A Role for miR-145 in Pulmonary Arterial Hypertension
Evidence From Mouse Models and Patient Samples

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Rationale: Despite improved understanding of the underlying genetics, pulmonary arterial hypertension (PAH) remains a severe disease. Extensive remodeling of small pulmonary arteries, including proliferation of pulmonary artery smooth muscle cells (PASMCs), characterizes PAH. MicroRNAs (miRNAs) are noncoding RNAs that have been shown to play a role in vascular remodeling.

Objective: We assessed the role of miR-145 in PAH.

Methods and Results: We localized miR-145 in mouse lung to smooth muscle. Using quantitative PCR, we demonstrated increased expression of miR-145 in wild-type mice exposed to hypoxia. PAH was evaluated in miR-145 knockout and mice treated with anti-miRs via measurement of systolic right ventricular pressure, right ventricular hypertrophy, and percentage of remodeling pulmonary arteries. miR-145 deficiency and anti-miR-mediated reduction resulted in significant protection from the development of PAH. In contrast, miR-143 anti-miR had no effect. Furthermore, we observed upregulation of miR-145 in lung tissue of patients with idiopathic and heritable PAH compared with unaffected control subjects and demonstrated expression of miR-145 in SMC of remodeled vessels from such patients. Finally, we show elevated levels of miR-145 expression in primary PASMCs cultured from patients with BMPR2 mutations and also in the lungs of BMPR2-deficient mice.

Conclusions: miR-145 is dysregulated in mouse models of PAH. Downregulation of miR-145 protects against the development of PAH. In patient samples of heritable PAH and idiopathic PAH, miR-145 is expressed in remodeled vessels and mutations in BMPR2 lead to upregulation of miR-145 in mice and PAH patients. Manipulation of miR-145 may represent a novel strategy in PAH treatment. (Circ Res. 2012;111:290-300.)

Key Words: pulmonary hypertension ▪ hypoxia ▪ molecular biology ▪ smooth muscle cells ▪ microRNA ▪ smooth muscle differentiation ▪ remodeling

Pulmonary arterial hypertension (PAH) is a disease of the small pulmonary arteries (PAs), characterized by an increase in PA pressure and vascular remodeling leading to a progressive increase in pulmonary vascular resistance.1 The consequence of vascular obliteration is right heart failure and high mortality.2,3 Germline mutations in the gene coding for the bone morphogenetic protein (BMP) type-2 receptor (BMPR2), a receptor for the transforming growth factor (TGF)-β superfamily, have been identified in approximately 70% of patients with the heritable form of PAH (hPAH).4 Moreover, BMPR2 expression is markedly reduced in PAH cases in the absence of mutations in this gene (idiopathic PAH, iPAH). In pulmonary artery smooth muscle cells (PASMCs), mutations in BMPR2 are associated with an abnormal growth response to BMPs and TGF-β.5 In pulmonary artery endothelial cells (PAECs), these mutations increase the susceptibility of cells to apoptosis.6,7 The absence of BMPR2 mutations in some families and in the majority of iPAH cases suggests that further pathological mechanisms still need to be identified. One of the main histopathologic features common to all forms of PAH is the accumulation of cells expressing smooth muscle specific α-actin (SMA) in

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peripheral pulmonary arteries. This includes the appearance of SMA-positive cells in the neointima and the extension of SMA-positive cells into precapillary pulmonary arterioles that are normally devoid of smooth muscle.7

