to 10-week-old *smmhc-PHD2^{fl/fl}* mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 2 weeks in a BioSpherix A chamber to induce PH. Then, 4-OHT was administered for 5 consecutive days to induce the knockout of PHD2, and mice were allowed 2 days of recovery. Mice injected with corn oil were used as controls. These mice were then exposed to room air or 10% oxygen for another 2 weeks. Mice were subjected to the measurement of RVSP (A), right ventricular hypertrophy (B), and pulmonary arterial wall thickness (C and D). Representative images of pulmonary arteries are shown in (D). The scale bar indicates 50 μ m of length. $n=5$ for each group. Data are presented as mean \pm SEM. OHT indicates hydroxytamoxifen; PH, pulmonary hypertension; PHD2, prolyl hydroxylase 2; RV, right-ventricular; RVSP, right-ventricular systolic pressure; SMC, smooth muscle cell. * $P<0.05$; ** $P<0.01$; ### $P<0.01$.

recruitment of endothelial progenitor cells (EPCs) and activation of endothelial nitric oxide synthase.⁵⁰ Thus, attenuation of EPO and HCT in *sm-17~92^{-/-}* mice represents an inhibitory effect on the protection from hypoxia-induced PH and may explain a rather moderate protection from PH in *sm-17~92^{-/-}* mice. Further studies are warranted to investigate whether restoration of EPO will augment protection from PH in *sm-17~92^{-/-}* mice.

Previous studies have shown abnormal miR-17~92 and HIF activation in PAH.^{4,51,52} Moreover, in a MCT-induced rat PH model, HIF and miR-17~92 are also upregulated.^{53,54} Therefore, although experimental PH models have their limitations and do not perfectly recapitulate human PAH,⁵⁵ activation of miR-17/20a and HIF may be a common phenomenon in human PAH and experimental PH models,^{4,51,52,55} indicating that the miR-17/20a–PHD2–HIF1 axis may be a potential therapeutic target for PAH. Indeed, both miR-17 inhibitors and the HIF inhibitor digoxin have been shown to inhibit or reverse

experimental PH.^{4,54,56} Accordingly, PHD2 activators may have a therapeutic role in PAH. A few reports indicate that KRH102140, KRH10253, and R59949 are small molecules that activate PHD2 and inhibit HIF activity.^{20,57,58} Thus, these compounds may be used for the treatment of PAH patients, and further investigations are warranted. In this study we show that R59949 is able to reverse the vessel remodeling in existing hypoxic mice (Figure 6). However, we need to be cautious about the specificity of R59949, as it demonstrates selective inhibitory effects on diacylglycerol kinase (DGK) isozymes.⁵⁹

Of the 3 PHDs, PHD2 is conserved throughout the animal kingdom, and the PHD2–HIF1 axis is present in all species and is widely expressed in mammalian cells.⁶⁰ Although PHD isoforms demonstrate certain functional redundancy in that all PHDs can target both HIF1 α and HIF2 α ,⁶⁰ they also exhibit isoform-specific roles: for example, total PHD2 knockout in mice is embryonically lethal, and conditional somatic inactivation of PHD2 causes polycythemia, whereas PHD1^{-/-} and

