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ORIGINAL ARTICLE

Loss of MicroRNA-17∼92 in Smooth Muscle Cells Attenuates Experimental Pulmonary Hypertension via Induction of PDZ and LIM Domain 5

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Abstract

Rationale: Recent studies suggest that microRNAs (miRNAs) play important roles in regulation of pulmonary artery smooth muscle cell (PASMC) phenotype and are implicated in pulmonary arterial hypertension (PAH). However, the underlying molecular mechanisms remain elusive.

Objectives: This study aims to understand the mechanisms regulating PASMC proliferation and differentiation by microRNA-17∼92 (miR-17∼92) and to elucidate its implication in PAH.

Methods: We generated smooth muscle cell (SMC)-specific miR-17∼92 and PDZ and LIM domain 5 (PDLIM5) knockout mice and overexpressed miR-17∼92 and PDLIM5 by injection of miR-17∼92 mimics or PDLIM5-V5-His plasmids and measured their responses to hypoxia. We used miR-17∼92 mimics, inhibitors, overexpression vectors, small interfering RNAs against PDLIM5, Smad, and transforming growth factor (TGF)-β to determine the role of miR-17∼92 and its downstream targets in PASMC proliferation and differentiation.

Measurements and Main Results: We found that human PASMC (HPASMC) from patients with PAH expressed decreased levels of the miR-17∼92 cluster, TGF-β, and SMC markers. Overexpression of miR-17∼92 increased and restored the expression of TGF-β_3, Smad3, and SMC markers in HPASMC of normal subjects and patients with idiopathic PAH, respectively. Knockdown of Smad3 but not Smad2 prevented miR-17∼92-induced expression of SMC markers. SMC-specific knockout of miR-17∼92 attenuated hypoxia-induced pulmonary hypertension (PH) in mice, whereas reconstitution of miR-17∼92 restored hypoxia-induced PH in these mice. We also found that PDLIM5 is a direct target of miR-17/20a, and hypertensive HPASMC and mouse PASMC expressed elevated PDLIM5 levels. Suppression of PDLIM5 increased expression of SMC markers and enhanced TGF-β/Smad2/3 activity *in vitro* and enhanced hypoxia-induced PH *in vivo*, whereas overexpression of PDLIM5 attenuated hypoxia-induced PH.

Conclusions: We provided the first evidence that miR-17∼92 inhibits PDLIM5 to induce the TGF-β_3/SMAD3 pathway, contributing to the pathogenesis of PAH.

Keywords: microRNA-17∼92; PDLIM5; transforming growth factor-β3; SMAD3; pulmonary arterial hypertension

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At a Glance Commentary

Scientific Knowledge on the Subject: Recent studies suggest that microRNA (miR)-17∼92 is implicated in pulmonary arterial hypertension. However, the molecular mechanisms underlying its role in the regulation of the pulmonary artery smooth muscle cell (PASMC) phenotype remain elusive.

What This Study Adds to the Field: This study shows that smooth muscle cell (SMC)-specific knockout of miR-17∼92 attenuates hypoxia-induced pulmonary hypertension (PH) in mice, whereas reconstitution of miR-17∼92 restores hypoxia-induced PH in these mice. We also identified PDZ and LIM domain 5 (PDLIM5) as a novel target of miR-17∼92, and loss of PDLIM5 up-regulates transforming growth factor-β/Smad2/3 signaling and induces expression of SMC markers. Our study provides the first evidence that miR-17∼92 can regulate PASMC differentiation through its novel target PDLIM5 and as a result contribute to vascular remodeling and the development of PH.

Pulmonary arterial hypertension (PAH) is a devastating disease that results in a progressive increase in pulmonary vascular resistance, right ventricular failure, and ultimately death of patients (1–3). PAH is characterized by excessive pulmonary vasoconstriction and abnormal vascular wall remodeling associated with proliferation of pulmonary artery smooth muscle cells (PASMC), indicating that there is a switch in the PASMC phenotype from a quiescent and differentiated state to a proliferative and dedifferentiated state during the development of PAH (4, 5). Although bone morphogenetic protein (BMP) signaling can modulate this phenotype switch via cross-talking with the Rho/MRTF pathway or cGMP/PKGI pathway, respectively (6, 7), the underlying molecular mechanisms involved in PASMC dedifferentiation remain elusive.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs that are evolutionarily conserved (8–11). Abnormal expression of a handful of miRNAs (12–18) has been implicated in regulation of PASMC phenotype and the pathogenesis of PAH, and inhibition of miR-17, a member of the miR-17∼92 cluster, induces p21 and prevents hypoxia-induced pulmonary hypertension (PH) (19). However, it is not clear to what extent PASMC-specific miR-17∼92 contributes to the pathogenesis of PH.

In this study, we found that smooth muscle cell (SMC)-specific knockout of miR-17∼92 attenuated hypoxia-induced PH in mice, whereas reconstitution of miR-17∼92 restored hypoxia-induced PH in these mice. We showed that miR-17∼92 was transiently up-regulated in early phase of hypoxia-induced PH and was later down-regulated in the late stage of PH. We identified PDZ and LIM domain 5 (PDLIM5) as a novel target of miR-17∼92 in human PASMC (HPASMC) and showed that miR-17∼92 regulates SMC phenotype via the PDLIM5/transforming growth factor (TGF)-β_3/Smad3 pathway. Loss of SMC-specific PDLIM5 enhanced hypoxia-induced PH in mice, whereas overexpression of PDLIM5 attenuated hypoxia-induced PH. Our study provides the first evidence that a novel pathway of miR-17∼92/PDLIM5/TGF-β/Smad is critical for vascular remodeling during the development of PAH. Some results from these studies have been reported in abstract form (20, 21).

Methods

Hypoxia-induced PH in C57BL/6 Mice, SMC-Specific Knockout of miR-17∼92, and PDLIM5 in Mice

We used a hypoxia-induced mouse PH model. Eight- to 10-week-old mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 3 or 5 weeks in a BioSpherix A-chamber (BioSpherix, Lacona, NY), and the oxygen concentration (10%) was monitored with a Proox Model P110 oxygen controller (BioSpherix). Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). We generated a strain of SMC-specific miR-17∼92 knockout (sm-17∼92$^{-/-}$) mice by crossbreeding the homozygous miR-17∼92$^{fl/fl}$ mouse with the homozygous sm22α-Cre mouse and subsequent backcrossing. We also crossbred the sm22α-Cre mouse with the PDLIM5$^{fl/fl}$ mouse (22) to generate a strain of SMC-specific PDLIM5 knockout (sm-PDLIM5$^{-/-}$) mouse. For the rescue experiments, miRNA mimics (either negative control mimic or combined mimics of miR-17, 18a, 19a, 92a) were injected into sm-17∼92$^{-/-}$ mice through the tail vein at a dose of 6.8 mg/kg body weight 1 day before exposure. Mice were exposed to either normoxia or 10% hypoxia for 5 weeks. During the exposure period, miRNA mimics were injected once a week at the same dose as mentioned above. One day after final injection of miRNA mimics, mice were used for PH parameter analysis. For the PDLIM5 overexpression experiment in mice, we mixed the vector or PDLIM5-V5-His plasmids (50 μg) with 100 μl of liposomes and injected it into the mouse tail vein (23–25) 1 day before exposure to normoxia or hypoxia for 3 weeks and then injected the plasmid/liposome mixture once a week during the experimental period. One day after the final administration of the plasmid/liposome mixture, we analyzed these mice for PH parameters. All animals were handled according to National Institutes of Health guidelines and the Institutional Animal Care and Use Committee–approved experimental protocols.

Isolation of Mouse PASMC

Mouse PASMC (mPASMC) were isolated from mouse lungs as described previously (26, 27) using a modification of the method of Marshall and colleagues (28). Briefly, after we performed surgery to expose trachea and aorta and drained the blood, we injected solution 1 (5 ml M199 medium [Sigma, St. Louis, MO] + 25 mg agarose + 25 mg iron filings) to blood circulation and solution 2 (5 ml M199 medium [Sigma] + 25 mg agarose) through the trachea. Then we removed the lungs and cut them into fine pieces. We collected lung vessels by passing lung tissues through Magnet column (DYNAL Invitrogen bead separations 1308; Invitrogen, Grand Island, NY). Blood vessels were filled with iron filings and were pulled down and enriched. After three washes, these vessels were digested by collagenase

A solution and disrupted by passing 16G and 21G blunt needles. These digested and disrupted vessels were enriched by Magnet column and washed, followed by culturing and maintaining in SmGM-2 medium.

Lentivirus-based miR-17∼92 Overexpression in HPASMC

For miRNA overexpression experiments, lentiviral vectors overexpressing the miR-17∼92 cluster were constructed by inserting the miRNAs into pLVX-Puro vector (Clontech, Mountain View, CA); pLVX-Puro vector without insertion of miRNAs was used as control. High-titer lentivirus was prepared using Lenti-X HT Packaging System (Clontech) in 293T cells following the user manual. HPASMC were infected by lentivirus supernatant and then selected with 1.5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO) for 3 to 4 days after 2 days of infection to remove uninfected cells. Overexpression of miRNAs was confirmed by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis.

Quantitative Mass Spectrometry Analysis to Identify miR-17∼92 Target Protein in HPASMC

We cultured HPASMC stably transduced with lentiviral vectors or lentiviruses encoding the miR-17∼92 cluster and extracted proteins, followed by the protein assay to determine protein concentration. We obtained 100 μg protein from each sample and precipitated proteins by a cold acetone/trichloroacetic acid method. Proteins were resuspended, reduced, and digested with trypsin. After desalting, these protein samples were labeled with differential Tandem Mass Tag labeling reagents (Thermo Fisher Scientific Inc., Rockford, IL), respectively. These samples were mixed, desalted, and analyzed by liquid chromatography–tandem mass spectrometry with data-dependent acquisition on the Orbitrap Velos. The data were used to search in Mascot database, and Mascot search results were imported into Scaffold Q+ to extract relative quantitation information for valid protein hits.