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MicroRNAs (miRNAs) are a class of small, endogenous and noncoding RNAs able to negatively regulate gene expression by targeting specific messenger RNAs (mRNAs) and induce their degradation or translational repression.8,9 A recent study has defined miRNA degradation as the predominant mechanistic effect of miRNA:mRNA targets.10 Several recent studies have assessed the direct role of miRNAs in vascular inflammation and in the development of vascular pathologies.11,12 In a recent study, miR-145 was shown to be abundantly expressed in the vessel wall.13 miR-145 is transcribed as a long pri-miRNA encoding both miR-143 and miR-145 on human chromosome 514 and on mouse chromosome 18, regulated by a conserved SRF-binding site.15 Localization of miR-145 to the vessel wall demonstrated high expression in the smooth muscle layer in comparison with adventitial fibroblasts and endothelial cells.15 For this reason, miR-145 is considered a smooth muscle cell phenotypic marker and modulator, able to regulate SMC through its target gene KLF-5 and its downstream signaling molecule, myocardin.13,16 Agonists within the TGF-β superfamily have been shown to activate miR-143/145 cluster via a Smad-dependent pathway.17,18 The analysis of miR-145, miR-143, and miR-143/145 knock-out (KO, −/−) mice showed a noticeably thinner smooth muscle layer of arteries.16 This leads to moderate systemic hypotension.15,16 Moreover, vascular smooth muscle cells (VSMCs) isolated from single and double knockout animals showed hyperproliferative activity and a higher ability to migrate toward platelet-derived growth factor, a known chemoattractant for VSMCs.15 A pharmacological analysis of the vasculature of miR-143/145 KO mice revealed a blunted response to vasopressive stimuli.15,16 miRNAs are therefore critical regulators of cardiovascular cell differentiation, growth, proliferation, and apoptosis. miRNAs are likely candidates as critical regulators of vascular remodeling in PAH, a topic that we have recently reviewed.19 In our previous publication20 we did not see any dysregulation of miR-145 in rat lung when assessed by quantitative PCR (q-PCR), but we did not assess this miRNA in mouse or human models, nor did we further investigate miR-145 in further detail in rat models. In the present study, in consideration of the importance of miR-145 in smooth muscle cell biology, we examined the expression and function of miR-145 in the development of PAH. We show that miR-145 is significantly upregulated in mice in response to chronic hypoxia and that genetic ablation or anti-miR–driven pharmacological reduction of miR-145 is protective against the development of PAH in mice. A whole transcriptome microarray study on isolated pulmonary arteries was used to quantify the transcriptional response to hypoxia. In human tissues, we show elevation of premature and mature forms of miR-145 in tissues and isolated PASMCs obtained from PAH patients with a mutation in the BMPR2 gene compared with controls. On the basis of these observations, we propose a critical role for miR-145 in the development of PAH.

Methods

An expanded Methods section is available in the Online Data Supplement.

Animal Models and In Vivo Modulation of miRNAs

miR-145 −/− mice have previously been described.15 Homozygous miR-145 −/− female mice or age-matched wild-type controls (strain C57BL6J/129SVEV, 8 weeks of age) were exposed to chronic hypoxia for 14 days or maintained in normoxic conditions and assessed at 10 weeks of age.

BMPR2 R899X Knock-In Mice

Knock-in mice harboring a heterozygous truncating mutation in BMPR2 were used to further determine the regulation of miR-145. These mice, similar to previously described R899X transgenic mice,5 develop spontaneous pulmonary hypertension. The LNA anti-miRs were 16 nucleotides in length targeting base 2 to 17 of mature miR-145/143 and were as fully phosphorothioated oligonucleotides perfectly complementary to the 5′ region of either miR-143 or miR-145 and were synthesized as a mixer of LNA and DNA. The LNA control oligonucleotide (scramble) consisted of a sequence directed against a Caenorhabditis elegans–specific miRNA with a comparable LNA/DNA content. Anti-miR and control oligonucleotides were administered to female C57Bl6 mice (8–10 weeks old) via subcutaneous injection (25 mg/kg in 0.2 mL saline). Mice were injected with oligonucleotide or vehicle on days 1 and 8 of the 14-day hypoxic exposure.

Statistical Analysis

For all the q-PCR experiments, values are expressed as fold change or mean ± SD. All data were analyzed using a 2-way ANOVA followed by Bonferroni post hoc test, 1-way ANOVA followed by
The right ventricle obtained from WT mice exposed to hypoxia for 14 days and compared this with normoxic controls to evaluate whether miR-145 was altered during response to injury in these tissues. Our analysis revealed a significant upregulation of miR-145 in response to hypoxia both in lung and the right ventricle (Figure 1B and 1C). miR-143 levels were also upregulated in the same tissues (Online Figure 1A and B). Analysis of miR-145 expression in the brain, kidney, and spleen of the same animals did not reveal any dysregulation (Online Figure II A, B, and C). Thus, miR-145 is expressed in smooth muscle cells in the lung of mice and is upregulated in response to hypoxia selectively in the lung.