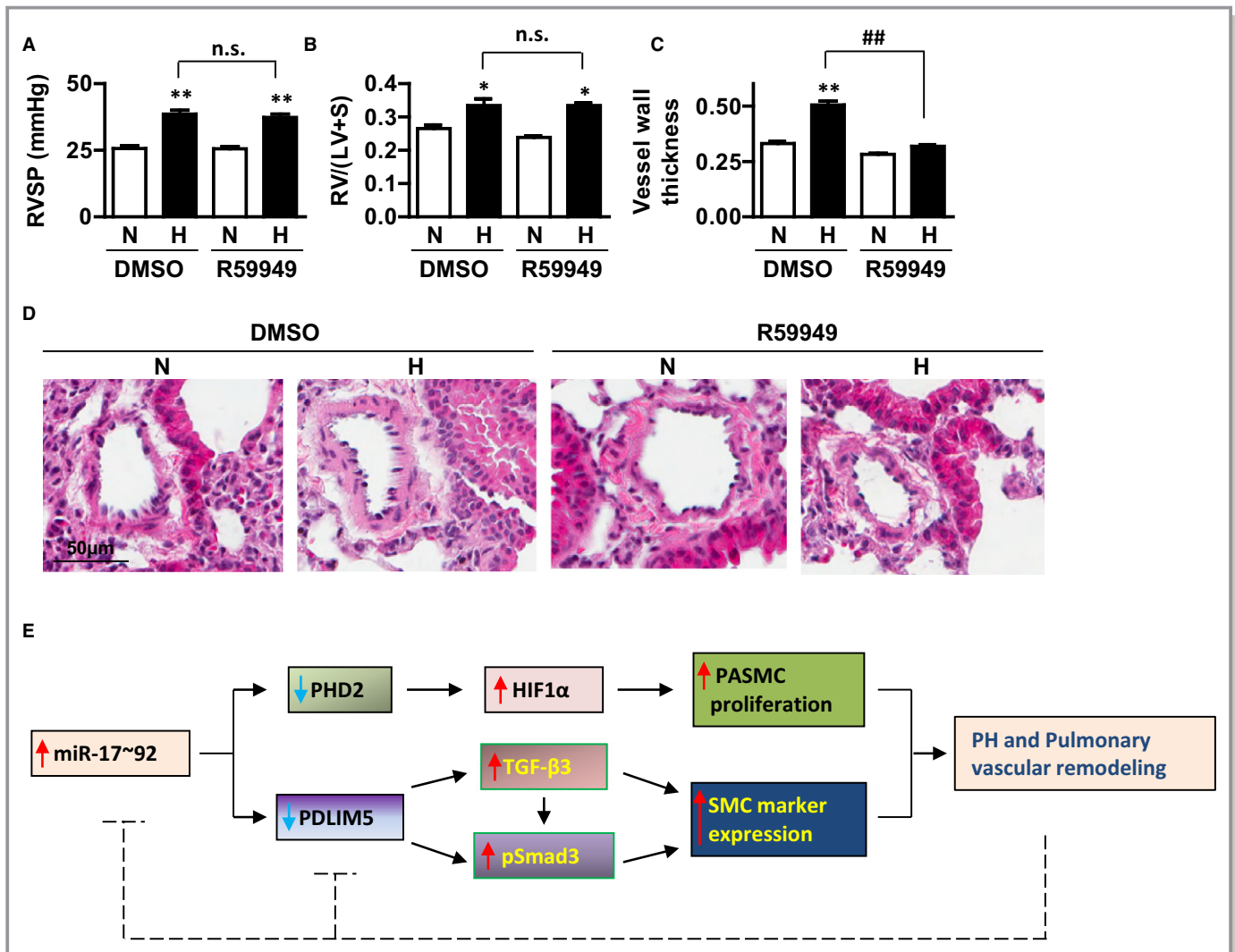


Figure 6. Activation of PHD2 by R59949 reverses vessel remodeling in existing hypoxia-induced PH in vivo. We exposed C57BL/6 mice to hypoxia (H) or normoxia (N) for 2 weeks, followed by injection of R59949 or DMSO once daily, 5 days a week, for another 2 weeks. After the completion of exposure, mice were anesthetized, RVPs were measured, and the quantification of RVSP (A), RV/(LV+S) (B), and arterial wall thickness (C) are shown. Representative images of pulmonary arteries are also shown (D). Data are presented as mean±SEM and compared to those from mice injected with DMSO and exposed to normoxia (DMSO+N). * $P<0.05$; ** $P<0.01$. $n=8, 7, 8, 7$ for each group. ## $P<0.01$, significant difference between DMSO+H and R59949+H groups; n.s., not significant. E, A schematic diagram depicts the dual roles of miR-17~92 in PASM proliferation and SMC contractile protein expression, its implication in the pathogenesis of PH, as well as its adaptive regulation in PH. HIF indicates hypoxia-inducible factor; PASM, pulmonary artery smooth muscle cell; PH, pulmonary hypertension; PHD2, prolyl hydroxylase 2; RV, right-ventricular; RVP, Right-ventricular pressure; RVSP, right-ventricular systolic pressure; SMC, smooth muscle cell.

PHD3^{-/-} mice are viable and manifest normal erythropoiesis.^{61,62} We find that although the individual siRNAs against PHDs are efficient in suppressing the cognate PHD proteins, a combination of PHD1/2/3 siRNA does not appear to achieve sufficient suppression of PHD1 and PHD2 (Figure S4A), suggesting a compensation of PHD isoforms.⁶³ Interestingly, a few studies have demonstrated that PHD2 is also a downstream target of HIF and acts to inhibit sustained HIF activation.^{27,28} Thus, it is possible that inhibition of PHDs may activate HIF and subsequently induce PHD2 as a negative feedback loop to limit sustained HIF activation.

In PASM, miR-17/20a, especially miR-17 (Figure 2), specifically targets and regulates PHD2 (Figure 2), thereby indicating the significance of PHD2 in this cell type. We also notice substrate selectivity of PHDs in PASM: PHD2 appears to act on HIF1 α only (Figure S4 and Figure 3); PHD1 can regulate both HIF1 α and HIF2 α ; PHD3 regulates HIF2 α only (Figure S4). This is consistent with earlier observations that PHD2 is most important in setting the levels of HIF1 α and that PHD1 and PHD3 are somewhat more active on HIF2 α (reviewed by Bishop and Ratcliffe⁶⁰). Under normoxic conditions, loss of PHDs appears to be sufficient to induce HIF

activity and cell proliferation (Figures S4 and S5), whereas gain of PHD2 is sufficient to decrease HIF1 α and cell proliferation but not enough to decrease expression levels of HIF targets (Figure 3). Thus, the HIF1-driven cell proliferation in normoxia may be dependent on other HIF targets.

Although loss of SMC-specific PHD2 is not sufficient to induce PH in normoxia, it did enhance the hypoxia-induced arterial vessel remodeling and RVSP (Figures 4 and 5), suggesting that loss of PHD2 exacerbates the response to hypoxia. This is consistent with the genome-wide association studies showing that populations adapted to the high altitudes on the Tibetan plateau have strongly selected haplotypes of the PHD2 gene.^{64,65}

Because HIF1 is ubiquitously expressed, we anticipate that the PHD2-HIF1 axis is likely functional in many cell types to contribute to PH. We show modest enhancement of the hypoxic response in *smmhc-PHD2^{fl/fl}* mice (Figures 4 and 5). This reflects the participation of the PHD2-HIF1 axis from other cell types. A recent study by Dai and colleagues reports that PHD2 deficiency in endothelial cells and hematopoietic cells causes spontaneous vascular remodeling in mice via HIF-2 α .⁶⁶ Thus, our study corroborates and complements theirs by providing a more complete and context-specific role of PHD2 in the development of PH: in PASMC, PHD2 inhibits PH via HIF1, whereas in endothelial cells, PHD2 inhibits PH via HIF2. Nonetheless, loss of SMC-specific PHD2 has little effect on RV hypertrophy in both experiments (Figures 4 and 5), suggesting that SMC-specific PHD2 mainly affects vessel function but has little effect on heart function.

