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Translational Physiology

## Micro-RNA-1 is decreased by hypoxia and contributes to the development of pulmonary vascular remodeling via regulation of sphingosine kinase 1

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### Abstract

Sphingosine kinase 1 (SphK1) upregulation is associated with pathologic pulmonary vascular remodeling in pulmonary arterial hypertension (PAH), but the mechanisms controlling its expression are undefined. In this study, we sought to characterize the regulation of SphK1 expression by micro-RNAs (miRs). In silico analysis of the SphK1 3'-untranslated region identified several putative miR binding sites, with miR-1-3p (miR-1) being the most highly predicted target. Therefore we further investigated the role of miR-1 in

primary artery smooth muscle cells (PASMCs) and the development of experimental pulmonary hypertension in vivo. Our results demonstrate that miR-1 is downregulated by hypoxia in PASMCs and can directly inhibit SphK1 expression. Overexpression of miR-1 in human PASMCs inhibits basal and hypoxia-induced proliferation and migration. Human PASMCs isolated from PAH patients exhibit reduced miR-1 expression. We also demonstrate that miR-1 is downregulated in mouse lung tissues during experimental hypoxia-mediated pulmonary hypertension (HPH), consistent with upregulation of SphK1. Furthermore, administration of miR-1 mimics in vivo prevented the development of HPH in mice and attenuated induction of SphK1 in PASMCs. These data reveal the importance of miR-1 in regulating SphK1 expression during hypoxia in PASMCs. A pivotal role is played by miR-1 in pulmonary vascular remodeling, including PASMC proliferation and migration, and its overexpression protects from the development of HPH in vivo. These studies improve our understanding of the molecular mechanisms underlying the pathogenesis of pulmonary

hypertension.

**Keywords:** hypoxia, micro-RNA-1, pulmonary hypertension, sphingosine kinase 1

## INTRODUCTION

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Pulmonary arterial hypertension (PAH) is a progressive and fatal disease for which the pathogenic mechanisms are poorly understood and no curative treatments are available (40). PAH is characterized by increases in pulmonary vascular resistance primarily due to uncontrolled pulmonary vascular remodeling, sustained vasoconstriction, or thrombosis in situ (15, 27). Rampant pulmonary artery smooth muscle cell (PASMC) proliferation and apoptosis resistance contribute to increases in pulmonary vascular resistance and pulmonary artery pressure (58), resulting in eventual right ventricular hypertrophy and heart failure, the major cause of death in PAH. Most current PAH therapies are limited to targeting pulmonary vasoconstriction, and patients continue to have a poor long-term prognosis (26). Thus there is an imperative need to understand the mechanisms contributing to pulmonary vascular remodeling to develop new therapies.

The significance of sphingolipid signaling in pulmonary disease has recently emerged. Our group and others have demonstrated that the bioactive lipid sphingosine-1-phosphate (S1P) is involved in the pathogenesis of pulmonary fibrosis and bronchopulmonary dysplasia and in regulation of the vascular endothelial barrier during acute lung injury (14, 18, 22, 41, 43, 48, 50). S1P mediates many important biological functions including cell proliferation, differentiation, motility, and resistance to apoptosis (12), and its synthesis in the lungs and blood is predominantly regulated by sphingosine kinase 1 (SphK1; 17). SphK1, a highly conserved, oncogenic enzyme, has itself been implicated in the promotion of cell proliferation, apoptosis resistance, and angiogenesis (36, 51), and preclinical studies have shown the efficacy of SphK1 inhibition in decreasing tumor size (16, 31). We have recently demonstrated that SphK1 and S1P are elevated in PAH and promote human PASMC (hPASMC) proliferation (7, 52). Additionally, we found that genetic deficiency or pharmacologic inhibition of SphK1 in vivo protects against the development of hypoxia-induced experimental pulmonary hypertension in mice (7). However, the mechanisms involved in the hypoxia-mediated upregulation of *SPHK1* expression in PAH and its contribution to hPASMC proliferation are presently not well understood. In this study, we aimed to identify novel molecular mechanisms of hypoxia-induced hPASMC proliferation, focusing on the role of SphK1 expression and its regulation by micro-RNAs (miRs).

As small, noncoding RNAs, miRs play important regulatory roles in animals and plants by base pairing with complementary sequences within mRNA molecules, resulting in cleavage, translational repression, or destabilization (1, 3, 42). The importance of miRs in modulating complex gene expression networks (32) and their contributions to human disease pathogenesis have emerged, including in the field of cardiovascular biology (49). Recent studies have identified several miRs associated with cell-specific phenotypes in PAH and experimental models of pulmonary hypertension (4, 63). A systems-level regulation of micro-RNA networks by miR-130/301 and their targeting of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) has been shown to promote pulmonary hypertension by distinct modulation of both PASMCs and pulmonary artery endothelial cells (PAECs; 4). Another recent study demonstrated that both miR-138 and miR-25 downregulate the mitochondrial calcium uniporter complex (MCUC), causing a cancerlike proliferative phenotype in PASMCs in PAH (25). In addition, the bone morphogenetic protein (BMP) signaling pathway, which has been widely shown to modulate vascular remodeling in PAH, was recently found to be controlled by miR-140-5p (46). The regulation of miR expression by hypoxia and their involvement in modulating the phenotype of smooth muscle cells (SMCs) have also been described (6, 8).

Arterial SMCs, unlike cardiac and skeletal myocytes, can switch to a highly proliferative and migratory

state under various stimuli, including hypoxia and vascular injury (21). We hypothesized that hypoxia induces downregulation of micro-RNAs targeting SphK1, contributing to enhanced hPASMCM proliferation and migration. In this study, we demonstrate that miR-1 is decreased in PASMCMs of PAH patients and is downregulated by hypoxia, a major contributor to PAH development, in both cultured PASMCMs and in lung tissues from experimental models of PH. In addition, overexpression of miR-1 protected mice from hypoxia-induced pulmonary hypertension (PH) and suppressed the expression of SphK1, a conserved lipid kinase that catalyzes formation of the proproliferative and promigratory lipid S1P.

## MATERIALS AND METHODS

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**Human pulmonary artery smooth muscle cells.** Human pulmonary artery smooth muscle cells (hPASMCMs) were isolated from six donors not suitable for lung transplantation and four patients with idiopathic PAH as previously described (2). The smooth muscle phenotype of cultured cells has been authenticated by immunohistochemistry and flow cytometric analysis with antibodies against smooth muscle  $\alpha$ -actin and calponin (2). In addition to these cell lines, a male primary hPASMCM cell line from Lonza (CC-2581) was used for cell transfection, migration, and proliferation assays at passages 4–8. The miR-1 mimics, inhibitors (antagomirs), or controls (50–100 nmol/l; Qiagen) were transfected using Lipofectamine (Thermo Fisher Scientific) per the manufacturer's instructions.

**Cell proliferation assays.** Cell proliferation was determined using a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (QIA58; Calbiochem) per the manufacturer's instructions in a 96-well format. Starting cell densities of 4,000 cells per well were used.

**Cell migration assays.** Cell migration was determined using a quantitative Transwell assay as previously described (35). In brief, cell migration across a Transwell membrane with 8- $\mu$ m pores was assessed following incubation in normoxic or hypoxic (3% O<sub>2</sub>) conditions. Cells were fixed and stained using Diff-Quik, and the ratio of migrated to total cells was assessed via five random fields per sample. For miR transfection studies, cells were transfected 24 h before transferring to the Transwell insert.

**Bioinformatics analysis and miRNA prediction.** In silico analysis for miR target prediction was completed using the microRNA.org target prediction resource (<http://34.236.212.39/microna/home.do>), utilizing miRanda sites and miR support vector regression (mirSVR) scoring (5). The target sites predicted using miRanda are scored for likelihood of mRNA downregulation using a regression model trained on sequence and contextual features of the predicted miRNA:mRNA duplex. Using these tools, hsa-miR-1-3p was selected as a miR candidate targeting *SPHK1*.