PDLIM5 3′-Untranslated Region Luciferase Reporter Assay

To construct the luciferase-PDLIM5 3′-untranslated region (UTR) (Wt-luc) reporter plasmid, a 0.3-kb 3′-UTR of human PDLIM5 gene containing the predicted miR-17/20a binding site was amplified from human genomic DNA and inserted into downstream of the luciferase reporter gene in the pGL3-Promoter vector (Promega, Madison, WI). We mutated the predicted miR-17/20a binding site on the Wt-luc reporter plasmid to generate the

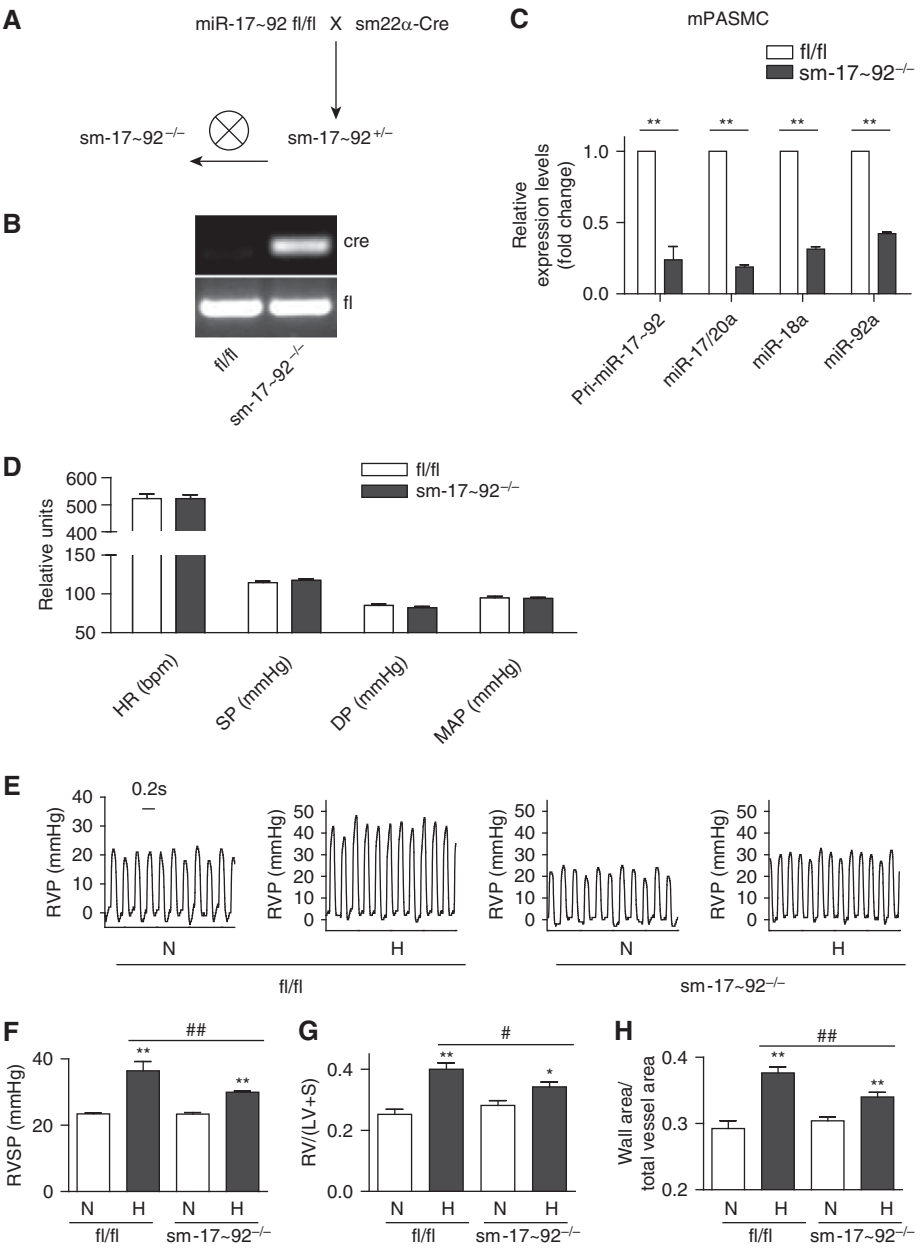

Figure 1. Smooth muscle cell (SMC)-specific knockout of microRNA (miR)-17∼92 is sufficient to attenuate hypoxia-induced pulmonary hypertension in mice. (*A*) The strategy to generate SMC-specific miR-17∼92 knockout (sm-17∼92$^{-/-}$) mice and mouse genotyping (*B*). (*C*) The expression levels of primary miR-17∼92 and mature miR-17∼92 members in freshly isolated mouse pulmonary artery smooth muscle cells (mPASMC) from sm-17∼92$^{-/-}$ mice (n = 3) and their wild-type littermates (n = 3). (*D*) sm-17∼92$^{-/-}$ mice (n = 14) and their wild-type littermates (n = 14) were subjected to the measurements of heart rate (HR), systolic pressure (SP), diastolic pressure (DP), and mean arterial pressure (MAP). (*E–H*) sm-17∼92$^{-/-}$ mice and their wild-type littermates were exposed to normoxia (N, ambient air) or hypoxia (H, 10% O_2) for 5 weeks. We measured right ventricular pressure (RVP) (*E*), calculated right ventricular systolic pressure (RVSP) (*F*), and determined right ventricle/(left ventricle + septum) [RV/(LV + S)] ratio (*G*) and pulmonary arterial wall thickness (*H*); n ⩾ 5 for each group. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.05$; $^{**}P < 0.01$; $^{\#\#}P < 0.01$.

Mut-luc reporter using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutated sequences are highlighted in Table E1 in the online supplement. All constructs were sequenced to confirm the DNA sequences.

The detailed methods are available in the online supplement.

Results

SMC-Specific Knockout of miR-17∼92 Is Sufficient to Attenuate Hypoxia-induced PH in Mice

Although Pullamsetti and colleagues reported that a chemical inhibitor of miR-17 prevents and reverses hypoxia-induced PH (19), the tissue-specific role of miR-17∼92 in the pathogenesis of PH is not clear. Systemic knockout of miR-17∼92 results in severe hypoplastic lungs in embryos, and these mice die shortly after birth (29). Thus, we generated a strain of SMC-specific miR-17∼92 knockout mice (sm-17∼92$^{-/-}$) by crossbreeding the homozygous miR-17∼92$^{fl/fl}$ mouse with homozygous sm22α-Cre mouse and subsequent backcrossing (Figure 1A) and confirmed their genotypes (Figure 1B). We isolated mPASMC from these mice and confirmed that the levels of the miR-17∼92 cluster were significantly reduced in PASMC of these mice (Figure 1C). sm-17∼92$^{-/-}$ mice were viable and fertile, did not display gross abnormalities, and showed no difference in heart rate, systolic pressure, diastolic pressure, and mean arterial pressure compared with wild-type mice (Figure 1D). We exposed 10-week-old sm-17∼92$^{-/-}$ and miR-17∼92$^{fl/fl}$ mice to hypoxia (10% O_2) or normoxia for 5 weeks and found that knockout of miR-17∼92 in SMC attenuated the hypoxia-induced increase in right ventricular systolic pressure (RVSP) (Figures 1E and 1F), RV hypertrophy measured by right ventricle/(left ventricle + septum) ratio [RV/(LV + S)] (Figure 1G), and pulmonary arterial wall thickness (Figure 1H; *see* Figure E1 in the online supplement), suggesting that miR-17∼92 is key to the development of hypoxia-induced PH.

miR-17∼92 Promotes PASMC Proliferation and SMC Marker Expression in Normal HPASMC

Because the miR-17∼92 cluster is known to regulate cell behavior (30, 31), we sought to investigate whether miR-17∼92 regulates SMC marker expression in HPASMC. We infected HPASMC with lentiviruses encoding miR-17∼92 to overexpress the miR-17∼92 cluster as a whole in normal HPASMC and confirmed the overexpression of the miR-17∼92 cluster by qRT-PCR (Figure 2A).

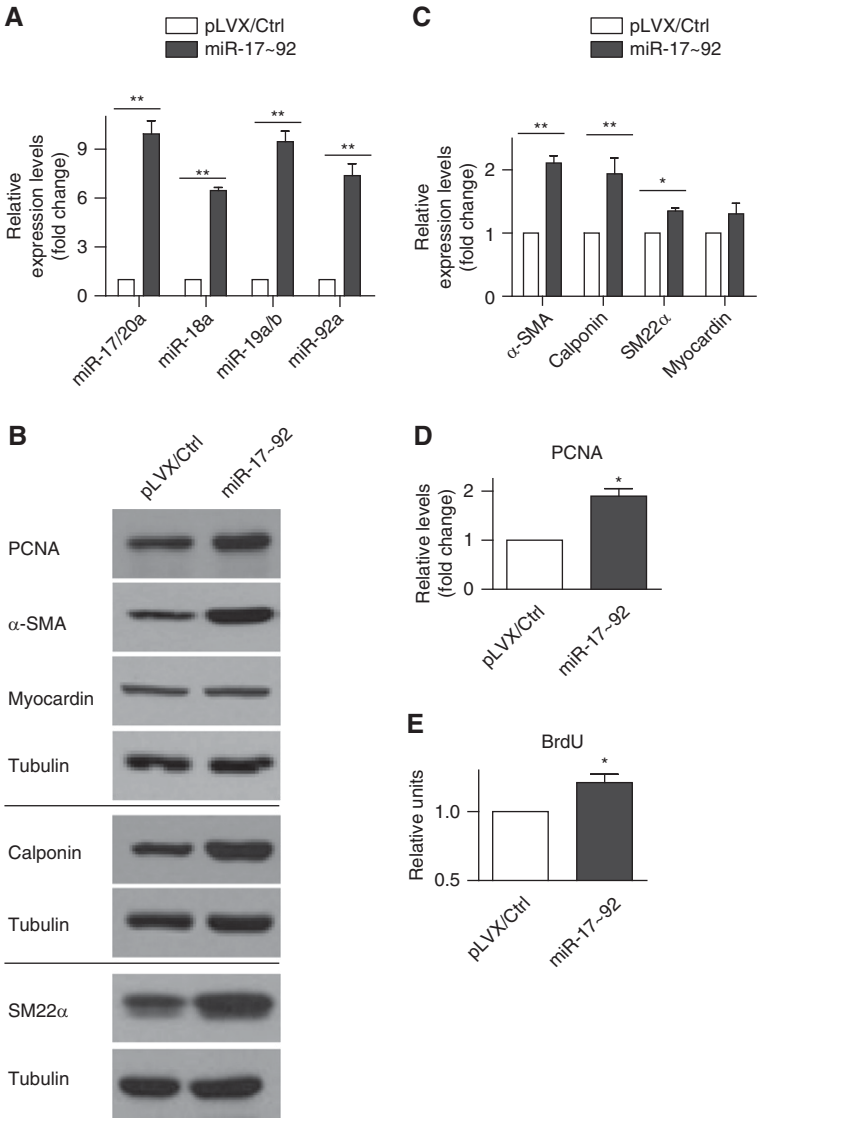

Figure 2. MicroRNA (miR)-17∼92 promotes proliferation and smooth muscle cell (SMC) marker expression in normal human pulmonary artery smooth muscle cells (HPASMC). (*A*) Normal HPASMC were infected with lentiviruses encoding the whole cluster of miR-17∼92. The expression of miR-17∼92 was determined by quantitative reverse transcriptase–polymerase chain reaction. miR-17∼92 levels in these cells were normalized to those of normal HPASMC infected with lentiviral vector (pLVX/Ctrl). Six independent preparations of lentiviral-infected cells for each virus were used in this assay, and quantitative reverse transcriptase–polymerase chain reaction was conducted in triplicates. (*B–D*) The expression levels of α-smooth muscle actin (α-SMA), calponin, SM22α, proliferating cell nuclear antigen (PCNA), and myocardin were determined in cell lysates of normal HPASMC infected with lentiviruses. The representative blots are shown in *B* and the quantification is shown in *C* and *D*. The expression levels of α-SMA, calponin, SM22α, myocardin (*C*), and PCNA (*D*) were normalized to those of normal HPASMC infected with lentiviral vector (pLVX/Ctrl) with tubulin as the internal control. Five independent preparations of lentiviral-infected cells for each virus were used in this assay. (*E*) The bromodeoxyuridine (BrdU) incorporation of normal HPASMC infected with lentiviral vector or lentivirus encoding the miR-17∼92 cluster. The assay was repeated five times independently and was performed in triplicates. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