Generally, pulmonary vascular remodeling precedes pulmonary arterial pressure changes in the pathogenesis of PH.⁶⁷ This notion is supported in our observation that, in the “prevention” experiment, we show that loss of PHD2 enhances hypoxia-induced vessel remodeling but not RVSP (Figure 4), whereas in the “reverse” experiment, loss of PHD2 enhances hypoxia-induced RVSP but not vessel remodeling (Figure 5). This also suggests that reversing of PH will likely be a progressive process.

Interestingly, in human IPAH PASMC samples, we do not observe the alteration in protein levels of PHD1, PHD2, and PHD3; however, PHD1 mRNA levels are downregulated in both IPAH and APAH PASMC, whereas PHD2 mRNA levels are upregulated in APAH PASMC (Figure S7). This suggests a likely distinct mechanism to regulate PHD1 and PHD2 in PAH. This mechanism of PHD1 regulation in APAH may be at the mRNA level as we show that, in both IPAH and APAH PASMC, the PHD1 mRNA levels are down. Our finding that PHD2 mRNA levels are up in APAH PASMC is consistent with the results in a previous study in which Edgar et al show that there is an upregulation of PHD2/EGLN1 in sporadic IPAH lungs as examined by suppression substrate hybridization.⁶⁸ In an earlier report, we have shown that miR-17~92 is

downregulated in PAH patients⁴; thus, upregulation of PHD2 mRNA in PAH is likely due to the downregulation of miR-17~92, which is consistent with our findings that miR-17~92 can directly target and inhibit PHD2 expression (Figures 1 and 2).

In our previous report, we demonstrate that miR-17~92 can target and suppress PDLIM5 to induce SMC contractile protein expression.⁴ In this study, we provide evidence that miR-17~92 directly targets and inhibits PHD2 to induce HIF1 and PASMC proliferation. Thus, we propose a dual function of miR-17~92 in the development of pulmonary hypertension (H in Figure 6): (1) suppression of PHD2 to induce HIF activity and PASMC proliferation, and (2) suppression of PDLIM5 to induce TGF- β 3/Smad3 signaling and SMC contractile protein expression. Both enhanced PASMC proliferation and contractile proteins expression contribute to pulmonary arterial remodeling and PH.

There is seemingly an inconsistency between our findings: (1) SMC-specific knockout of miR-17~92 attenuates and partially reverses hypoxia-induced PH⁴ (Figure 1), and (2) miR-17~92 is downregulated in PAH patient PASMC.⁴ Along with the biphasic regulation of miR-17~92 (early induction of miR-17~92 in PH and reduction of miR-17~92 in late stage of PH),⁴ we previously proposed an adaptive response regarding the role of miR-17~92 in the development of PH: upregulation of miR-17~92 may be the first common step in the development of PH, whereas the later decrease in miR-17~92 expression and an increase in PDLIM5 expression may be an adaptive response to inhibit further progression of PH.⁴ Our findings of the miR-17~92-PHD2-HIF1 α link and the known negative feedback between PHD2 and HIF1 provide a key piece to support this intrinsic adaptive response to limit PH progression. During hypoxia, in the presence of miR-17~92, low levels of PHD2 and PDLIM5 induce HIF1 and TGF- β 3/Smad3, leading to PASMC proliferation and SMC contractile protein expression and, ultimately, pulmonary vessel remodeling and PH. At the later stage of PH, an adaptive response inhibits miR-17~92 expression, leading to higher levels of PHD2 and PDLIM5 that will limit HIF activity and TGF- β 3/Smad3 signaling as well as progression of PH (Figure 6H). Further studies into the decreased miR-17~92 levels in late stage of PH are warranted.

Taken together, our study demonstrates for the first time that miR-17 directly targets PHD2 to induce HIF1 in PASMC and provides a mechanism by which miR-17~92 promotes PASMC proliferation. Given the importance of PHD2 in the miR-17-HIF pathway, therapies targeting PHD2 may provide novel insights into the treatment of pulmonary arterial hypertension—and, indeed, we show that a PHD2 activator R59949 reverses vessel remodeling in hypoxic mouse lungs (Figure 6). In addition, our finding links miR-17~92, PHD2, and HIF, which may have broad implications in several human diseases, including cancer, because miR-17~92 is known to

be oncogenic^{4,5,38} and HIF is implicated in cancer progression.^{15,39}

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Disclosures

None.