**Transient transfections and reporter assays.** For all miR transfection studies, miScript miRNA-1 mimics or antagomirs were used (Qiagen) per the manufacturer's guidelines, with AllStars negative control small interfering RNA (siRNA) used to account for nonsequence effects. The reporter construct containing the firefly luciferase gene fused to the full-length human *SPHK1* 3'-untranslated region (UTR) within the pMirTarget vector was ordered from OriGene Technologies. This vector was cotransfected with a control vector expressing a *hRluc Renilla* luciferase reporter gene and a herpes simplex virus thymidine kinase (HSV-TK) promoter for normalization of transfection efficiency (pGL4.74[hRluc/TK]; Promega). All transfections were performed in triplicate. At passages 4–8, hPASMCMs were grown to 75% confluence before transfection with miR mimics (50–100 nmol/l) or antagomirs (100 nmol/l) with controls of the same concentrations using Lipofectamine (Thermo Fisher Scientific) per the manufacturer's instructions. Cells were harvested for downstream analysis 24–72 h posttransfection. For luciferase reporter assays, cells were transfected concurrently with reporter vectors or controls. Cells were harvested at 24 h, and luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) per the manufacturer's protocol, using a GloMax luminometer (Promega). Transfection efficiency was determined by normalizing firefly luciferase light units to *Renilla* luciferase light units. Results are representative of at least three

independent experiments.

**Immunoblot analysis.** Lung tissues were perfused with PBS to remove blood and homogenized using radioimmunoprecipitation assay buffer (Sigma) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem). Cells were washed with PBS, and protein was extracted using the same modified radioimmunoprecipitation assay buffer solution. Proteins (10–25  $\mu\text{g}$ ) were separated on Mini-Protean TGX precast gels (Bio-Rad), transferred to nitrocellulose membranes, and blocked in 5% nonfat dry milk. Membranes were then probed with primary and secondary antibodies, and bands were visualized by enhanced chemiluminescence (Pierce) per the manufacturer's instructions. Densitometry was used to quantify protein levels using ImageJ software, and expression levels were normalized to GAPDH or  $\beta$ -actin. The primary antibodies used were SphK1, GAPDH, and horseradish peroxidase-conjugated  $\beta$ -actin (Cell Signaling Technology) with specificity previously determined (7, 59). An anti-rabbit IgG, horseradish peroxidase-linked secondary antibody was used (Cell Signaling Technology).

**Lung tissue immunofluorescence microscopy.** Paraffin-embedded mouse lung tissue sections were used for immunostaining following antigen retrieval, using rabbit SphK1 (cat. no. ab71700; Abcam) and cyanine 3 (Cy3)-labeled mouse smooth muscle actin antibodies as previously described (30). The secondary antibody used for SphK1 was Alexa Fluor 488 donkey anti-rabbit IgG (Thermo Fisher Scientific). Mounting media with 4',6-diamidino-2-phenylindole (DAPI; Life Science) were used to fix the coverslip to a slide. Slides were examined using a Nikon Eclipse E800 fluorescence microscope.

**RNA extraction and quantitative real-time PCR analysis.** Total RNA containing miRNA was isolated from hPASC and lung tissue samples using a miRNeasy kit (Qiagen) and reverse transcribed with the miScript II RT kit (Qiagen). For real-time PCR, miRNA-specific miScript primer assays (Qiagen) were used with RNU6-2 as an internal control. Expression of mRNAs was determined using specific TaqMan primer assays (Applied Biosystems) with GAPDH used as an internal control. Relative changes in mRNA and miRNA expression were calculated using the comparative cycle threshold method.

**Measurement of intracellular S1P generation.** S1P levels from hPASCs were measured using an established method previously described (60). Briefly, total lipid extracts were extracted from cell cultures after exposure to experimental conditions and then subjected to thin-layer chromatography and autoradiography. The areas corresponding to labeled [ $^{32}\text{P}$ ]S1P were excised, and radioactivity was determined by scintillation counting. The data were normalized to total radioactivity in  $10^5$  cells.

**Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay.** Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were performed as previously described (28). In brief, hPASCs at passages 4–8 were grown to 75% confluence before transfection with miR mimics (50–100 nmol/l) with controls of the same concentrations using Lipofectamine (Thermo Fisher Scientific) per the manufacturer's instructions. TUNEL assays were then performed at 24 h with nuclear DAPI costaining. For quantitative analysis, TUNEL-positive apoptotic cells in  $\geq 5$  high-power fields per condition were expressed as percent apoptotic cells compared with controls.

**Animal model of hypoxia-mediated pulmonary hypertension.** All experimental protocols using vertebrate animals were reviewed and approved by the University of Illinois at Chicago Animal Care and Use Committee. All experiments were performed in accordance with the approved guidelines and regulations for the use of laboratory animals. In the mouse model of hypoxia-mediated pulmonary hypertension (HPH), 8-wk-old male C57BL/6 mice were exposed to normoxia or hypoxia (10%  $\text{O}_2$  in a ventilated chamber) for 1 day to 4 wk ( $n = 3$ –5 per group). HPH development was assessed by measuring right ventricular systolic pressure via a pressure transducer catheter (Millar) and right ventricular hypertrophy as a weight ratio of the right ventricle divided by the sum of left ventricle and septum [ $\text{RV}/(\text{LV} + \text{S})$ ]. Pulmonary artery vessel thickness was calculated as [(external vessel area – internal vessel

area)/external vessel area], as previously described (7), using images of lung histological sections (Aperio ImageScope). Lung and heart tissues were collected for further analysis.

For mouse HPH prevention studies, animal-grade *mirVana* miRNA mimics or negative miRNA control no. 1 (Thermo Fisher) were prepared with InvivoFectamine 3.0 reagent (Thermo Fisher) per the manufacturer's instructions and injected retroorbitally (7.8 mg/kg body wt) weekly for 4 wk starting 1 day before exposure to normoxia or hypoxia (10% O<sub>2</sub>). HPH development was assessed as described above.

**Statistical analysis.** Results are shown as means ± SE from at least three experiments, and statistical significance was calculated with Student's *t*-test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. controls) using GraphPad Prism software.

## RESULTS

**miR-1 targets and regulates the expression of SphK1 in hPASMCs.** To investigate the potential regulation of SphK1 by miRs, we performed in silico analysis of the human SphK1 3'-UTR to identify predicted miR response elements. This approach identified several putative binding sites, including miR-1-3p (miR-1) as the most highly predicted target of SphK1 with a high probability of downregulation (7-mer-m8 canonical site type, mirSVR score = -0.6270; 5). Suggesting its importance in gene regulation, miR-1 and its SphK1 3'-UTR binding site are highly evolutionarily conserved across species. As bioinformatics analysis identified the putative binding site of miR-1 within the 3'-UTR of *SPHK1*, we hypothesized that this miR-1 may be a negative regulator of SphK1 expression in hPASMCs. To test this hypothesis, we utilized a luciferase reporter vector containing a sequence-verified clone of the SphK1 3'-UTR designed for micro-RNA target validation. To determine the interaction of miR-1 with the SphK1 3'-UTR, we overexpressed or inhibited miR-1 expression using human miR-1 mimics or antagomirs, respectively, in hPASMCs cotransfected with the SphK1 3'-UTR reporter vector. Overexpression of miR-1 resulted in reduced luciferase activity due to SphK1 3'-UTR binding, while transfection of miR-1 antagomirs had no effect (Fig. 1, A and B). In addition, both protein and mRNA expression levels of SphK1 were significantly decreased in hPASMCs following transfection with the miR-1 mimics (Fig. 1, C and D, respectively), while miR-1 antagomirs did not alter SphK1 expression (data not shown). Transfection of hPASMCs with miR-1 mimics also prevented hypoxia-induced generation of intracellular S1P generation at 48 h but did not alter basal levels of S1P under normoxic conditions (Fig. 1E), demonstrating a mechanistic role of miR-1 in limiting S1P production in hypoxia.