We found that overexpression of the miR-17∼92 cluster induced expression of α-smooth muscle actin (α-SMA) and calponin by twofold and SM22α by 1.5-fold, without altering myocardin expression levels (Figures 2B and 2C). Overexpression of miR-17∼92 induced proliferating cell nuclear antigen (PCNA) expression and bromodeoxyuridine (BrdU) incorporation (Figures 2B, 2D, and 2E). In contrast, treatment with a mixture of miR-17, miR-18a, miR-19a, and miR-92a inhibitors dramatically inhibited the expression levels of the miR-17∼92 cluster (Figure E2A) and suppressed expression levels of α-SMA, calponin, and PCNA in a dose-dependent manner (Figure E2B). These results suggest that miR-17∼92 is a positive regulator of PASMC proliferation and SMC marker protein expression.

Reconstitution of miR-17∼92 Restores Hypoxia-induced PH in SMC-Specific miR-17∼92 KO Mice

To address whether reconstitution of miR-17∼92 restores hypoxia-induced PH in sm-17∼92$^{-/-}$ mice, we first treated normal HPASMC with the same amount of miR-17∼92 mimics individually or a mixture of two, four, or six mimics. A mixture of miR-17/18a/19a/92a achieved the best induction of PCNA and α-SMA while maintaining the levels of calponin and SM22α (Figure E3). Therefore, we injected a mixture of miR-17/18a/19a/92a mimics via tail vein to reconstitute miR-17∼92 in sm-17∼92$^{-/-}$ mice (Figure 3A). We found that reconstitution of miR-17∼92 restored the increase in RVSP, RV/(LV + S) ratio, and pulmonary artery remodeling in sm-17∼92$^{-/-}$ mice exposed to hypoxia without changes in basal levels of RVSP, RV/(LV + S), and pulmonary arterial wall thickness (Figures 3B–3D). These results suggest that miR-17∼92 is critical for the pathogenesis of PH.

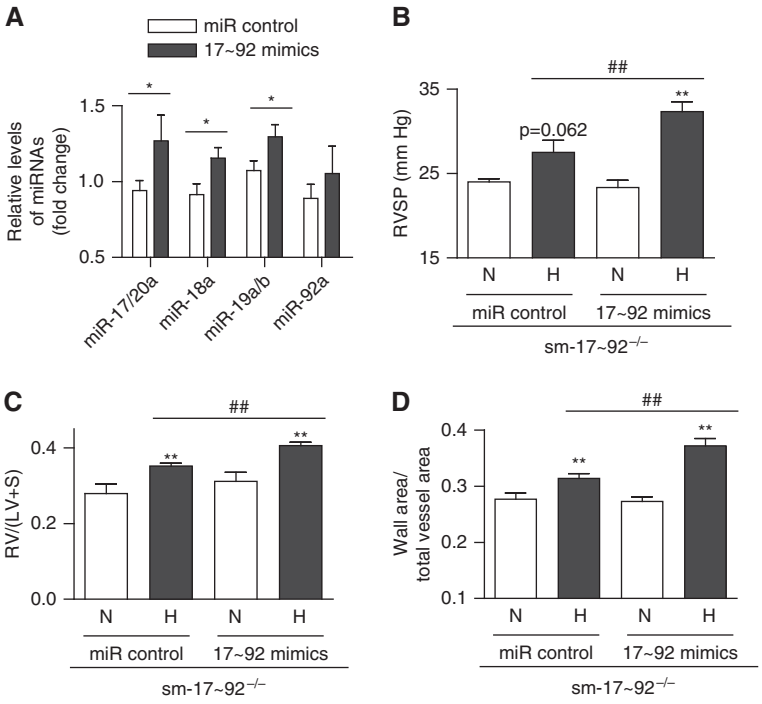

Figure 3. Reconstitution of microRNA (miR)-17∼92 restores hypoxia-induced pulmonary hypertension in smooth muscle cell (SMC)-specific miR-17∼92 knockout (sm-17∼92$^{-/-}$) mice. sm-17∼92$^{-/-}$ mice were injected with control miR or miR-17∼92 mimics once a week and were exposed to normoxia (N) or hypoxia (H) for 5 weeks, followed by measurement of levels of miR-17∼92 in mouse lungs (*A*), right ventricular systolic pressure (RVSP) (*B*), right ventricle/(left ventricle + septum) [RV/(LV + S)] (*C*), and pulmonary arterial wall thickness (*D*). Each group has at least five mice. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#\#}P < 0.01$; * and ** indicate the comparison between normoxic mice injected with control miRNA and other groups; ## indicates the comparison between hypoxic groups.

PDLIM5 Is a Novel miR-17∼92 Target in PASMC

To identify novel targets of miR-17∼92 in human PASMC, we conducted a quantitative mass spectrometry analysis (32, 33). We labeled stable normal HPASMC overexpressing miR-17∼92 or control vector with differential Tandem Mass Tag labeling reagents, followed by OrbiTrap Velos liquid chromatography–tandem mass spectrometry and data analysis with Scaffold Q+ software (Figure E4A). We found 14 proteins that were down-regulated by at least 1.2-fold (Figure E4B) in HPASMC overexpressing miR-17∼92. We found that PDLIM5 contains a potential binding site for miR-17 and miR-20a in its 3′-UTR (Figure E4B). We confirmed the reduction of PDLIM5 in HPASMC overexpressing miR-17∼92 in our mass spectrometry analysis (Figure E5). Four unique peptides of PDLIM5 were identified, and a representative peptide and its mass are shown in Figure 4A.

Figure 4B shows the conserved 8-mer seed sequence of miR-17 and miR-20a in the 3′-UTR of PDLIM5. We further confirmed the decreased PDLIM5 levels in HPASMC that overexpress miR-17∼92 by Western blotting (Figure 4C). We also confirmed the reduction of SERPINE1 and ZNF185 (Figure E6), two proteins that are identified in quantitative proteomics (Figure E4B). In addition, we found that treatment with miR-17 mimics decreased PDLIM5 levels (Figure 4D), whereas a mixture of miR17/20a inhibitors increased PDLIM5 levels (Figure 4E). We constructed a luciferase reporter (Wt-Luc) containing a 0.3 KB 3′-UTR of PDLIM5 and a mutant reporter (Mut-Luc) in which the putative miR-17/20a site was mutated (Figure 4F; Table E1). We found that overexpression of miR-17∼92 suppressed luciferase activity of Wt-Luc but not Mut-Luc (Figures 4G and 4H). Mimics of miR-17 and miR-20a inhibited the luciferase activity of Wt-Luc PDLIM5 3′-UTR reporter (Figure 4I), whereas mimics of miR-20a slightly increased the luciferase activity of Mut-Luc (Figure 4J). MiR-17/20a inhibitors increased the luciferase activity of Wt-Luc (Figure 4K) without affecting the luciferase activity of Mut-Luc (Figure 4L). Furthermore, mPASMC isolated from sm-17∼92$^{-/-}$ mice expressed higher levels of PDLIM5 than those of miR-17∼92$^{fl/fl}$ mice (Figures 4M and 4N). These results suggest that miR-17 and miR-20a directly