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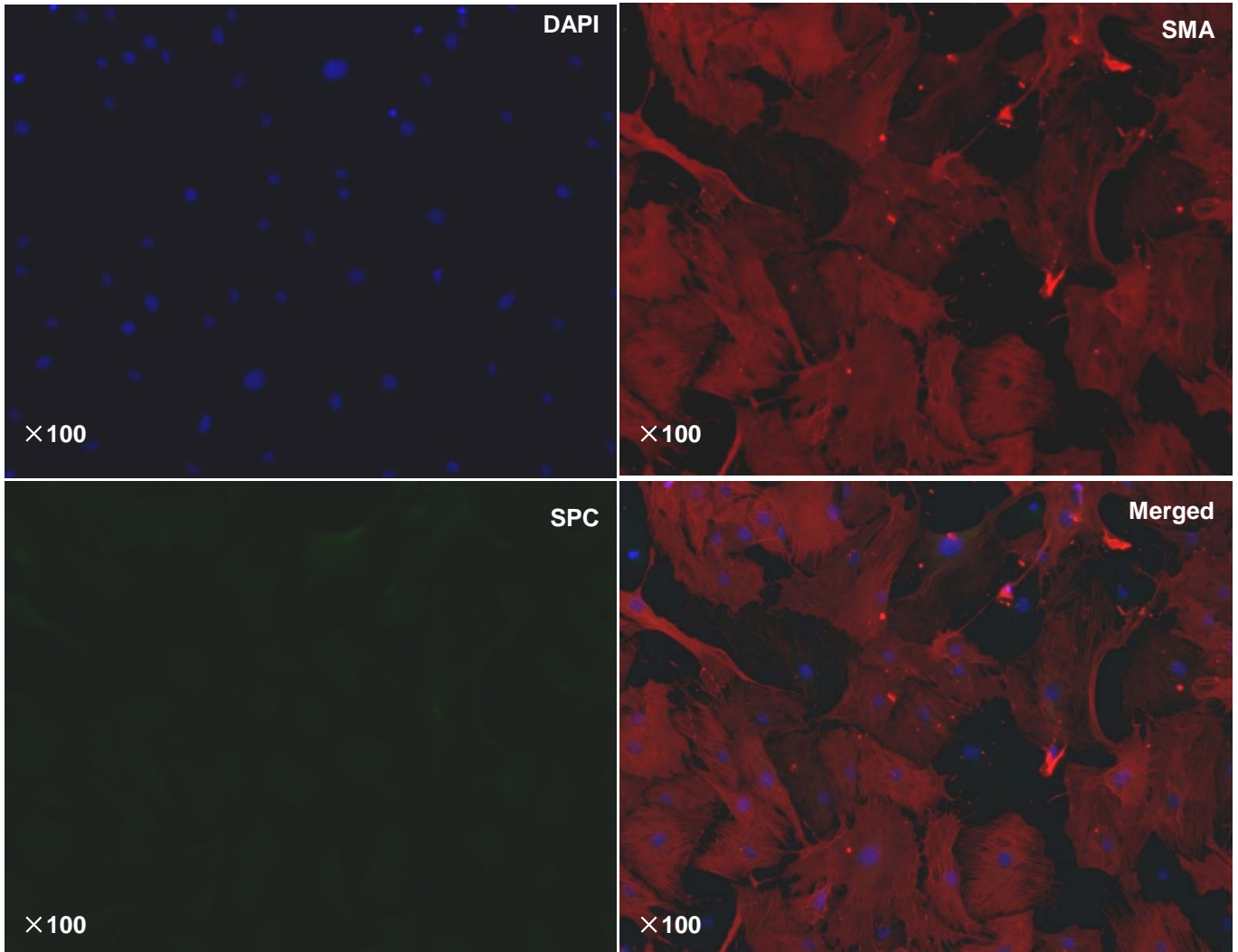
SUPPLEMENTAL MATERIAL

Table S1. Primer sequences for PCR and qRT-PCR

Gene	Forward 5' to 3'	Reverse 5' to 3'	Application
VEGF	ggaggcgcagcggttag	aaccggatcaatgaatatcaaa	qRT-PCR
EDN1	aaggcaagccctccagaga	ccgaaggtctgtcaccaatgt	qRT-PCR
Glut1	tgctcatggccttctcgaa	aagcgcccaggatcag	qRT-PCR
HK2	gagccatcctgcaacactagg	cagtgcacacctcctaacaatg	qRT-PCR
PDK1	tatggattgccatatacag	catctgtcccgtaacctct	qRT-PCR
VHL	cgtagcggttggtgactg	ccctggttgttcctctgac	qRT-PCR
PHD1	cctgaatcagaactgggacgtt	cggccctcaggggaagatc	qRT-PCR
PHD2	gcttggttgcctccagagtatt	gaatgtccctccaatcctaata	qRT-PCR
PHD3	gccagcggttacctgatagat	ttcctcgctgtgctccta	qRT-PCR
PHD2 3'-UTR	gctctagaggttctgtattgacaatgct	gctctagactaaactgaattgag	Luciferase Reporter Plasmid Cloning
PHD2 3'-UTR mut17/20a	tttatttactaaaataagaaaagg ^{tcgag} ^{gta} aattacaactgaaaaatcagattgt	aacaatctgattttcagttgtaatt ^{acctcga} cctt ttctattttaagtaaaataaa	Luciferase Reporter Plasmid Mutagenesis
hsa-miR-20b-5p	caaagtgctcatagtcaggta	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-106a-5p	caaagtgctaacagtcaggta	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-106b-5p	taaagtgctgacagtcagaga	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-93-5p	caaagtgctgttcgtgc	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-18b-5p	taaggtgcatctagtcaggt	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-25-3p	cattgcacttgtctcgggt	gcgagcacagaattaatacgactcac	qRT-PCR
hsa-miR-363-3p	aattgcacggtatccatctgtaa	gcgagcacagaattaatacgactcac	qRT-PCR

Figure S1.

A



B

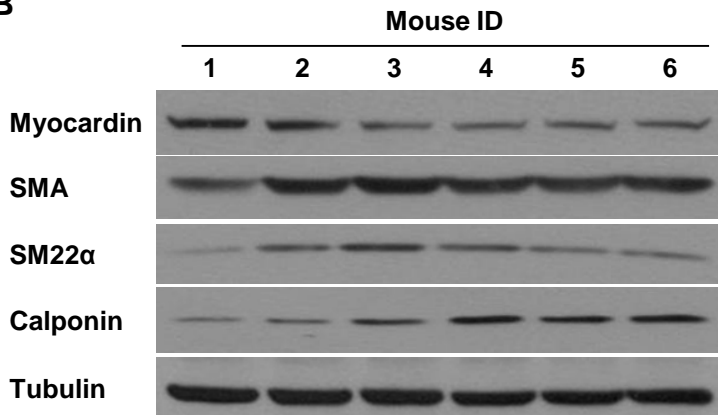


Figure S1. Validation of freshly isolated mouse PASC (mPASC). (A) co-immunofluorescence staining of mPASC with DAPI, SMA and Surfactant protein C (SPC). Representative images were shown. 10X objectives. (B) Western blot analysis of SMC markers in mPASC. Each lane represents mPASC from one mouse.