Quantification of the Development of PAH in miR-145−/− Mice Compared With Controls

We next evaluated the effect of genetic ablation of miR-145 on the development of PAH. Eight-week-old miR-145−/− mice and control age-matched mice were exposed to chronic hypoxia or maintained in normoxic conditions for 14 days and evaluated for the development of PAH using a series of in vivo measurements. The absence of miR-145 expression in the lungs of the KO animals used in the study was confirmed by q-PCR and in situ hybridization (Online Figure IIIA and B). Because miR-145 is transcribed in its pri-form clustered with miR-143, we also analyzed WT and KO animals for the expression of miR-143 under both normoxic and hypoxic conditions to ensure that miR-143 levels were not substantially altered in the lung after genetic ablation of miR-145 and in response to hypoxic insult. q-PCR analysis of miR-143 expression in RNA extracted from the total lung of WT and miR-145−/− mice showed no difference in response to hypoxia between WT and miR-145−/− animals (Online Figure IV). Thus, any changes in the development of PAH in response to hypoxia in miR-145−/− are specific to the selective loss of miR-145.

We next quantified the development of PAH in WT and miR-145−/− mice in response to chronic hypoxia. In WT mice, we observed a significant and expected increase in systolic right ventricular pressure (RVP) and right ventricular hypertrophy (RVH) (Figure 2A and 2B). In contrast, miR-145−/− animals displayed no increase in systolic RVP or RVH (Figure 2A and 2B). Interestingly, there was no difference in baseline systolic RVP or RVH between WT and miR-145−/− mice (Figure 2A and 2B). Measurements are also presented in Online Tables I and II. Histological analysis showed the presence of pulmonary vascular remodeling in small PAs of WT animals after exposure to hypoxia but this was reduced in lungs harvested from miR-145−/− animals, a finding confirmed by quantitative scoring (Figure 2C and 2D). Therefore, genetic ablation of miR-145 protects mice against the development of PAH in response to hypoxia. The mean systemic arterial pressure (SAP) was lower in miR-145−/− mice compared with controls, as expected and consistent with previously published work.15,16 Neither the WT nor miR-145−/− mouse SAP were altered by exposure to hypoxia (Online Figure VA), and no changes were observed in the heart rate of the WT or miR-145−/− mice exposed to hypoxia (Online Figure VB).
Analysis of miR-145 Predicted Targets in the Lungs of miR-145−/− Mice Compared With Controls

To verify the effect of miR-145 ablation on gene and protein expression, several miR-145 gene targets, already validated in the literature or identified using TargetScan and PicTar prediction algorithms and selected for their potential involvement in PAH, were analyzed by q-PCR in lung tissue of WT and miR-145 KO animals. They included KLF4 and KLF5 (Kruppel-like factor 4 and 5), both involved in SMC proliferation and differentiation, and SMAD4 and SMAD5, signaling intermediaries for the TGF-β superfamily. Analysis of RNA revealed a significant upregulation of both SMAD4 and SMAD5 in miR-145−/− normoxic mice in comparison with WT normoxic mice (Online Figure VIA and B). KLF4 was elevated both in normoxic and hypoxic miR-145−/− mice, whereas KLF5 expression was significantly upregulated in WT hypoxic versus miR-145−/− hypoxic mice (Online Figure VIC and D). The Western blot analysis of KLF4 protein expression in WT and KO animals in hypoxic conditions confirmed the significant upregulation of this target, whereas no significant changes were observed in normoxia (Online Figure VII). In contrast, the KLF5 protein level was not significantly upregulated in the same samples both in normoxic and hypoxic conditions (Online Figure VII).