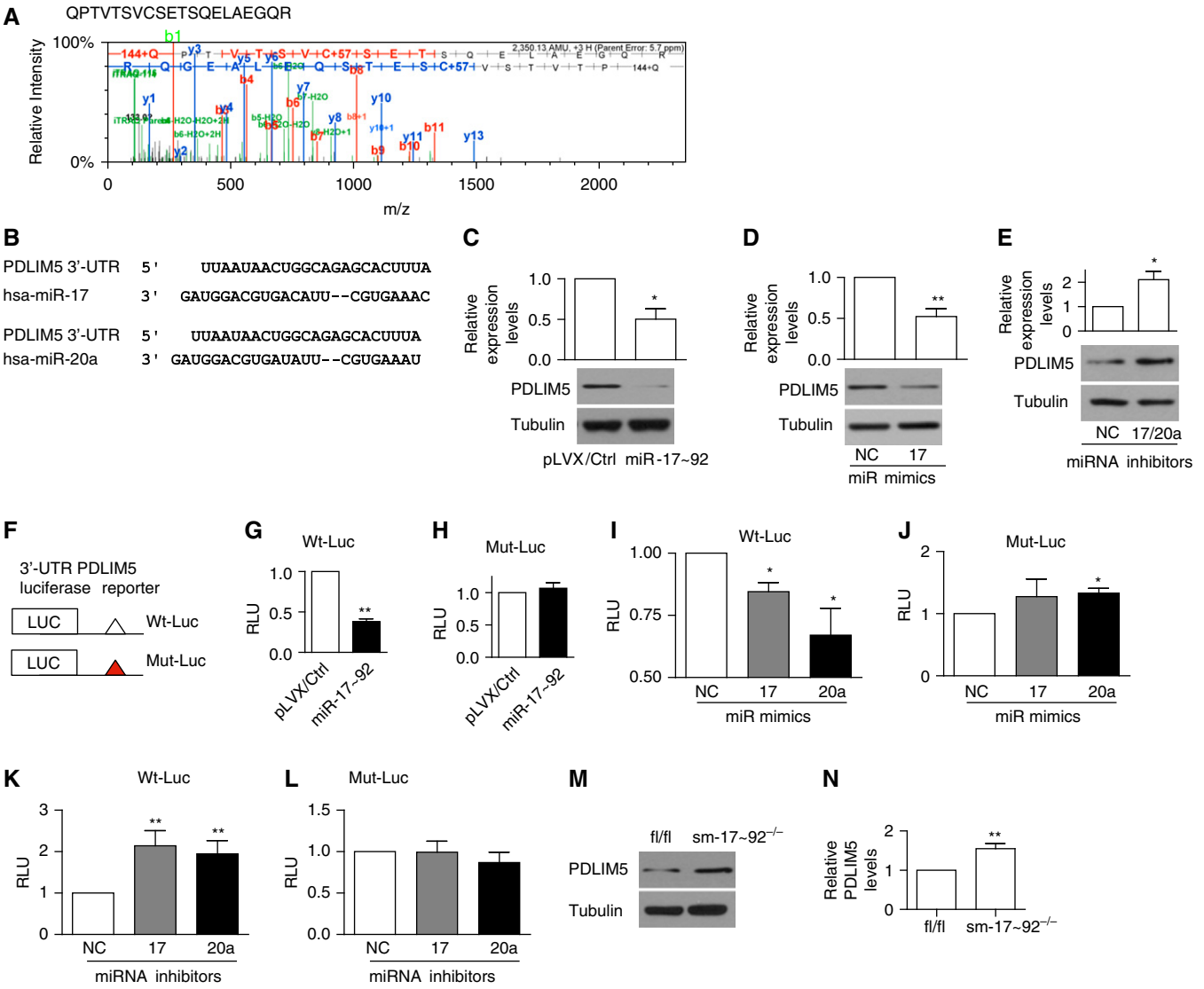

Figure 4. PDZ and LIM domain protein 5 (PDLIM5) is a novel microRNA (miR)-17∼92 target in pulmonary artery smooth muscle cells (PASMC). (*A*) A representative peptide and mass of PDLIM5 identified by quantitative proteomics. (*B*) The putative binding site of miR-17 and miR-20a at the 3′-untranslated region (UTR) of PDLIM5. (*C–E*) Normal human PASMC (HPASMC), either infected with lentiviral vector (pLVX/Ctrl) and miR-17∼92 encoding lentiviruses (*C*, n = 5) or treated with miR-17 mimics (*D*, n = 6) or miR-17/20a inhibitors (*E*, n = 5) were harvested 48 hours after transfection to determine the levels of PDLIM5. The amount of tubulin was used as loading control. (*F*) Diagram of the wild-type (Wt-Luc) and mutated (Mut-Luc) 3′-UTR of PDLIM5 luciferase reporter constructs. *Open triangle* represents the binding site of miR-17/20a, and the *red triangle* represents the mutated miR-17/20a binding site. (*G–H*) Wt-Luc (*G*) or Mut-Luc (*H*) reporter plasmids were transfected into normal HPASMC infected with lentiviruses containing pLVX/Ctrl or miR-17∼92. The luciferase activity (relative light units [RLU]) of normal HPASMC infected with pLVX/Ctrl transfected with Wt-Luc (*G*) or Mut-Luc (*H*) was designated as 1 and used as control for normalization. Data are presented as mean ± SEM from five independent experiments that were performed in triplicate. (*I–L*) Normal HPASMC were cotransfected with the Wt-Luc reporter (*I*, *K*) or Mut-Luc (*J*, *L*) and mimics (*I*, *J*) or inhibitors (*K*, *L*) of miR-17 or miR-20a, and we determined the luciferase activity as described above. n = 5 for each graph. (*M*, *N*) We determined the protein levels of PDLIM5 in freshly isolated mouse PASMC from miR-17∼92fl/fl and smooth muscle cell–specific miR-17∼92 knockout (sm-17∼92$^{-/-}$) mice. In each strain, mouse PASMC were pooled from four mice, and the Western blotting was repeated four times. Representative blots were shown in *M* and quantification in *N*. **P* < 0.05; ***P* < 0.01. NC = negative control.

target the 3′-UTR of PDLIM5 to suppress its expression.

PDLIM5 Suppresses Expression of Contractile Proteins in PASMC

To investigate effects of PDLIM5 on SMC marker expression and PASMC proliferation, we suppressed PDLIM5 with small interfering RNA (siRNA) and found that suppression of PDLIM5 increased the expression of SM22α, α-SMA, myosin heavy chain (MHC), and calponin, but not myocardin (Figures 5A and 5C) and increased α-SMA staining and fiber formation in human PASMC (Figure 5E). However, suppression of PDLIM5 did not change the expression levels of PCNA and BrdU incorporation (Figures 5B–5D). Thus, PDLIM5 is a negative regulator of SMC-differentiated phenotype.

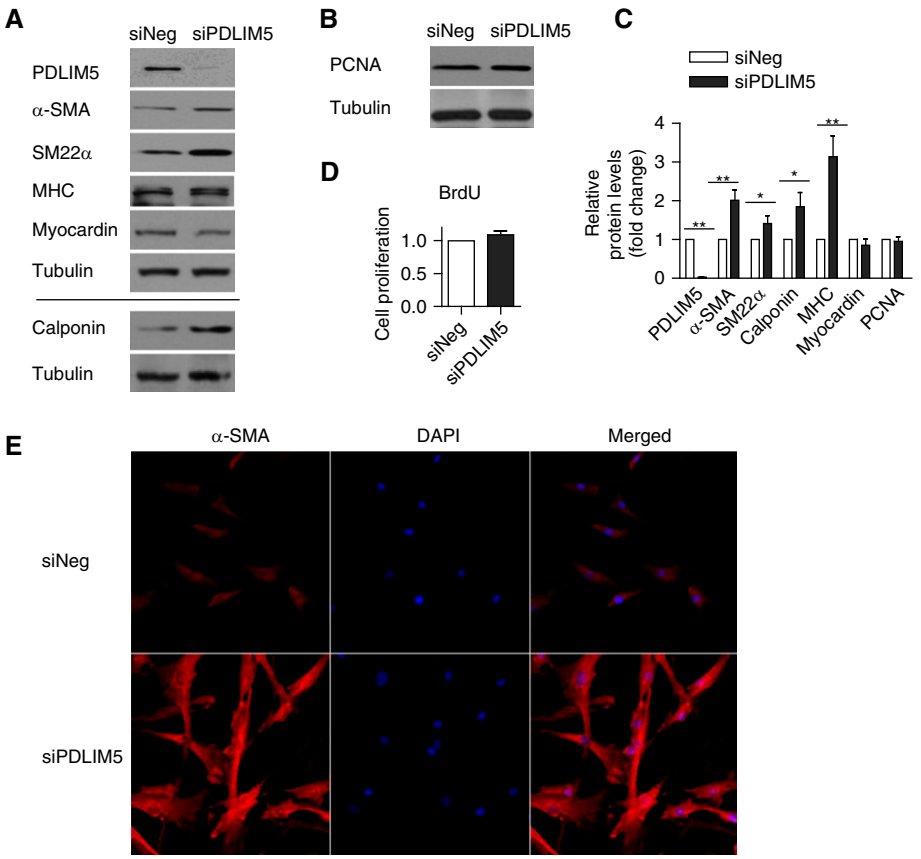

Figure 5. PDZ and LIM domain protein 5 (PDLIM5) negatively regulates expression of smooth muscle cell (SMC) contractile proteins. (*A*, *B*) Normal human pulmonary artery smooth muscle cells (HPASMC) were transfected with siRNA against PDLIM5 (siPDLIM5) or a negative control siRNA (siNeg) and incubated for 48 hours. The protein levels of PDLIM5, myocardin, SM22α, calponin, myosin heavy chain (MHC), α-smooth muscle actin (α-SMA) (*A*), and proliferating cell nuclear antigen (PCNA) (*B*) were determined. The representative blots are shown in *A* and *B*, and the quantification of the results are shown in (*C*). n = 5. (*D*) Normal HPASMC transfected with siPDLIM5 or siNeg were used for the bromodeoxyuridine (BrdU) incorporation assay. The assay was repeated five times independently and was performed in triplicates. (*E*) Representative images of immunofluorescence costaining of α-SMA and 4′,6-diamidino-2-phenylindole (DAPI) in HPASMC transfected with siPDLIM5 or siNeg. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

Dysregulation of the miR-17∼92 Cluster in Lungs of Patients with PAH and Lungs of Mice Exposed to Hypoxia

We obtained HPASMC samples from normal donors, patients with idiopathic PAH (IPAH), and PAH associated with other diseases (APAH) (Table E2) and found that IPAH and APAH HPASMC expressed lower levels of the miR-17∼92 cluster than normal HPASMC (Figure E7A). In normal HPASMC, levels of miR-17∼92 were induced after exposure to 3% O_2 for 6 hours but decreased after 24 hours of hypoxic exposure (Figures E7B and E7C). In lungs of C57BL/6 mice, prolonged hypoxic exposure (10% O_2 for 3 wk) induced PH (Figure E7D) but repressed miR-17∼92 expression (Figure E7F), whereas short-term exposure to hypoxia (10% O_2 for 1 wk) caused an induction of miR-17∼92 (Figure E7E). In mPASMC, hypoxia (3% O_2) induced miR-17∼92 expression (except miR-92a) at 6 hours of exposure, whereas it decreased miR-17∼92 levels at 24 hours (Figure E7G). Hypoxia reduced levels of miR-143 and miR-139-3p and induced miR-210 expression (34) without altering miR-145 levels (Figure E8). These results suggest a biphasic regulation of miR-17∼92 expression during hypoxia and PH: an early transient induction and a late phase reduction.

Overexpression of miR-17∼92 Restores SMC Phenotype in HPASMC Isolated from Patients with IPAH

Previous reports suggest a dedifferentiated PASMC in PAH (4, 7, 35). We found that the expression levels of α-SMA, calponin, SM22α, and serum response factor were significantly decreased in IPAH HPASMC, myocardin levels were not significantly different (Figures E9A and E9B), and MHC levels were up-regulated (Figures E9C and E9D). There was no significant difference in PCNA expression levels, BrdU incorporation, and cell cycle parameters between normal and IPAH HPASMC (Figures E9E–E9H and E10), suggesting that cell proliferation rate is not altered in IPAH HPASMC. Taken together, these results suggest that the reduced expression of miR-17∼92 correlates with a dedifferentiated phenotype in IPAH HPASMC.