### Lung expression of miR-1 and SphK1 is dysregulated in the development of hypoxia-mediated pulmonary hypertension.

Hypoxia is associated with the pathogenesis of PAH and contributes to pulmonary vascular remodeling. We utilized a mouse model of experimental HPH to investigate the development of disease progression following 1, 7, 14, and 28 days of 10% O<sub>2</sub> exposure, assessed by the measurement of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH). Mice developed significant elevations in RVSP by *day 14* and increased RVH and heart weight-to-body weight ratio by *day 7*, with increased severity of these parameters by *day 28* (Fig. 2, A–C). Interestingly, the expression of miR-1 in whole lung was significantly reduced over time following hypoxia exposure (Fig. 2D), and this paralleled an increase in lung SphK1 protein expression (Fig. 2E). Significant elevation in vessel wall thickness of small pulmonary arteries, due to medial hypertrophy of PASMCs, was also observed by *day 28* (data not shown), consistent with prior reports (7). These results demonstrate the utility of the HPH mouse model in studying the role of miR-1 expression.

### miR-1 expression is reduced in PASMCs from PAH patients and is decreased by hypoxia in hPASMCs.

Since miR-1 was reduced in lungs from HPH mice, we investigated whether PASMCs isolated from



patients with PAH had altered levels of miR-1. Expression of miR-1 was significantly reduced in PASMCs from PAH patients vs. controls (Fig. 3A). We then tested whether hypoxia could alter miR-1 expression in hPASMCs. Hypoxia exposure (3% O<sub>2</sub>) increased the proliferative phenotype of hPASMCs over time (Fig. 3B) and significantly reduced the expression of miR-1 normalized to RNU6-2 expression (Fig. 3C). These data are consistent with our hypothesis that hypoxia may downregulate miR-1, leading to increased SphK1 expression and enhanced cell proliferation. Since overexpression of miR-1 mimics in hPASMCs can reduce SphK1 protein expression under normoxic conditions (Fig. 1C), we tested whether it could also prevent the induction of SphK1 expression by hypoxia. As shown in Fig. 3, D and E, miR-1 overexpression attenuates SphK1 expression under hypoxic conditions in hPASMCs over time, suggesting a significant role of miR-1 in directly targeting the 3'-UTR of SphK1. The transient transfection of miR-1 mimics sustained expression at 72 h, with the most robust expression at 24 h (data not shown).

**miR-1 regulates the proliferation of hPASMCs.** Because miR-1 is significantly decreased in the hypoxia-induced proliferative state of hPASMCs, we investigated whether altering miR-1 levels with the transfection of miR-1 mimics could alter the proliferative phenotype in these cells. Overexpression of miR-1 mimics at increasing concentrations reduced the proliferative capacity of hPASMCs in both normoxic and hypoxic conditions, respectively (Fig. 4, A and B). Differences in proliferation were also observed by light microscopy, with a substantial reduction in the number of hPASMCs in both normoxia and hypoxia following miR-1 overexpression (Fig. 4, C and D). The proliferative changes induced by miR-1 were not caused by enhanced cytotoxicity, as measured via lactate dehydrogenase activity in cell supernatants (data not shown). TUNEL assays demonstrated an ~11% induction of apoptosis in hPASMC transfection with miR-1 mimics at the highest dose of 100 nmol/l (Fig. 4, E and F). Although this finding may contribute to the antiproliferative effects of miR-1 in hPASMCs, the low level of apoptosis induced by miR-1 mimics is unlikely to account for the significant impact on proliferation observed.

**miR-1 regulates the migration of hPASMCs.** Both hPASMC proliferation and migration contribute to pathogenic pulmonary vascular remodeling in PAH. On the basis of the findings that miR-1 overexpression greatly reduced hPASMC proliferation in vitro, we tested whether miR-1 overexpression could also attenuate normoxic or hypoxia-induced hPASMC migration. In a Transwell assay, hPASMCs migrated over time under normoxic conditions, and as expected, migration increased when these cells were exposed to hypoxia for 12–48 h (Fig. 5, A and B). Overexpression of miR-1 inhibited the migration of hPASMCs at normoxia and further reduced the migration under hypoxic conditions (Fig. 5, C and D). This significant reduction in migratory capacity under hypoxic conditions demonstrates the importance of diminished miR-1 expression during this synthetic state of hPASMCs. Thus reduced miR-1 expression in hypoxia can enhance both proliferation and migration in this cell type during in vitro conditions that contribute to pulmonary vascular remodeling.

**Systemic miR-1 delivery protects from the development of HPH.** To test whether increasing expression of miR-1 in vivo could prevent HPH development, we delivered either nontargeting or miR-1 mimics via retroorbital injection to mice weekly throughout their 4-wk hypoxia course. Compared with controls, mice receiving miR-1 mimics were protected against the development of HPH, with significant attenuation of RVSP elevation and RVH (Fig. 6, A–C). Hypoxia-induced pulmonary vascular remodeling was also reduced with miR-1 mimic treatment (Fig. 6, D–F). No changes were observed in any of these parameters under normoxic conditions with miR-1 administration. Systemic administration of miR-1 mimics prevented the reduction in miR-1 expression induced by hypoxia, which resulted in a fourfold to fivefold increase in expression in the lung (Fig. 6G). To test whether increased miR-1 expression altered SphK1 expression in this model, we measured SphK1 protein in lung tissues. Overexpression of miR-1 had no significant effect on SphK1 under normoxic conditions but reduced SphK1 expression induction with hypoxia exposure (Fig. 7, A and B). To test whether SphK1 expression was altered in mouse PASMCs receiving miR-1 mimics, we measured both SphK1 and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), a marker for smooth muscle cells,

by immunofluorescence imaging in histological lung sections. SphK1 expression colocalized with  $\alpha$ -SMA and was significantly reduced in mice receiving miR-1 mimics in both normoxic and hypoxic conditions (Fig. 7C). These results indicate that overexpression of miR-1 mimics reduces the development of experimental HPH and, together with the *in vitro* data presented in this study, suggest that altered expression of SphK1 expression by miR-1 may contribute to disease pathogenesis.

## DISCUSSION

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PAH is a devastating lung disease for which curative treatments are unavailable and patient morbidity and mortality remain high (26, 40). Despite active research in uncovering the pathogenic mechanisms resulting in this disease, therapies targeting the prevention or reversal of pulmonary vascular remodeling and subsequent elevations in pulmonary arterial pressure have not been successful. The present study provides evidence of the role of miR-1, a muscle-specific micro-RNA, and its posttranscriptional regulation of SphK1 in the pathogenesis of PAH and highlights its potential as a novel therapeutic target.

In the present study, using an *in silico* approach, we identify miR-1 as a potential regulator of SphK1 expression. We found that miR-1 is highly downregulated by hypoxia in hPASMCs, as well as in the lungs of mice throughout the progression of experimental HPH, coincident with increased SphK1 expression. Expression of miR-1 was also significantly reduced in PASMCs isolated from patients with PAH. Hypoxia-mediated decreases in miR-1 expression were associated with enhanced hPASMC proliferation and migration *in vitro*, and overexpression of miR-1 inhibited these effects. Additionally, we provide *in vivo* evidence that miR-1 overexpression prevents the development of experimental HPH and reduces the overexpression of SphK1 in PASMCs in this model. Although there are likely multiple mRNA targets of miR-1, the regulation of SphK1 expression is consistent with our findings that this kinase is highly influential in promoting PASMC proliferation and the development of experimental pulmonary hypertension (7). These experiments describe a novel role of miR-1 in regulating the intricate molecular mechanisms underlying pathogenic pulmonary vascular remodeling in PAH.