Figure S2.

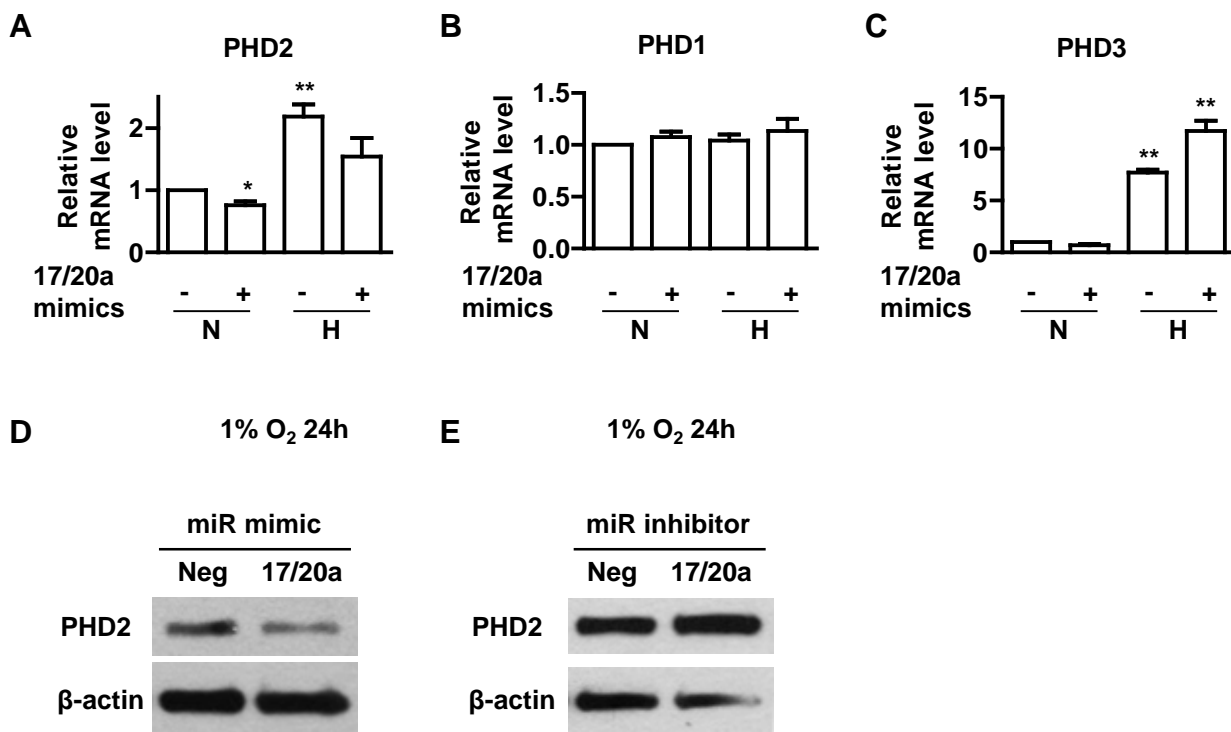


Figure S2. miR-17/20a is sufficient to inhibit PHD2 in both normoxic and hypoxic conditions.

(A-C) hPASCs were transfected with miR-17/20a mimics or a control miRNA and incubated for 24 hours in prior to exposure to normoxic (N) or hypoxic (H, 1% O₂) conditions for another 24 hours, followed by the measurement of mRNA levels of PHD2 **(A)**, PHD1 **(B)**, and PHD3 **(C)**. n = 3. *, p < 0.05; **, p < 0.01. Data are presented as mean ± SEM. **(D-E)** hPASCs were transfected with miR-17/20a mimics **(D)** or miR inhibitors **(E)**, were incubated for 24 hours, and were exposed to hypoxic (H, 1% O₂) conditions for another 24 hours, followed by the measurement of protein levels of PHD2 with β-actin as the loading control.

Figure S3.

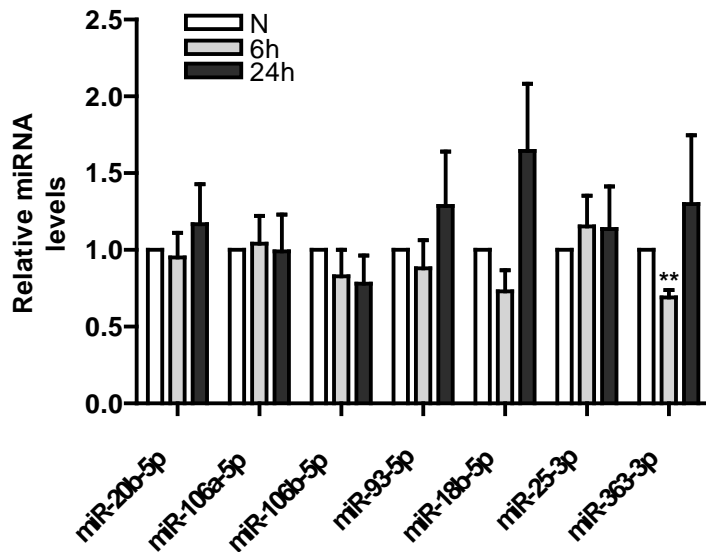


Figure S3. Expression levels of the members of the miR-106a/363 and miR-106b/25 clusters in PASMC exposed to hypoxia. hPASMC were exposed to normoxia (N) or hypoxia (H) for 6 or 24 hours, followed by the measurement of the expression levels of individual miRNA of the miR-106a/363 and miR-106b/25 clusters. We calculated the ratio of miRNA levels under hypoxic and normoxic conditions at each time point with the values at normoxia set as 1. Four independent experiments were performed. Data are presented as mean \pm SEM. **, $p < 0.01$.