To investigate whether down-regulation of miR-17∼92 is responsible for the altered phenotype of IPAH HPASMC, we infected IPAH HPASMC with lentiviruses encoding the miR-17∼92 cluster to overexpress the miR-17∼92 cluster and confirmed the overexpression of the miR-17∼92 cluster by qRT-PCR (Figure E9I). We found that miR-17∼92 overexpression restored the expression of α-SMA, SM22α, and calponin in IPAH HPASMC without changing myocardin levels (Figures E9J and E9K) or cell proliferation (Figure E9L), suggesting that miR-17∼92 overexpression can rescue SMC phenotype in IPAH HPASMC.

PDLIM5 Is Up-regulated in Hypertensive Human and Mouse PASMC

Next we investigated the levels of PDLIM5 in APAH and IPAH HPASMC. We found that HPASMC of patients with APAH but not IPAH expressed significantly elevated levels of PDLIM5 compared with control subjects (Figures 6A–6C; Figure E11). We did not detect obvious difference in PDLIM5 levels between sm-17∼92$^{-/-}$ mice and their wild-type littermates exposed to normoxia and hypoxia using two commercial PDLIM5 antibodies and two staining methods (immunohistochemistry and immunofluorescence staining) (Figure E12), possibly due to the limitation of the

antibodies. Nonetheless, mPASMC isolated from hypoxic mice had elevated PDLIM5 levels (Figure 6D), and hypoxic exposure for 1 week or 3 weeks increased PDLIM5 in lungs of C57BL/6 mice (Figures 6E–6H). Hypoxia increased the expression of PDLIM5 in normal mPASMC and HPASMC (Figures 6I and 6J). $CoCl_2$, which stabilizes hypoxia-inducible factor (HIF) in normoxic conditions, also induced PDLIM5 expression in HPASMC (Figure 6K; Figures E13A and E13B). HIF1α silencing but not HIF2α silencing decreased PDLIM5 levels (Figures 6L and 6M; Figures E13C and E13D) (36), suggesting a specific role of HIF1 in the induction of PDLIM5.

miR-17∼92 Regulates SMC Marker Expression Mainly via TGF-β_3/Smad3 Signaling

Because TGF-β is also known to maintain the differentiated phenotype of SMC (37–41), we investigated whether miR-17∼92 regulates SMC phenotype via a TGF-β–dependent pathway. In normal HPASMC, overexpression of miR-17∼92 up-regulated mRNA levels of TGF-β_1, TGF-β_2, TGF-β_3, and TGF-β receptor 1 (TβR1), decreased the levels of TβR2 and TβR3 (Figure 7A), and increased TGF-β activity in the culture media (Figure 7B). IPAH HPASMC expressed significantly lower mRNA levels of TGF-β_2, TGF-β_3, TβR1, and

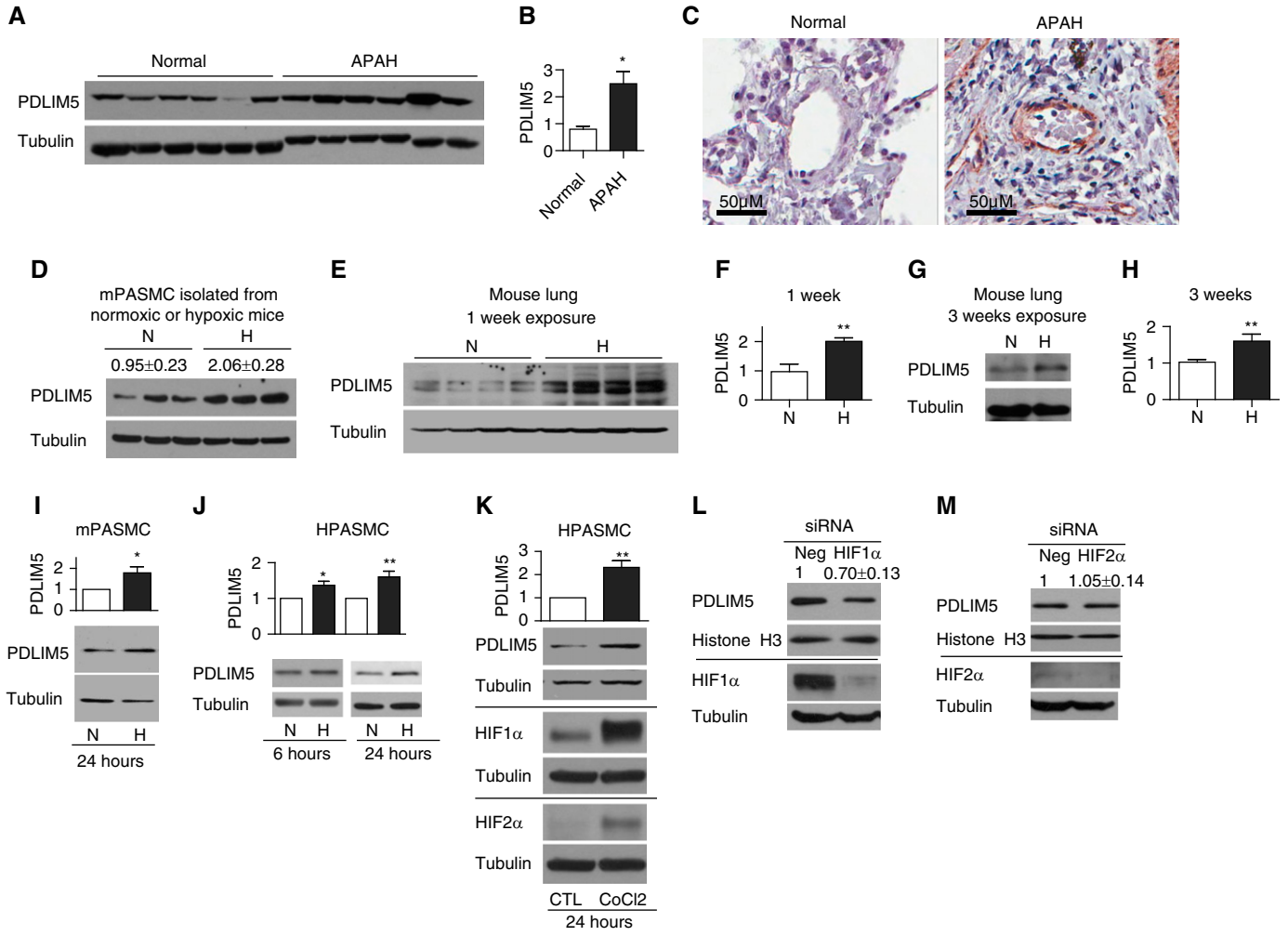

Figure 6. PDZ and LIM domain protein 5 (PDLIM5) is up-regulated in human and mouse hypertensive pulmonary artery smooth muscle cells (PASMC). (*A*, *B*) Protein levels of PDLIM5 in human PASMC (HPASMC) samples (normal HPASMC, n = 6; pulmonary arterial hypertension associated with other diseases [APAH] HPASMC, n = 6) (*A*). PDLIM5 levels were normalized to tubulin, and the relative amount of PDLIM5 in APAH HPASMC was compared with that in normal HPASMC. The quantification of PDLIM5 is shown in (*B*). (*C*) Normal and APAH lung sections were stained with PDLIM5, and the representative images are shown. (*D*) C57BL/6 mice were exposed to normoxia (N) or hypoxia (10% O_2, H) for 3 weeks, and mouse PASMC (mPASMC) were isolated (three mice each group) to measure the levels of PDLIM5. Representative blots are shown in (*D*), and quantification is shown above the blots. (*E–H*) PDLIM5 levels in the whole lung tissue of mice exposed to normoxia or hypoxia for 1 week (*E*, *F*) or 3 weeks (*G*, *H*). Representative blots are shown in *E* and *G* and quantification in *F* and *H*, respectively. There were four mice in each group. (*I*) mPASMC isolated from C57BL/6 mice were exposed to normoxia or hypoxia (3% O_2) for 24 hours and used to determine protein levels of PDLIM5. n = 5. (*J*, *K*) Normal HPASMC were exposed normoxia or hypoxia (3% O_2) for 6 or 24 hours (*J*) or treated with 250 μM $CoCl_2$ for 24 hours (*K*). Protein levels of PDLIM5, hypoxia-inducible factor 1α (HIF1α), or HIF2α were measured. n = 5. (*L*, *M*) Normal HPASMC were transfected with siRNAs against HIF1α or HIF2α, and the protein levels of PDLIM5 were determined as above. n = 3. Tubulin was used as the loading control in these blots. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. CTL = control.

TβR3; however, the mRNA levels of TGF-β_1, $T\beta R_2$, and BMP receptor 2 were unchanged (Figure 7C). Overexpression of miR-17∼92 in IPAH HPASMC up-regulated the TGF-β_3 mRNA level and TGF-β activity in the culture media, did not change mRNA levels of TGF-β_1 and TβR1, and decreased levels of TGF-β_2, TβR2, and TβR3 (Figures 7D and 7E). In IPAH HPASMC, treatment of TGF-β_3 with 5 ng/ml TGF-β_3 for 24 hours significantly induced the expression of α-SMA, SM22α, and calponin, whereas myocardin levels remained unchanged (Figures 7F and 7G). Conversely, TGF-β_3 silencing reduced expression levels of calponin but not those of α-SMA and SM22α (Figure E14). Taken together, these results suggest that although miR-17∼92 can act on multiple TGF-β and TGF-β receptors, it mainly induces TGF-β_3 signaling to restore the differentiated SMC phenotype in IPAH HPASMC.

TGF-β initiates signal transduction via TGF-β receptor-mediated activation of the Smad proteins (42–44). We showed that in IPAH HPASMC, overexpression of miR-17∼92 significantly increased phosphorylation of Smad2 and phosphorylated and total Smad3 levels (Figures 8A and 8B). Consistently, in mPASMC, knockout of miR-17∼92 slightly decreased PCNA expression and significantly decreased expression levels of total Smad2 and Smad3 (Figures E15A–E15D). However, Smad2/3 levels were not significantly different between normal and IPAH HPASMC (Figure E16).