Our previous work has demonstrated that elevated levels of both S1P and SphK1 in the lung are associated with PAH (7). These studies showed that both genetic knockout of SphK1 in mice and pharmacologic SphK1 inhibition in rats could prevent the development of hypoxia-mediated PH. Inhibiting the S1P receptor 2 (S1PR2) in mice also prevented PH development, and mice heterozygous for S1P lyase, which catabolizes S1P, were more susceptible to PH (7). Together, these studies define the role of SphK1-S1P signaling in PAH. Here we identified miR-1 as a negative regulator of SphK1 expression. Under basal conditions, normal miR-1 expression limits SphK1 expression, whereas in hypoxia the reduction in miR-1 allows for SphK1 upregulation at both mRNA and protein levels.

Unrestrained PASMC proliferation and migration, as well as hypoxic vasoconstriction, are major contributors to pulmonary vascular remodeling in PAH, and upregulation of the SphK1-S1P signaling axis has been implicated in these pathogenic processes (7, 11, 53). We have previously demonstrated that both overexpression of SphK1 and stimulation with S1P promote the proliferative phenotype of hPASMCs. Several studies have also reported that miRNAs can regulate cell proliferation and migration associated with vascular remodeling by several mechanisms, including targeting of ion channels, mitochondrial function, and BMP receptor 2 (BMPR2) signaling pathways (19). Here, we report that miR-1 is downregulated in PASMCs isolated from PAH patients and overexpression of miR-1 can reduce hypoxia-induced SphK1 expression and attenuate PASMC proliferation and migration. These studies indicate the critical role of miR-1 in regulating pulmonary vascular remodeling in PAH. The relevance of these findings also extends beyond PAH, as SphK1 and S1P are well-known mediators of cell proliferation, apoptosis resistance, and angiogenesis in myriad cell types, including cancer cells (36, 51).

Shared features of cancer development with the unrestricted vascular cell proliferation, apoptosis

resistance, and glycolytic shifts of PAH have recently been appreciated (20, 44). In these studies, a “pseudohypoxic environment” is proposed where glycolysis is predominant and hypoxia-inducible factor 1 $\alpha$  is activated under normoxia (13, 47). These observations are consistent with our findings of reduced miR-1 expression in PSMCs isolated from PAH patients in normoxic conditions. Importantly, inhibiting the oncogenic properties of SphK1 has shown efficacy in preclinical studies to decrease tumor growth (16, 31), and therefore induction of miR-1 to limit SphK1 expression may be beneficial. Recent reports have also associated downregulation of miR-1 with cancer development in various cell types (38, 39, 55, 56) via its modulation of cell proliferation, migration, and invasion. These studies suggest the ubiquitous role of miR-1 in the regulation of cell proliferation and are consistent with our findings that miR-1 is antiproliferative in PSMCs.

In SMCs, miR-1 has also been reported to regulate gene expression. It is an important modulator of cardiac and skeletal muscle proliferation, with excess expression leading to a reduced pool of proliferating ventricular cardiomyocytes in vivo (9, 61, 62). Expression of miR-1 can be induced by myocardin, a transcriptional activator of serum response factor (SRF), to inhibit cell proliferation and contractility in human vascular SMCs (8, 29), potentially through regulation of proviral integration site for Moloney murine leukemia virus-1 (Pim-1), a recently identified biomarker in PAH (45). Downregulation of miR-1 has also been observed in vascular SMCs isolated from spontaneously hypertensive rats and may regulate proliferation by targeting insulin-like growth factor I (IGF-I; 37), a growth factor involved in neonatal HPH (57). In addition, genetic knockout of miR-1 in mice results in uniform lethality before weaning due to cardiac dysfunction, and hearts from these mice exhibit gene expression characteristics more similar to vascular smooth muscle (24). This suggests that the influence of miR-1 on the proliferation and migration of PSMCs likely functions through multiple parallel mechanisms to achieve the resulting cellular phenotype.

Micro-RNAs have shown promise as potential therapeutic targets and biomarkers for many diseases, including PAH. Interestingly, a recent study exploring the role of circulating miRNAs in human PH demonstrated reduced expression of miR-1 in the buffy coat of patients with both moderate and severe PH (54). Here, we have demonstrated that miR-1 is strongly downregulated early in and throughout the course of experimental hypoxia-induced PH in the lungs and that this downregulation is retained in PSMCs from PAH patients. This suggests that miR-1 plays a role in early disease pathogenesis rather than having an end-stage disease effect. Overexpression of miR-1 can reduce the pulmonary vascular remodeling phenotype both in vitro and in vivo; therefore identifying mechanisms to elevate or sustain miR-1 expression in PAH may be beneficial.

There are several limitations to the present study that warrant further investigation. The ability of miR-1 to decrease SphK1 levels in PSMCs is one mechanism through which it regulates vascular remodeling, but other direct targets of miR-1 involved in PAH development may contribute to our findings here and would be important to identify in future studies. Table 1 provides a list of the most highly predicted mRNA targets of miR-1. This list includes two genes, brain-derived neurotrophic factor (*BDNF*) and gap junction  $\alpha$ -1 protein/connexin 43 (*GJA1/Cx43*), that have been previously associated with PSMC pathobiology (10, 23, 33). Another potential limitation is that miR-1 overexpression induces apoptosis in hPSMCs, though the low level of induction even at high mimic concentrations is unlikely to account for the significant impact on proliferation we observed. Furthermore, in addition to unrestrained PSMC proliferation and apoptosis resistance, pulmonary vascular remodeling involves neointimal formation due to PAEC dysfunction and proliferation (34, 58). The studies described here did not investigate the role of the SphK1-S1P axis on the phenotype of PAECs; this would provide valuable insight into disease mechanisms and deserves exploration in future studies. Finally, though we have used idiopathic PAH samples in this study and demonstrated a role of miR-1 under normoxic conditions in PSMCs, several of our experiments utilize hypoxia as a model of disease, which more classically parallels group 3 PH. As discussed above, we



believe that hypoxia and normoxic activation of hypoxia-related signaling pathways are fundamental to the development of PAH and that our studies therefore offer important insights into this group of PH as well.

Together, these novel data demonstrate that miR-1 can target SphK1 to regulate molecular mechanisms in PH with effects on PASMCs and pulmonary arterial remodeling. The activation of miR-1 in the pulmonary vasculature may therefore provide therapeutic benefit in PH, a group of diseases in significant need of new treatments.

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## DISCLOSURES

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

## AUTHOR CONTRIBUTIONS

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J.R.S., V.N., and R.F.M. conceived and designed research; J.R.S., J.C., S.S., S.Z., and S.A.C. performed experiments; J.R.S., J.C., S.S., S.Z., and R.F.M. analyzed data; J.R.S., J.C., S.S., V.N., and R.F.M. interpreted results of experiments; J.R.S. and S.Z. prepared figures; J.R.S. drafted manuscript; R.F.M. edited and revised manuscript; J.R.S., J.C., S.S., S.A.C., V.N., and R.F.M. approved final version of manuscript.