Figure S4.

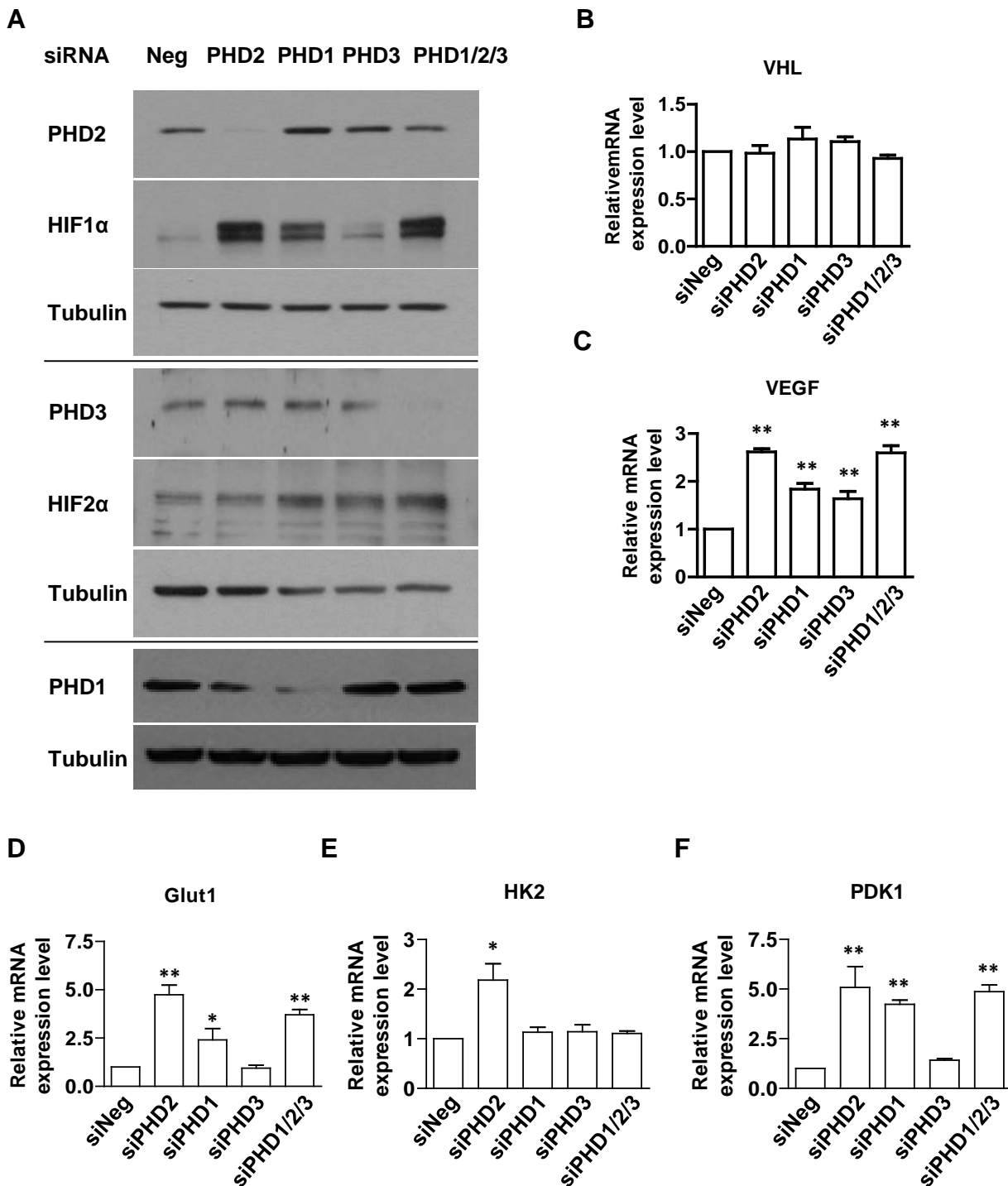


Figure S4. Loss of PHD2 induces the HIF1/VEGF signaling pathway in PASC. hPASC were transfected with siRNA against PHDs (siPHDs) or a negative control siRNA (siNeg) and incubated for 48 hours. **(A)** Protein levels of PHD1, 2, 3 and HIF1/2 α are shown. **(B)** The mRNA levels of VHL were detected by qRT-PCR (n = 5). **(C-F)** mRNA levels of VEGF **(C)**, Glut1 **(D)**, HK2 **(E)**, and PDK1 **(F)** were measured by qRT-PCR after silencing of PHDs, n = 5. *, p < 0.05; **, p < 0.01. Data are presented as mean \pm SEM.

Figure S5.