Knockdown of Smad3 repressed miR-17∼92-induced expression of α-SMA and SM22α (Figures 8C and 8E), whereas knockdown of Smad2 had no effect on miR-17∼92-induced expression of α-SMA and SM22α (Figures 8D and 8F). Neither Smad2 siRNA nor Smad3 siRNA changed myocardin levels (Figures 8C–8F). These results suggest that miR-17∼92 can act at multiple levels in the TGF-β_3/Smad3 signaling pathway and that the TGF-β_3/Smad3 pathway is the main pathway that is involved in the rescue of SMC differentiated phenotype by miR-17∼92 in IPAH HPASMC.

PDLIM5 Suppresses SMC Marker Expression via TGF-β_3/Smad3 Signaling

To investigate whether PDLIM5 regulates TGF-β/Smad signaling in

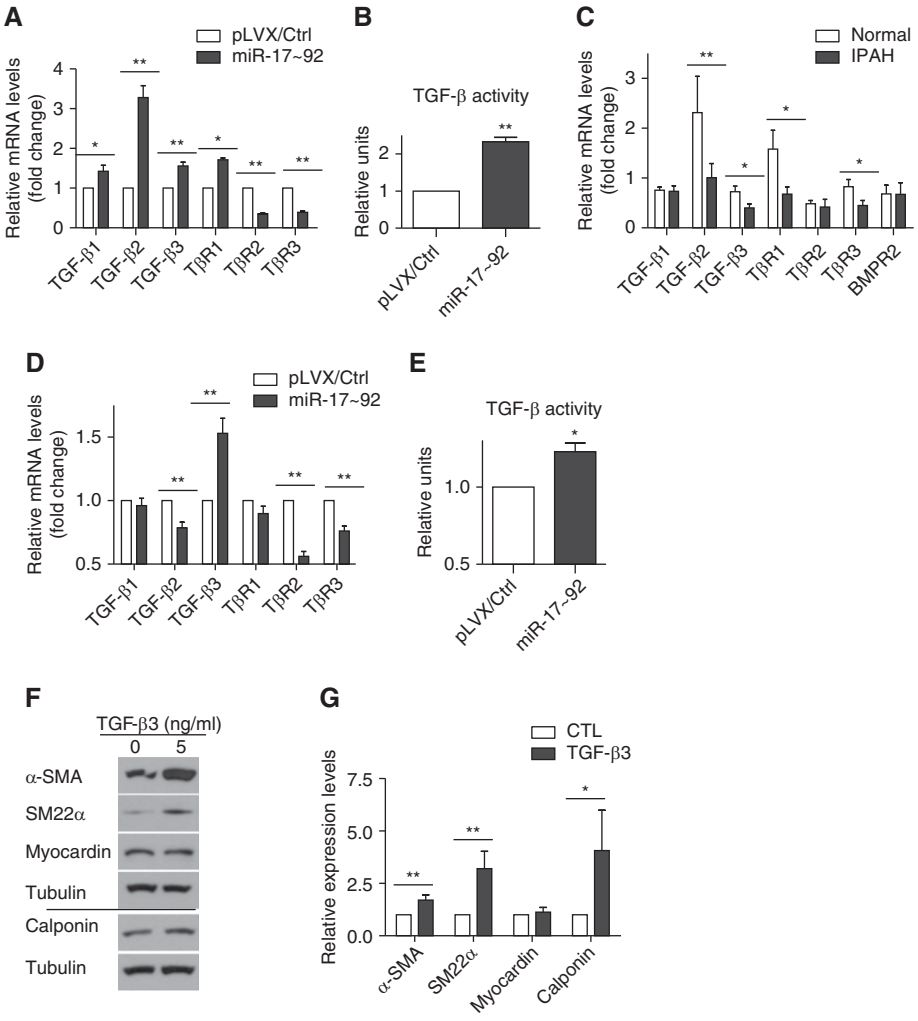

Figure 7. MicroRNA (miR)-17∼92 regulates smooth muscle cell (SMC) marker expression mainly via transforming growth factor (TGF)-β_3 signaling. (*A*) The expression levels of TGF-β and their receptors were determined by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis using normal human pulmonary artery smooth muscle cells (HPASMC) infected with lentiviruses containing pLVX/Ctrl or miR-17∼92. The levels of these mRNAs were normalized to those of normal HPASMC infected with lentiviral vector (pLVX/Ctrl). Five independent preparations of lentiviral-infected cells for each virus were used in this assay, and qRT-PCR was conducted in triplicates. (*B*) The same number of normal HPASMC infected with lentiviruses containing pLVX/Ctrl or miR-17∼92 were cultured with the same volume of media. The conditioned media was used to culture the mink lung epithelial cell luciferase reporter cells for the measurement of TGF-β activity. Three independent experiments were repeated and conducted in triplicates. (*C*) mRNA levels of the indicated genes between HPASMC samples (normal HPASMC, n = 9; idiopathic pulmonary arterial hypertension [IPAH]: n = 13). The expression levels of those genes in IPAH HPASMC were compared and normalized to those of normal HPASMC. (*D*) IPAH HPASMC were infected with lentiviruses containing pLVX/Ctrl or miR-17∼92 and were selected. The expression levels of TGF-$\beta_{1/2/3}$ and TβR1/2/3 were determined by qRT-PCR analysis and normalized to those of IPAH HPASMC infected with lentiviral vector (pLVX/Ctrl). At least three independent preparations of lentiviral-infected cells for each virus were used in this assay, and qRT-PCR was conducted in triplicates. (*E*) We collected conditioned media from IPAH HPASMC infected with lentiviruses containing pLVX/Ctrl or miR-17∼92 and determined TGF-β activity as described in *B*. Three independent experiments were repeated and conducted in triplicates. (*F*, *G*) IPAH HPASMC were treated with TGF-β_3 (5 ng/ml) for 24 hours, and cell lysates were used to determine the expression levels of α-smooth muscle actin (α-SMA), SM22α, calponin, and myocardin by Western blot analysis. Tubulin was used as the loading control. The representative blots are shown in *F* and the quantification in *G*. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. CTL = control.

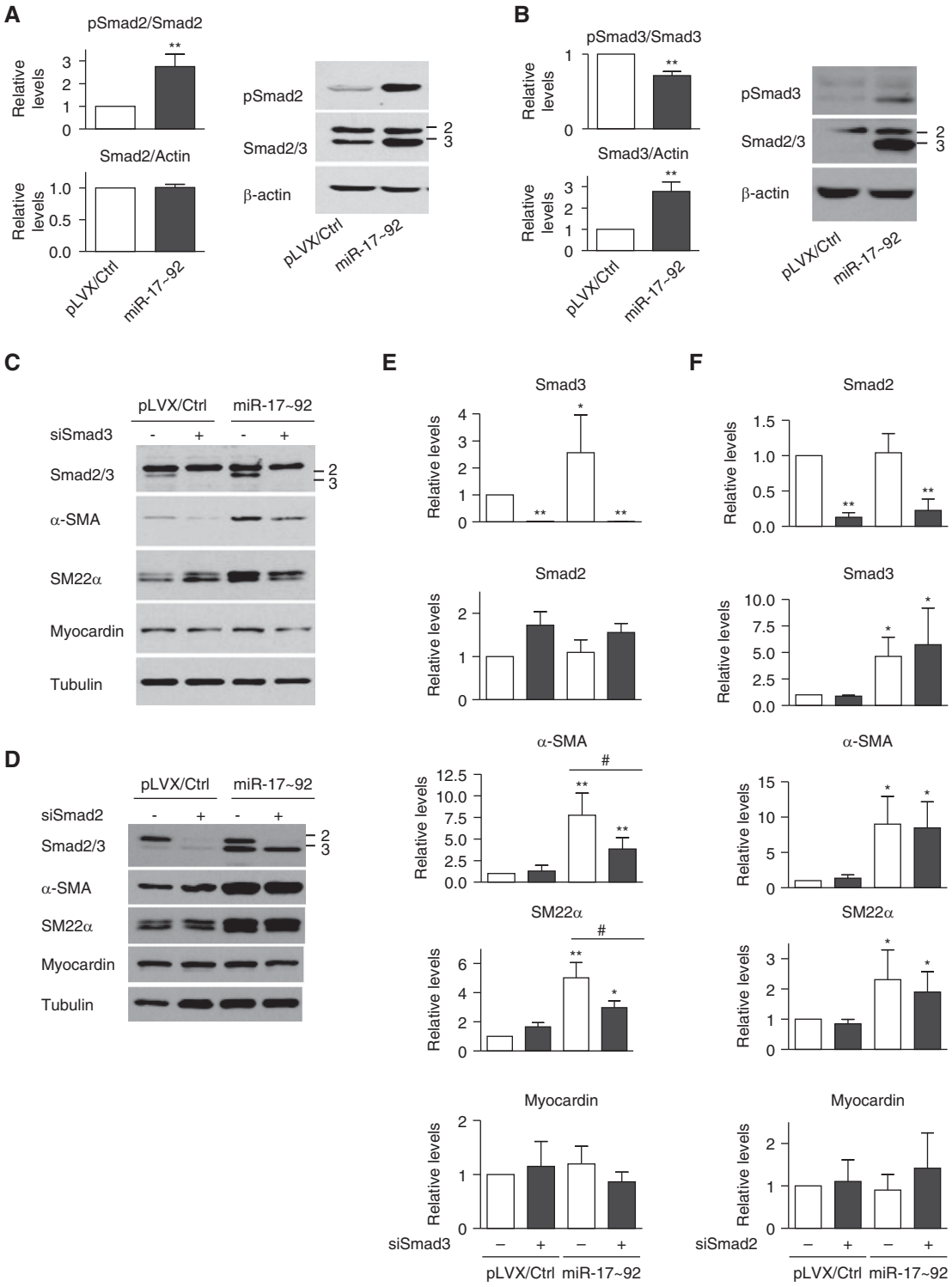

Figure 8. MicroRNA (miR)-17∼92 regulates smooth muscle cell (SMC) marker expression mainly via Smad3 signaling. (*A*, *B*) Idiopathic pulmonary arterial hypertension (IPAH) human pulmonary artery smooth muscle cells (HPASMC) infected with lentiviruses containing pLVX/Ctrl or miR-17∼92 were lysed, and the pSmad2 and total Smad2 (*A*) and the pSmad3 and total Smad3 (*B*) were determined by Western blot analysis with β-actin as the loading control. The ratios of pSmad2/Smad2, Smad2/β-actin (*A*), pSmad3/Smad3 and Smad3/β-actin (*B*) were calculated and compared with that of IPAH HPASMC infected with lentiviral vector (pLVX/Ctrl). Five independent preparations of lentiviral-infected cells for each virus were used in this assay. (*C*) IPAH HPASMC infected with lentiviruses containing pLVX/Ctrl or miR-17∼92 were transfected with negative control siRNA or siRNA against Smad3 (siSmad3). Forty-eight hours after transfection, the cell lysates were collected and used to determine the amount of the indicated proteins by Western blot analysis. Tubulin was used as the loading control. The representative blots are shown in *C* and the quantification in *E*. (*D*) We repeated the experiments

HPASMC, we suppressed PDLIM5 with siRNA and measured the expression levels of TGF-$\beta_{1/2/3}$ and their receptors. We found that suppression of PDLIM5 induced TGF-β_3 and TβR1, whereas levels of TGF-β_1, TGF-β_2, TβR2, and TβR3 remained unchanged (Figure 9A). Furthermore, suppression of PDLIM5 increased TGF-β activity in the culture media (Figure 9B) and induced expression levels of total Smad2 but not Smad3 and phosphorylated Smad2 and Smad3 (Figures 9C and 9D). Moreover, suppression of PDLIM5 enhanced nuclear staining of Smad2/3 (Figure E17). These results suggest that PDLIM5 negatively regulates TGF-β_3/Smad2/3 signaling.