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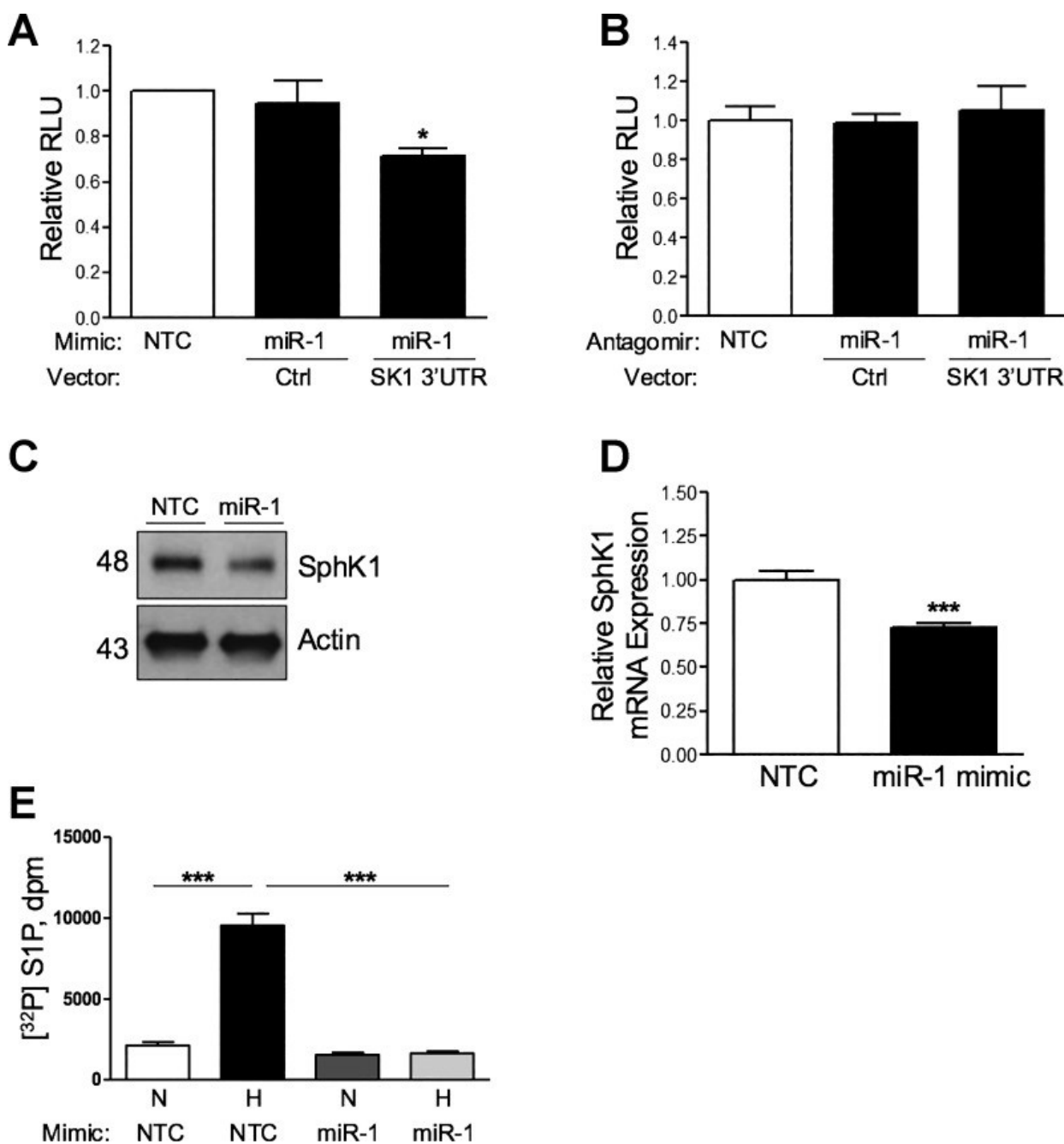
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## Figures and Tables

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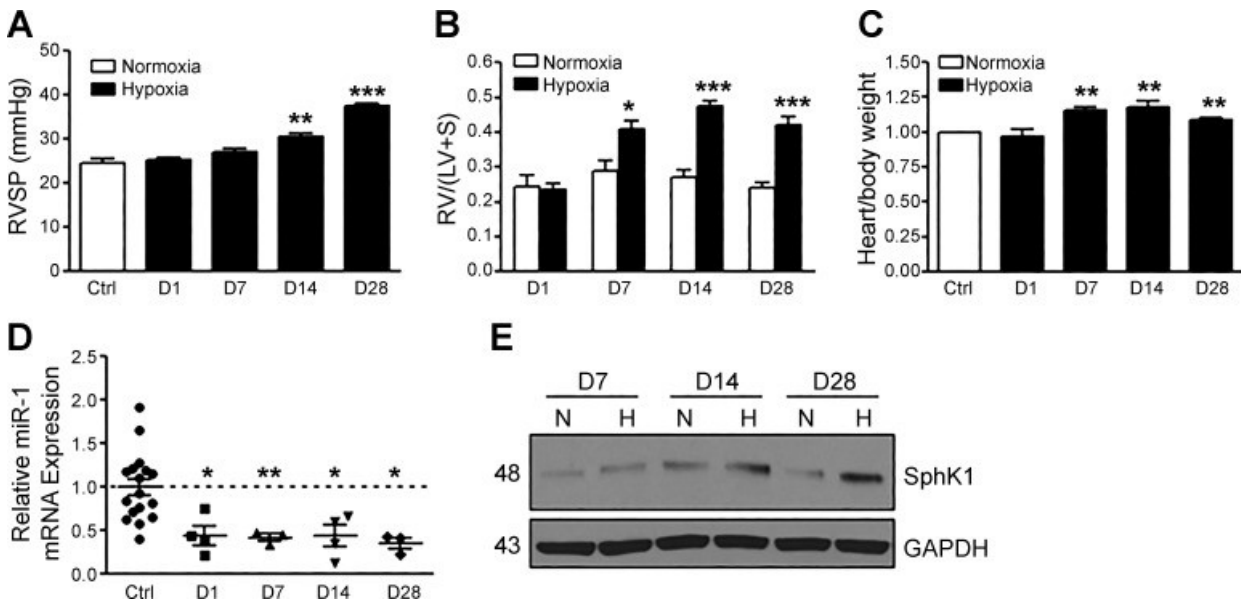
**Fig. 1.**



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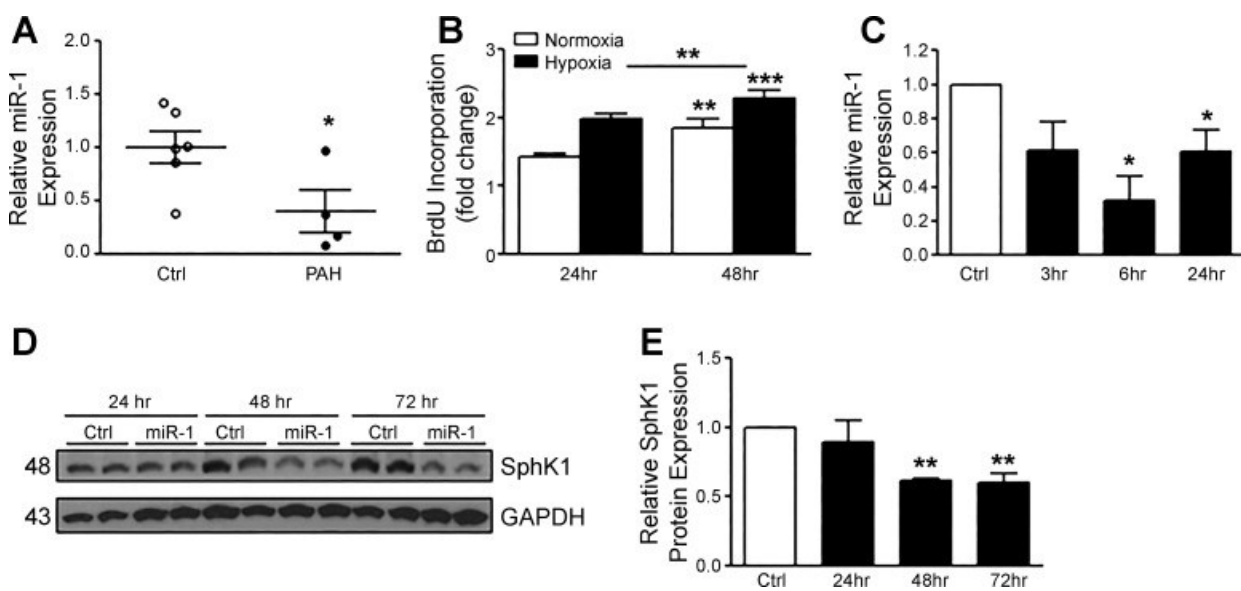
Micro-RNA-1 (miR-1) binds to the sphingosine kinase 1 (SphK1) 3'-untranslated region (SK1 3'UTR) and inhibits SphK1 expression. *A* and *B*: transfection of human pulmonary artery smooth muscle cells (hPASCs) with miR-1 mimics (50 nmol/l), but not antagonirs (100 nmol/l), significantly reduces luciferase activity of a SphK1 3'-UTR reporter construct relative to control (Ctrl). Overexpression of miR-1 mimic (50 nmol/l) in hPASCs significantly decreases SphK1 protein expression (*C*) and mRNA expression (*D*) at 48 h. *E*: transfection of hPASCs with miR-1 mimics (50 nmol) prevents hypoxia-induced generation of intracellular [<sup>32</sup>P]sphingosine-1-phosphate ([<sup>32</sup>P]S1P) generation at 48 h. Here, *n* ≥ 5 per condition. \**P* < 0.05, \*\*\**P* < 0.001 relative to control. NTC, nontargeting control; RLU, relative light units; N, normoxia; H, hypoxia (3% O<sub>2</sub>); dpm, disintegrations per minute.