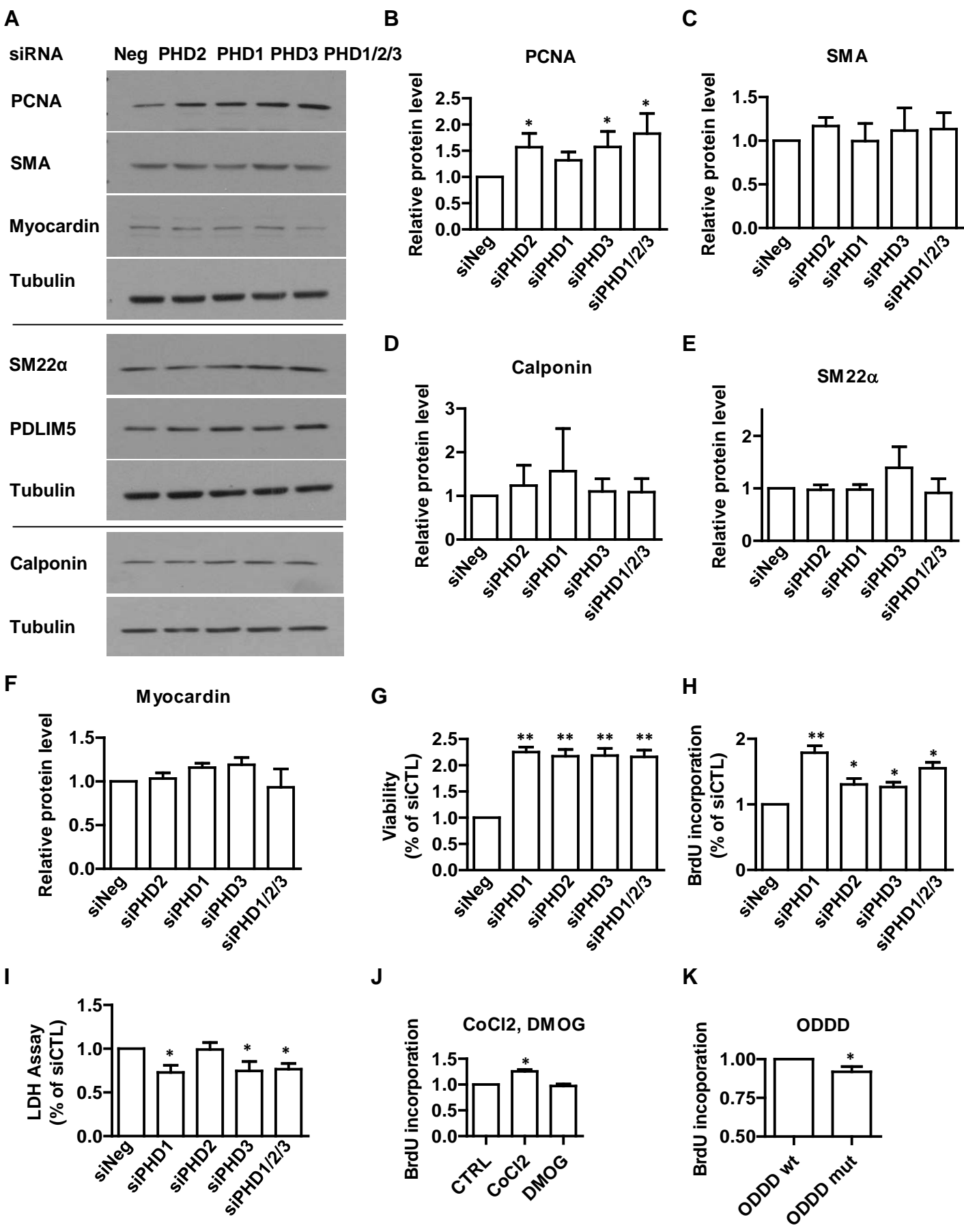


Figure S5. Loss of PHD2 induces PASC proliferation. (A-F) hPASC were transfected with siRNA against PHDs (siPHDs) or a negative control siRNA (siNeg) and incubated for 48 hours. Protein levels of PCNA, SMA, Calponin, SM22 α , and Myocardin are shown in **(A)** and quantified in **(B-F, n = 5)**. The representative blots are shown in **(A)**. **(G-I)** After transfection with siRNA against PHDs (siPHDs) or a negative control siRNA (siNeg), hPASC were used to perform the viability assay **(G)**, the BrdU incorporation assay **(H)**, and the LDH assay **(I)**. n = 6. **(J-K)** hPASC were treated with CoCl₂ (250 μ M) or DMOG (500 μ M) and infected with wild-type or mutated HIF1 α -ODDD adenoviruses (100 pfu/cell). We measured the BrdU incorporation in these cells. n = 6. Data are presented as mean \pm SEM. *, p < 0.05; **, p < 0.01.

Figure S6.