SMC-Specific Knockout of PDLIM5 Enhances Hypoxia-mediated Vascular Remodeling

To investigate the role of PDLIM5 *in vivo*, we generated a strain of SMC-specific PDLIM5 knockout (sm-PDLIM5$^{-/-}$) mouse (Figure 10A) by crossbreeding the sm22α-Cre mouse with the PDLIM5$^{fl/fl}$ mice (22). sm-PDLIM5$^{-/-}$ mice and their wild-type littermates were exposed to normoxia or hypoxia for 4 weeks. We found that SMC-specific knockout of PDLIM5 did not affect hypoxia-induced RVSP (Figure 10B) or RV hypertrophy (Figure 10C) but enhanced hypoxia-mediated vascular remodeling (Figure 10D), suggesting that PDLIM5 is a negative regulator of hypoxia-induced PH.

Overexpression of PDLIM5 Reduces Hypoxia-induced PH

To address whether induction of PDLIM5 can be used as a strategy to treat PH, we explored the effect of overexpression of PDLIM5 in hypoxia-induced PH. We constructed a PDLIM5-V5-His plasmid in which the epitope-tagged PDLIM5 was readily detectable when transfected into HEK293 cells (Figure 11A). We adopted a liposome-plasmid mixture delivery system for the vasculature as described previously (45–47). We mixed the vector or PDLIM5-V5-His plasmids (50 μg) with 100 μl of liposomes and injected it into the tail vein of the mouse (23–25) 1 day before exposure to normoxia or hypoxia and then injected the plasmid/liposome mixture once a week during the experimental period. Overexpression of PDLIM5 was confirmed in the whole lung of these mice (Figure 11B). We found that overexpression of PDLIM5 attenuated hypoxia-induced elevation of RVSP, RV hypertrophy, and arterial wall remodeling (Figures 11B–11E), confirming results in Figure 10.

Discussion

In this study, we investigated the contribution of SMC-specific miR-17∼92 in the pathogenesis of PH using a genetic approach. We report here that SMC-specific knockout of miR-17∼92 attenuates hypoxia-induced PH in mice (Figure 1), and reconstitution of miR-17∼92 restores hypoxia-induced PH in miR-17∼92 knockout mice (Figure 3). However, overexpression of miR-17∼92 mimics was not sufficient to induce PH under normoxic conditions (Figure 3). These results suggest that SMC-specific miR-17∼92 is necessary but not sufficient in the development of PH. Our study expands and complements the previous study by Pullamsetti and colleagues (19). These data also suggest that miR-17∼92 in other cells may also contribute to PH. Brock and colleagues reported that miR-17∼92 directly suppress BMP receptor 2 in endothelial cells and increase endothelial cell proliferation and resistance to apoptosis (48), suggesting a potential contribution of endothelial miR-17∼92 in PH.

Interestingly, miR-17∼92 expression is biphasic in hypoxia: an early transient induction and a late reduction in expression in mouse lungs, mPASMC, and HPASMC (Figure E7), which is also consistent with a previous report (19). This biphasic expression of miR-17∼92 in PAH is similar to its expression pattern during development (29) and T-cell activation and differentiation (49). Thus, the early transient induction of miR-17∼92 may reflect the reactivation of this developmental program in the pathogenesis of PAH. Similarly, reactivation of Rho/Rho-kinase signaling (50) and Notch3 in PASMC is reported to promote PAH (51). It was a bit puzzling how the reduction in miR-17∼92 levels during the later stage of hypoxic exposure fit into the etiopathology of PH, given that miR-17∼92 inhibitors can reverse existing PH (19). However, we found that miR-17/20a directly suppressed PDLIM5 (Figure 4; Figures E4 and E5), and in the late stage of hypoxia, miR-17∼92 is down-regulated, whereas PDLIM5 is up-regulated concomitantly (Figures E6 and E7). Moreover, overexpression of PDLIM5 inhibited hypoxia-induced PH (Figure 11). These results provide an explanation for the biphasic regulation of miR-17∼92 and reduced levels of miR-17∼92 in patients with PAH (Figure E7): up-regulation of miR-17∼92 may be the first common step in the development of both clinical and experimental PH, and 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.

Although others have reported that IPAH PASMC are in a dedifferentiated and proliferative state (7), we did not observe any difference in proliferation measured by multiple methods between normal and IPAH PASMC (Figures E9 and E10). This seeming discrepancy between our findings and those of others emphasizes the complexity involved in the study of PAH. Some possible explanations are: (*1*) variability in culture conditions of PASMC studies, such as growth factors, passage, etc.; (*2*) high diversity of patients with IPAH and therefore in the PASMC isolated from them, as evidenced by the high level of variability among cells from different individuals (Figures E9, E11, and E16). We used cells at five to seven passages in studies comparing protein expression between normal, IPAH, or APAH PASMC to maintain PASMC phenotype as in similar studies (52, 53). We found that normal PASMC maintain expression levels of vimentin and calponin well through p12 (there is an increase of calponin from p10) with loss of MHC at p11. APAH PASMC demonstrate a gradual decrease in expression of these proteins from p5 to p7. From p5 to p8, IPAH PASMC appears to

Figure 8. (Continued). in *C* with negative control siRNA or siRNA against Smad2. The representative blots are shown in *D* and the quantification in *F*. Five independent experiments were conducted (*C–F*). Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.05$; $^{**}P < 0.01$. α-SMA = α-smooth muscle actin.

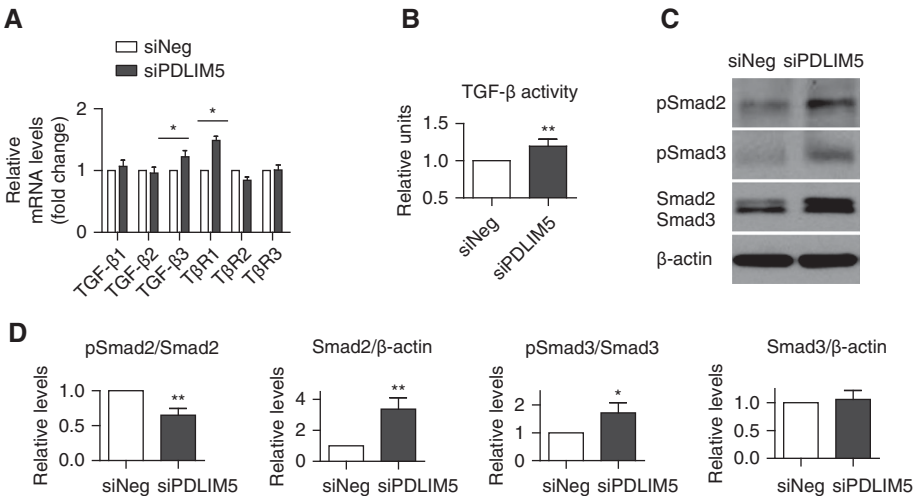

Figure 9. PDZ and LIM domain protein 5 (PDLIM5) negatively regulates transforming growth factor (TGF)-β_3/Smad3 signaling. Normal human pulmonary artery smooth muscle cells (HPASMC) were transfected with siRNA against PDLIM5 (siPDLIM5) or negative control (siNeg) and incubated for 48 hours. The mRNA expression levels of TGF-β and their receptors were determined using normal HPASMC transfected with siNeg as control (*A*). n = 9. The culture media was used to culture the mink lung epithelial cell luciferase reporter cells for the measurement of TGF-β activity (*B*). The levels of pSmad2, pSmad3, and total Smad2/3 were determined by Western blot analysis with β-actin as loading control (*C*). The quantification of 10 independent experiments is shown in (*D*). Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

maintain MHC levels and gradually lose vimentin and calponin (although there is an increase from p5 to p6) (Figure E18). Thus, we need to be cautious when interpreting any results from HPASMC samples. On the other hand, these observations suggest that normal, IPAH, and APAH PASMC are very different from each other, as also reported by Morrell and colleagues (53). This notion is also supported by our results reported here that overexpression of miR-17∼92 induces proliferation and expression of SMC markers in normal PASMC, whereas in IPAH PASMC, overexpression of miR-17∼92 increases SMC marker expression but has no effect on proliferation (Figure 2; Figure E9). These results suggest that there are additional mechanisms controlling the proliferation of IPAH PASMC, largely due to genetic and epigenetic changes that occur in PAH (54).

Our results suggest that miR-17∼92 regulates SMC phenotype via a myocardin-independent but TGF-β/Smad-dependent pathway (Figures 2, 7, and 8; Figures E7, E9, and E15). Interestingly, overexpression of miR-17∼92 only rescues TGF-β_3 expression in IPAH HPASMC (Figure 7D), and TGF-β_3 is sufficient to restore the expression of SMC markers in IPAH HPASMC (Figure 7F), suggesting that TGF-β_3 is primarily responsible for the dedifferentiation of IPAH HPASMC. Moreover, overexpression of miR-17∼92 induces Smad3 expression (Figure 8B). Suppression of Smad3 but not Smad2 prevents miR-17∼92-mediated restoration of SMC markers (Figures 8C–8F), suggesting a specific role of TGF-β_3/Smad3 in the altered phenotype of diseased HPASMC. Indeed, silencing of TGF-β_3 reduces expression levels of calponin (Figure E14). Interestingly, although IPAH PASMC express less miR-17∼92 and TGF-β (Figures 6 and 8), IPAH PASMC do not have a concomitant decrease in Smad2/3 (Figure E16), suggesting that multiple signaling pathways may contribute to the regulation of TGF-β/Smad signaling, as also evidenced in other cells (55, 56). Although our results suggest a positive regulation of TGF-β/Smad by miR-17∼92, recently there were reports suggesting that miR-17∼92 negatively regulates TGF-β_1/Smad pathway in gastrointestinal tumors and neuroblastoma (55, 56). Thus, miR-17∼92 may regulate TGF-β/Smad in a cell type–specific, disease-specific, or isoform-specific fashion. Nonetheless, our study indicates for the first time that miR-17∼92 is also a key regulator of cell differentiation, particularly PASMC differentiation.