**Fig. 2.**



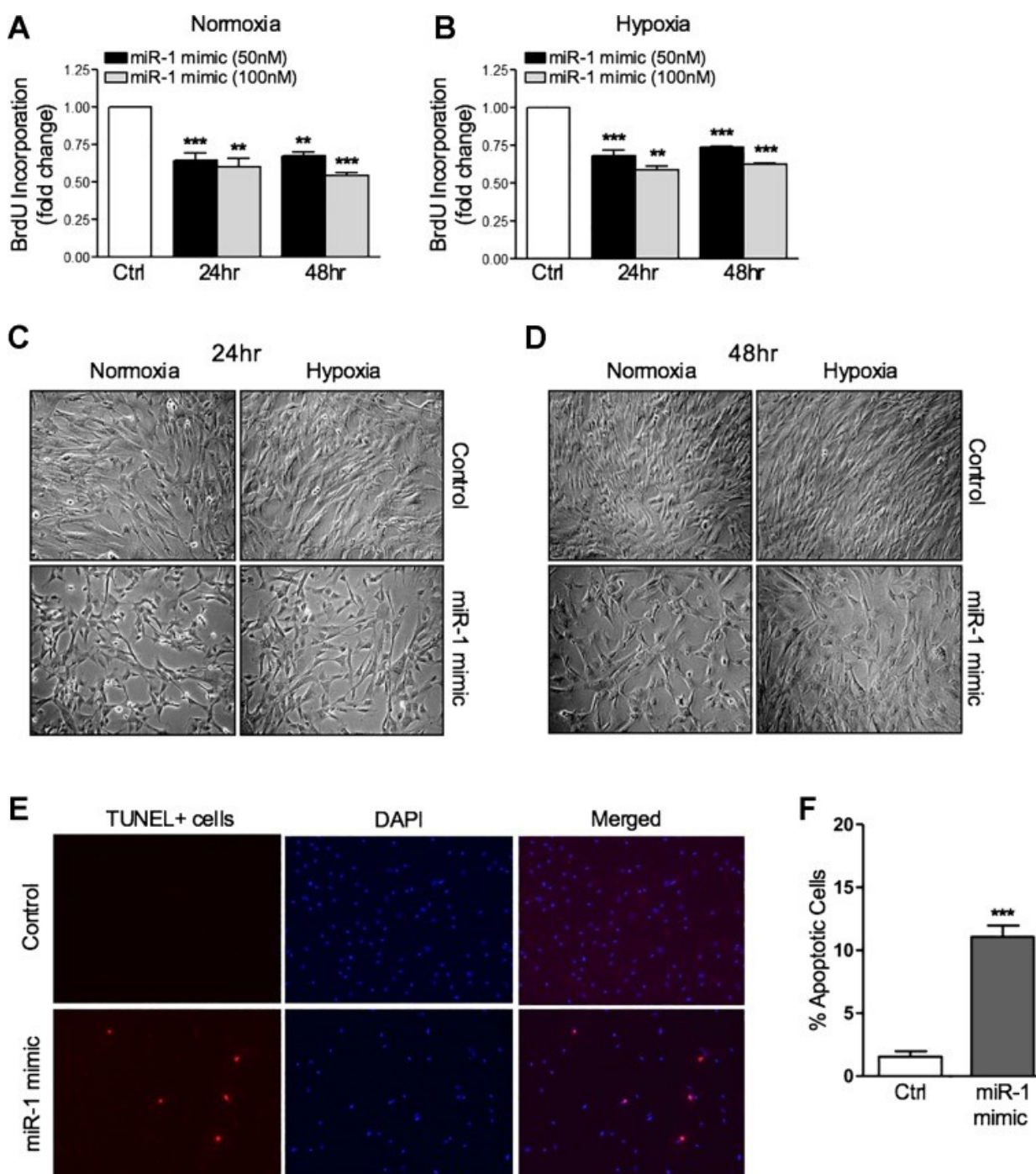
Micro-RNA-1 (miR-1) is downregulated in mouse lungs during the progression of experimental hypoxia-mediated pulmonary hypertension (HPH). *A* and *B*: HPH development in mice is demonstrated by elevations in right ventricular systolic pressure (RVSP) and right ventricular hypertrophy [measured as a weight ratio of the right ventricle divided by the sum of the left ventricle and septum, RV/(LV + S)] over time [*day* (D) 1–28 of hypoxia]. *C*: change in mouse heart weight-to-body weight ratio over the course of HPH development. Expression of miR-1 normalized to RNU6-2 expression is reduced (*D*) and sphingosine kinase 1 (SphK1) protein expression is increased (*E*) in mouse lungs during HPH development. Here,  $n \geq 5$  per condition. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  relative to control (Ctrl). N, normoxia; H, hypoxia (3% O<sub>2</sub>).