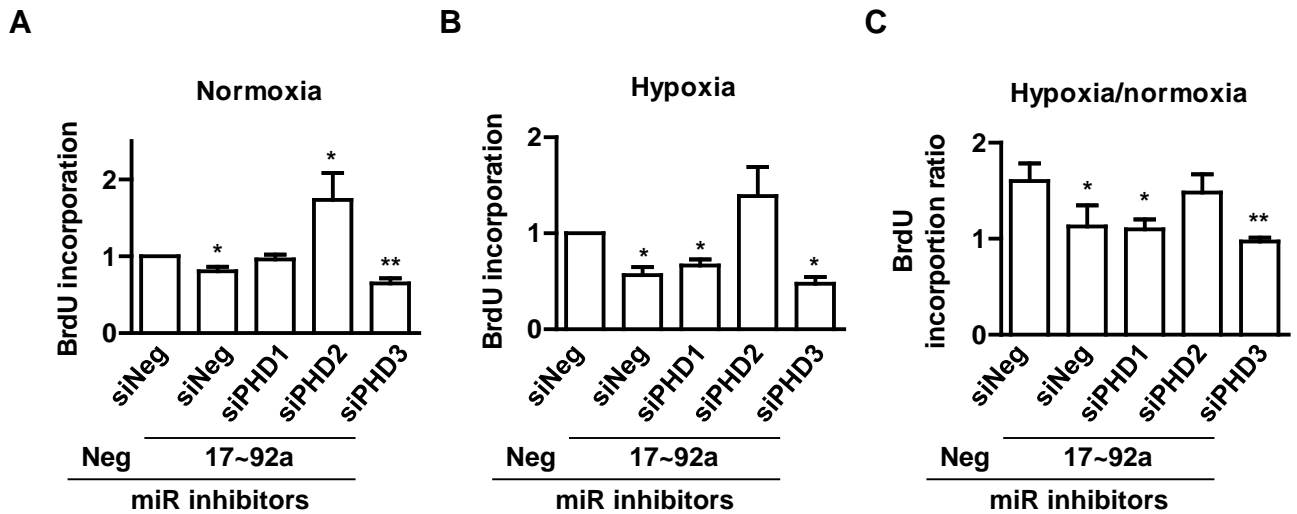


Figure S6. Silencing of PHD2 prevents inhibition of PASC proliferation mediated by miR-17~92 inhibitors. hPASCs were transfected with siRNA against PHDs (siPHDs) or a negative control siRNA (siNeg) in conjunction of miR-17~92a inhibitors or a negative control miRNA inhibitor (Neg) and incubated for 24 hours, followed by exposure to normoxia **(A)** or hypoxia **(B)** for an additional 24 hours. hPASC proliferation rates were measured by the BrdU incorporation assay as described in the manufacturer's manual. The BrdU incorporation of hPASCs transfected with siNeg and control miRNA was set as 1. **(C)** the hypoxia-induced human PASC proliferation was calculated as the ratio of BrdU incorporation of hypoxic cells over that of normoxic cells. $n = 3$. Data are presented as mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$.

Figure S7.

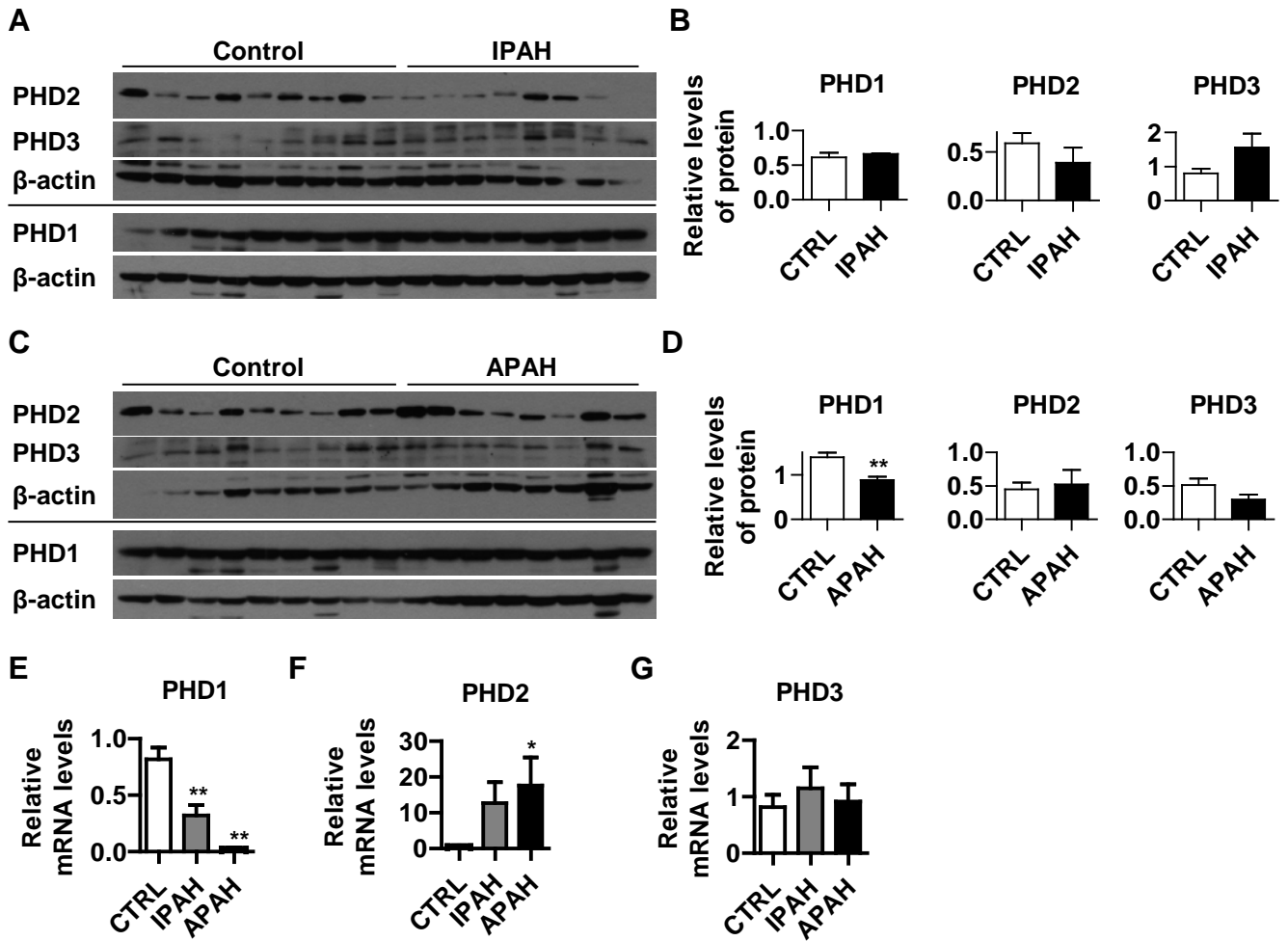


Figure S7. Dysregulation of PHDs in lungs of PAH patients. hPASMCM samples (normal, $n = 9$; IPAH, $n = 8$; APAH, $n = 8$) were cultured to extract proteins (**A-D**) or RNA (**E-G**) for the analysis of PHD1, PHD2, and PHD3 expression levels. We set the value of one normal human PASMCM sample as 1, and the expression levels of PHD1, PHD2, and PHD3 in other normal, IPAH, APAH human PASMCM samples were compared and normalized to that one. Data are presented as mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$.