In an attempt to identify the mechanism underlying the positive regulation of TGF-β_3/Smad3 by miR-17∼92 in PASMC, we found that miR-17/20a directly suppressed PDLIM5 (Figure 4; Figures E4 and E5), a member of the PDZ-LIM protein family, that acts as a signal modulator to influence organ development and different disease states (57, 58). PDLIM5 acts as an adaptor protein to

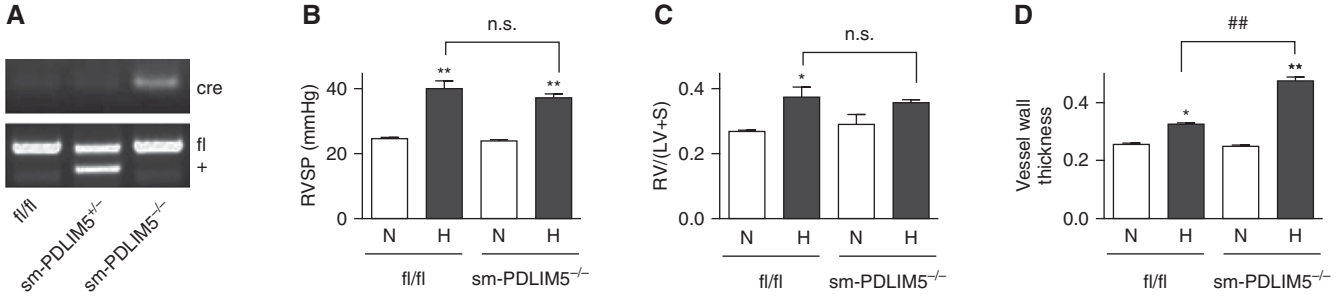

Figure 10. Smooth muscle cell (SMC)-specific knockout of PDZ and LIM domain protein 5 (PDLIM5) enhances hypoxia-induced pulmonary vascular remodeling in mice. (*A*) Genotyping of SMC-specific PDLIM5 knockout mice and their wild-type littermates. (*B–D*) sm-PDLIM5$^{-/-}$ mice (n = 4) and their wild-type littermates (n = 4) were exposed to normoxia (N) or hypoxia (H, 10% O_2) for 4 weeks, and then we measured right ventricular systolic pressure (RVSP) (*B*), right ventricle/(left ventricle + septum) [RV/(LV + S)] ratio (*C*), and pulmonary arterial wall thickness (*D*) in these mice. At least 15 vessels were studied in each mouse for the wall thickness analysis. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#\#}P < 0.01$; n.s. = not significant.

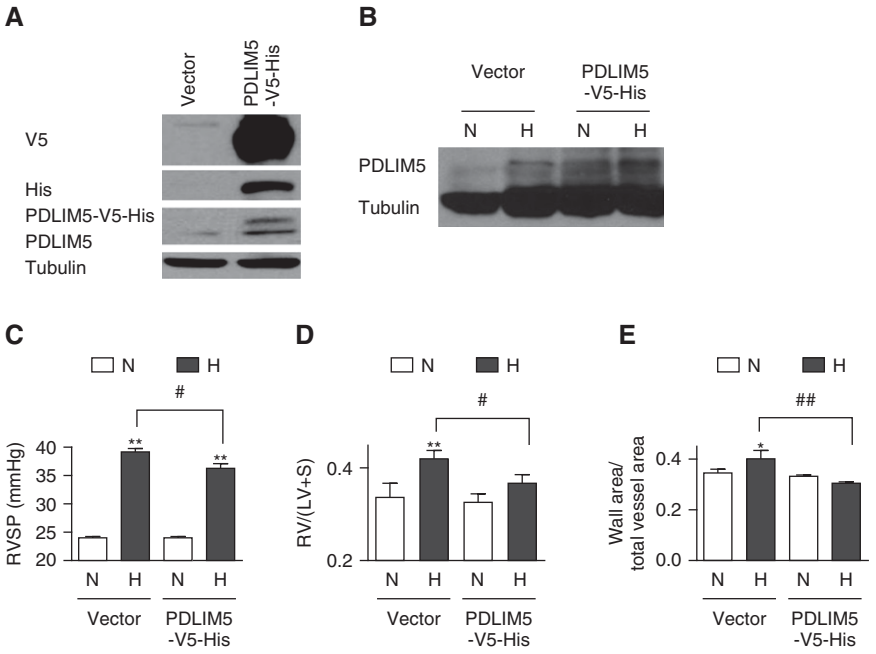

Figure 11. Overexpression of PDZ and LIM domain protein 5 (PDLIM5) inhibits hypoxia-induced pulmonary hypertension. (*A*) PDLIM5-V5-His plasmid and vector were transfected to HEK293 cells, and the expression levels of endogenous and epitope-tagged PDLIM5 were determined by Western blot analysis. (*B–E*) We injected the mixture of the vector or PDLIM5-V5-His plasmids with liposomes into the mouse tail vein 1 day before exposure. Additional injections were given once a week during the experimental period. One day after the final administration of plasmid/liposome mixture, we measured the expression levels of PDLIM5 in mouse lungs by Western blot analysis; representative blots (*B*), right ventricular systolic pressure (RVSP) (*C*), RV hypertrophy (*D*), and pulmonary arterial wall thickness (*E*). There were at least five mice in each group. H = hypoxia; LV = left ventricle; N = normoxia; RV = right ventricle; S = septum. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.05$; $^{**}P < 0.01$; $^{\#\#}P < 0.01$; * and ** indicate the comparison between normoxic mice injected with vector and other groups. # and ## indicate the comparison between hypoxic groups.

sequester transcription factors in the cytoplasm or interacts with kinases to exert its effects (57, 59–62). Consistent with decreased miR-17∼92 in APAH PASMC, PDLIM5 is up-regulated in APAH but not in IPAH PASMC (Figure 6; Figure E11). Lack of significant elevation of PDLIM5 in IPAH PASMC may be due to the higher variability in the IPAH pool (Figure E11). Although miR-17∼92 can directly target and suppress PDLIM5 (Figure 4), the expression levels of miR-17∼92 and PDLIM5 do not appear to be inversely related all the time: although in the late stage of hypoxia, miR-17∼92 is down-regulated and PDLIM5 is up-regulated, in the early stage of hypoxia, both miR-17∼92 and PDLIM5 are up-regulated (Figures E6 and E7). This discrepancy suggests an miR-17∼92-independent mechanism for the induction of PDLIM5, but which may be HIF dependent (Figures 6L and 6M). Although the involvement of HIF (63–65) and miR-17∼92 (19) in PAH is clear, the understanding of the contribution of PDLIM5 in PAH needs further studies.

PDLIM5 negatively regulates TGF-β_3/Smad3 pathway and expression of SMC markers (Figures 5 and 9). Interestingly, suppression of PDLIM5 induces expression levels of total Smad2 but not Smad3 and phosphorylated Smad2 and Smad3 (Figures 9C and 9D), which is different from overexpression of miR-17∼92-mediated induction of total Smad3 and phosphorylated Smad2 and Smad3 (Figure 8), suggesting that although loss of PDLIM5 and overexpression of miR-17∼92 both lead to an increase in phosphorylated Smad2 and Smad3, they also have distinct roles in specifically regulating Smad2 or Smad3, respectively. Overall, our results suggest that miR-17∼92 up-regulates TGF-β_3/Smad3 via suppression of PDLIM5. Thus, miR-17∼92 regulates SMC phenotype mainly via a PDLIM5/TGF-β_3/Smad3-dependent pathway. Although the mechanism underlying PDLIM5-mediated TGF-β/Smad signaling is not clear, we found that suppression of PDLIM5 increases nuclear staining of Smad2/3 (Figure E17). Thus, PDLIM5 may act as an adaptor protein to regulate Smad nuclear/cytosol shuttle and subsequent expression of SMC markers. Interestingly, PDLIM5 can bind to Inhibitors of Differentiation 2 (ID2) and sequester it in the cytoplasm, inhibiting Id2 activity (59, 60). Because ID family proteins are well implicated in BMP signaling and PAH (66, 67), it will be of interest to investigate whether PDLIM5 regulates Smad nuclear/cytosol in an ID-dependent pathway. Alternatively, PDLIM5 may regulate Smad phosphorylation by protein kinase C, as PDLIM5 also recruit activated protein kinase C and its substrates to promote phosphorylation (57, 61, 62). Further studies are needed to address these possibilities.

TGF-β/Smad2/3 signaling is known to promote PH and inhibit BMP/Smad1/5/8 signaling, which is abnormal in hereditary PAH (68–70). Thus, loss of PDLIM5, a negative regulator of TGF-β/Smad2/3, should enhance PH, and overexpression of PDLIM5 should inhibit PH. Indeed, our results showed that SMC-specific knockout of PDLIM5 enhanced hypoxia-induced pulmonary vascular remodeling, and overexpression of PDLIM5 inhibited hypoxia-induced PH (Figures 10 and 11). However, it appears that induction of endogenous PDLIM5 may not be strong enough to inhibit hypoxia-induced PH (Figures 6 and 11), which is consistent with the notion that PAH is a progressive disease. Importantly, by enhancing this endogenous adaptive induction of PDLIM5, we can inhibit hypoxia-induced PH. Thus, agents that amplify PDLIM5 induction or PDLIM5 signaling may be used as novel therapeutic agents for the treatment of PAH.

In summary, miR-17∼92, particularly miR-17/20a, directly targets and suppresses PDLIM5, leading to increased expression of TGF-β and Smad activation, resulting in pulmonary hypertension. These data are supported by a recent report in which inhibition of miR-17 inhibits hypoxia-induced PH by induction of p21 (19). miR-17∼92/PDLIM5/TGF-β/Smad may represent a novel signaling pathway and may serve as a therapeutic target for the treatment of PAH. Further studies on how miR-17∼92 expression is regulated and how PDLIM5 regulates TGF-β/Smad signaling are warranted. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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