**Fig. 3.**



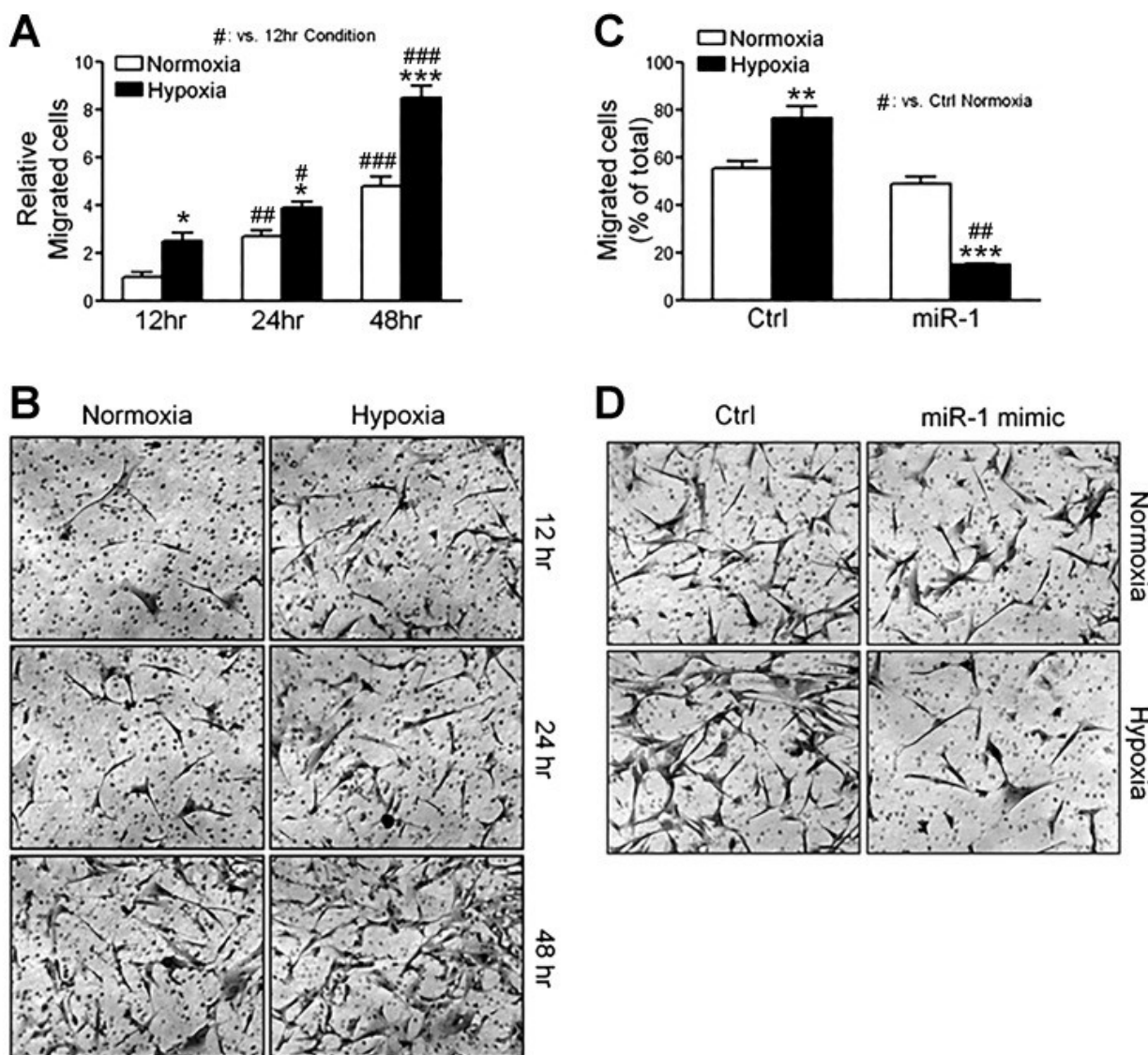
Micro-RNA-1 (miR-1) is decreased in human pulmonary artery smooth muscle cells (hPASCs) from pulmonary arterial hypertension (PAH) patients, is downregulated by hypoxia, and prevents hypoxia-induced sphingosine kinase 1 (SphK1) expression. *A*: miR-1 expression normalized to RNU6-2 expression is reduced in hPASCs isolated from PAH patients ( $n = 4$ ) vs. controls (Ctrl;  $n = 6$ ). *B*: stimulation of hPASCs with hypoxia induces proliferation (24–48 h, 3% O<sub>2</sub>). *C*: hypoxia (3% O<sub>2</sub>) induces downregulation of miR-1 expression over time in hPASCs. *D* and *E*: miR-1 overexpression in hPASCs attenuates hypoxia-induced SphK1 protein expression (24–72 h, 50 nmol/l mimics). Here,  $n \geq 5$  per condition, unless otherwise indicated. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  relative to control. BrdU, 5-bromo-2'-deoxyuridine.



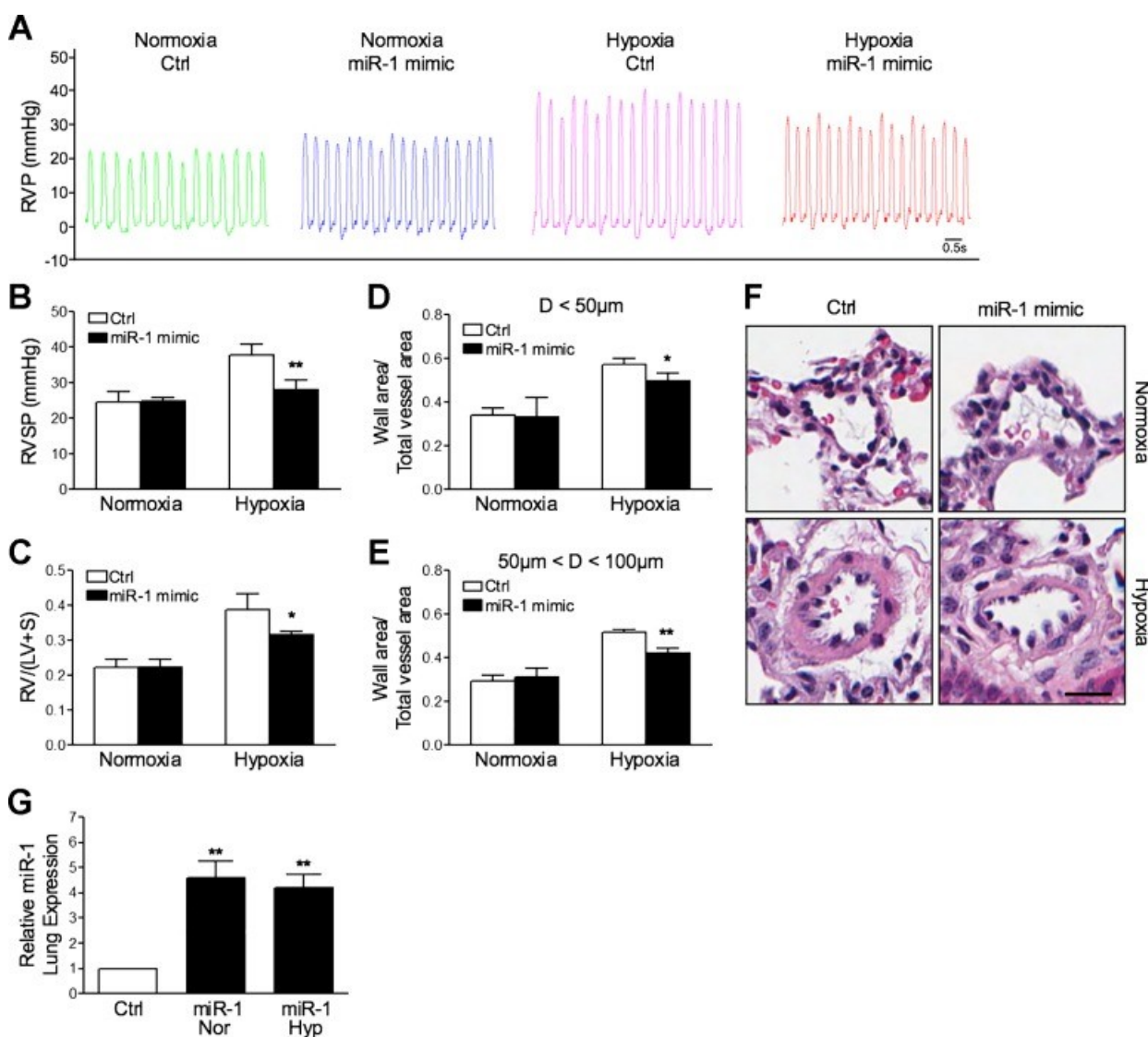
**Fig. 4.**[Open in a separate window](#)

Micro-RNA-1 (miR-1) overexpression inhibits human pulmonary artery smooth muscle cell (hPASMC) proliferation in normoxia and hypoxia. Overexpression of miR-1 mimics in hPASMCs (50–100 nmol/l) significantly reduces proliferation in normoxia (*A*) and hypoxia (3% O<sub>2</sub>, 24–48 h; *B*). *C* and *D*: representative images of hPASMCs demonstrating miR-1 mimic overexpression in normoxia and hypoxia reduces the rate of proliferation (24 h, ×10 magnification). Representative staining images (*E*) and quantification of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (*F*) demonstrating induction of apoptosis at low levels by overexpression of miR-1 mimics (100 nmol/l) in hPASMCs (24 h, ×10 magnification). Data are represented as percentage of TUNEL-positive cells of total 4',6-diamidino-2-phenylindole (DAPI)-positive cells per high-power field per condition (≥5 fields counted). Here,  $n \geq 5$  per condition. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  relative to control (Ctrl). BrdU, 5-bromo-2'-deoxyuridine.

**Fig. 5.**

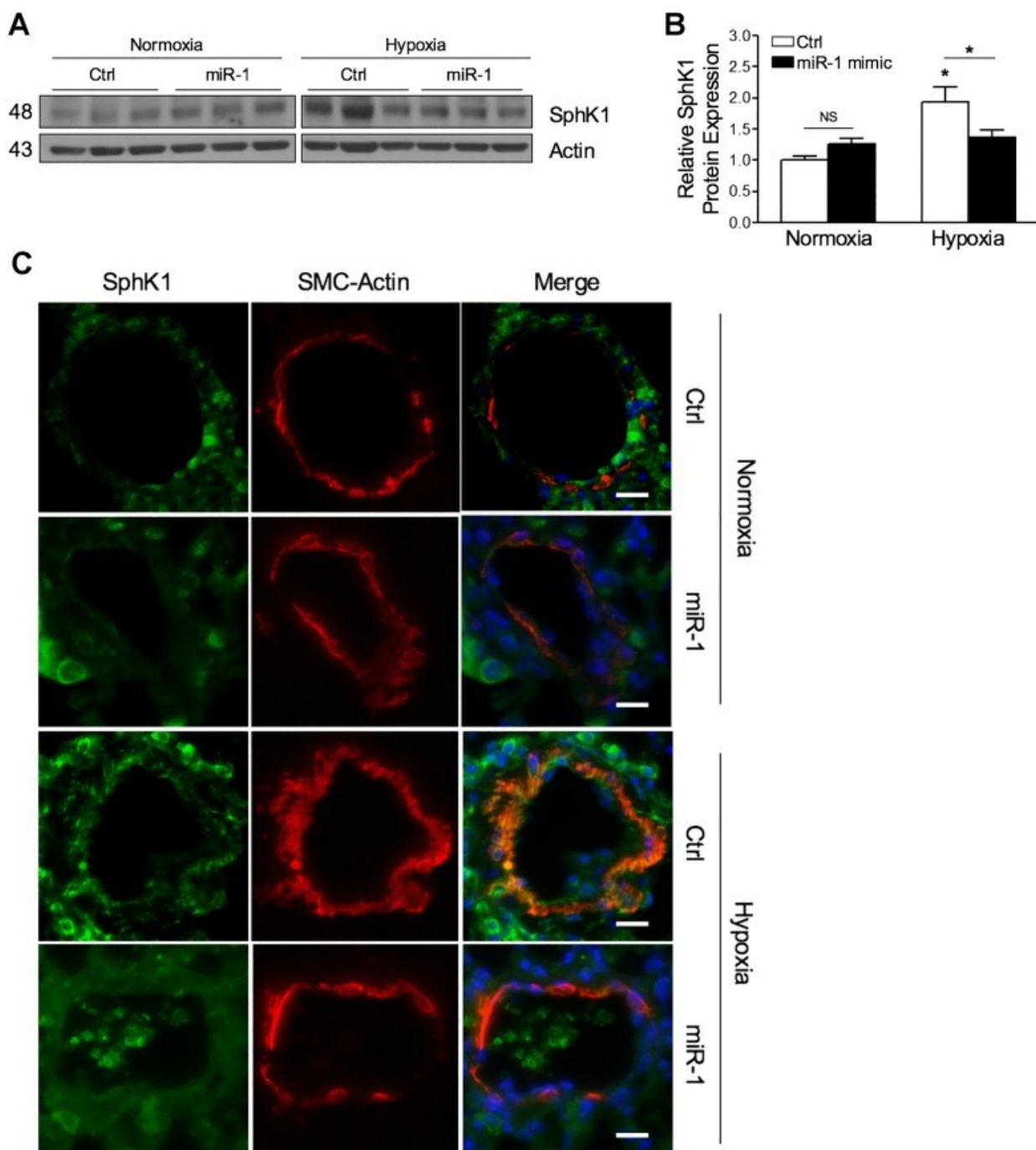


Micro-RNA-1 (miR-1) overexpression inhibits human pulmonary artery smooth muscle cell (hPASMC) migration in hypoxia. Stimulation of hPASMCs with hypoxia induces cell migration in a Transwell assay (12–48 h, 3% O<sub>2</sub>; *A*), with representative images ( $\times 10$  magnification; *B*). Overexpression of miR-1 mimics in hPASMCs (50 nmol/l) significantly reduces hypoxia-induced cell migration in a Transwell assay (3% O<sub>2</sub>, 24 h; *C*), with representative images ( $\times 10$  magnification; *D*). Here,  $n \geq 5$  per condition. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  relative to control (Ctrl), unless otherwise indicated. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ .

**Fig. 6.**

Micro-RNA-1 (miR-1) overexpression *in vivo* prevents the development of hypoxia-mediated pulmonary hypertension (HPH) in mice. Compared with controls (Ctrl), mice in the HPH model receiving systemic miR-1 mimics (7.8 mg/kg) develop less severe right ventricular systolic pressure (RVSP) elevation (*A* and *B*), right ventricular hypertrophy [calculated as a weight ratio of the right ventricle divided by the sum of left ventricle and septum,  $RV/(LV + S)$ ; *C*], and pulmonary vascular remodeling in arteries with diameters  $D < 50\ \mu\text{m}$  and  $50\text{--}100\ \mu\text{m}$ , respectively (*D* and *E*). *F*: representative histological images of small pulmonary arteries in normoxia and hypoxia, with and without treatment of systemic miR-1 mimics. Scale bar = 20  $\mu\text{m}$ . *G*: systemic administration of miR-1 mimics (7.8 mg/kg) in mice resulted in a fourfold to fivefold increase in lung expression in both normoxic (Nor) and hypoxic (Hyp) conditions, normalized to RNU6-2 expression (24 h following final injection). Here,  $n \geq 5$  per condition. \* $P < 0.05$ , \*\* $P < 0.01$  relative to control. RVP, right ventricular pressure.



**Fig. 7.**[Open in a separate window](#)

Micro-RNA-1 (miR-1) overexpression in vivo reduces induction of sphingosine kinase 1 (SphK1) in the lungs and pulmonary arteries. Representative Western blotting in whole lung tissues from mice with hypoxia-mediated pulmonary hypertension (HPH) demonstrates attenuation of SphK1 expression following systemic miR-1 mimic administration (7.8 mg/kg; *A*), with data quantification relative to actin expression (*B*). *C*: immunofluorescence staining demonstrates that hypoxia-induced SphK1 protein expression in small pulmonary arteries is reduced in miR-1 mimic-treated mice compared with controls (Ctrl). Scale bars = 20  $\mu$ m. \* $P$  < 0.05 relative to control. NS, not significant; SMC, smooth muscle cell.

**Table 1.**

Predicted mRNA targets of miR-1 in human cells

<b>Gene Symbol</b>	<b>Gene Description</b>	<b>mirSVR Score</b>
<i>ZNF280C</i>	Zinc finger protein 280C	-3.21
<i>BDNF</i>	Brain-derived neurotrophic factor	-3.19
<i>GJA1</i>	Gap junction $\alpha$ -1 protein [connexin 43 (Cx43)]	-3.03
<i>KIF2A</i>	Kinesin family member 2A	-2.42
<i>SLC44A1</i>	Solute carrier family 44 member 1	-2.29
<i>FAM104B</i>	Family with sequence similarity 104 member B	-2.19
<i>YWHAQ</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein- $\theta$	-2.19
<i>HACD3</i>	3-Hydroxyacyl-CoA dehydratase 3	-2.11
<i>MEX3C</i>	Mex-3 RNA-binding family member C	-2.07
<i>GLCC11</i>	Glucocorticoid-induced 1	-2.07
<i>MGC70870</i>	COOH-terminal binding protein-2 pseudogene	-1.93
<i>RP2</i>	RP2, ARL3 GTPase-activating protein	-1.92
<i>UST</i>	Uronyl 2-sulfotransferase	-1.89
<i>CDK14</i>	Cyclin-dependent kinase 14	-1.85
<i>CHSY1</i>	Chondroitin sulfate synthase 1	-1.85

Data were collected from microRNA.org (<http://34.236.212.39/microrna/home.do>); miR-1, micro-RNA-1; mirSVR, miR support vector regression; ARL3, ADP-ribosylation factor-like protein-3; RP2, retinitis pigmentosa protein-2.

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