Microarray Analysis of Gene Expression Profile in the PAs of miR-145−/− Mice in Comparison With WT Animals

Global transcriptome analysis was performed with 6 samples from each of the miR-145−/− hypoxic, WT hypoxic miR-145−/− normoxic and WT normoxic groups. Potential miR-145 targets with 3′UTR miRNA binding regions as predicted in at least 3 of the databases in miRWalk and significant false discovery rates in the miR-145−/− hypoxic versus WT hypoxic comparison were selected for further analysis. Ingenuity pathway analysis software and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) were used to select targets that are present in pathways relevant to pulmonary hypertension. A total of 13 targets were selected for validation by real-time PCR, using these selection criteria (Online Table III). The validated miR-145 targets FSCN1, DAB2, and ACE were selected for further analysis.13,22–24 Real-time PCR validated 13 of the targets selected from the microarray (Online Figure VIII). The WT hypoxic group showed significantly increased expression versus all other groups for 7 of the targets TGFβ2, FRZB, ACE, ANGPTL4, AP2B1, and ITGB1 (Online Figure VIII). Further, the q-PCR data reflected the changes between groups observed in the microarray for WIFI1, CAMK2A, TTN, ACE, DAB2, and FSCN1 (Online Table III) and showed that these changes were significant (Online Figure VIII). The changes observed in the microarray were not validated for TMOD1 and APH1A (not shown). The validated targets were categorized into 5 groups: inhibitors of Wnt signaling (WIF1, FRZB, DAB2), regulation of actin cytoskeleton (FSCN, TTN), transcriptional regulation (CAMK2A), cell adhesion (ITGB1, CTGF), and endothelial function (ACE, ANGPTL4). Further analysis from the array data revealed marked changes in many components of the WNT signaling pathway (Online Table IV). We used primary human PASMC cultures to manipulate miR-145 using anti-miR and pre-miR strategies. Concordant regulation of targets was observed by miR145 manipulation in vitro (Online Figures IX and X). Since previous studies have demonstrated that p53 can regulate miR-143 and miR-145,25 we assessed p53 levels in WT and KO animals. Although p53 mRNA levels were enhanced in response to hypoxia in both WT and
KO animals, no differences between WT and KO animals were observed (Online Figure XI).

Quantification of the Development of PAH in WT Mice Treated With an miR-145 Anti-miR in Comparison With Controls and WT Mice Treated With an miR-143 Anti-miR

To determine if the protective effect against the development of PAH observed in miR-145 ablated mice in response to chronic hypoxia can be replicated by pharmacological manipulation of miR-145, 8-week-old female C57Bl6 mice were injected subcutaneously with an anti-miR specific for miR-145 or miR-143 and then exposed for 2 weeks to chronic hypoxia and compared with vehicle- and scramble-treated mice. A second dose was injected at day 8 of hypoxic exposure. The selective and substantial downregulation of miR-145 or miR-143 in the treated animals was confirmed in the lung by q-PCR (Figure 3A and 3B). Scramble-treated mice showed no change in the expression of both miR-143 and miR-145 (Figure 3A and 3B). Northern blot confirmed selective downregulation (Figure 3C through 3F). We next evaluated the development of PAH in treated mice in comparison with controls. In vehicle-treated hypoxic mice, we observed a significant and expected elevation in systolic RVP and RVH in comparison with vehicle-treated normoxic mice (Figure 4). The same effect was observed in scramble-treated and anti-miR-143 animals; however, mice treated with anti-miR to miR-145 showed a significant reduction in systolic RVP (Figure 4A). No changes were observed in RVH and SAP in anti-miR-145–treated or anti-miR-143–treated mice (Figure 4B), despite substantially reduced levels of the miR-145 in the right ventricle and left ventricle plus septum observed by q-PCR (Online Figure XII). Histological analysis showed a significant reduction of pulmonary vascular remodeling in small PAs of anti-miR-145–treated mice exposed to chronic hypoxia in comparison with the percentage of remodeling observed in vehicle- or scramble-treated mice exposed to hypoxia (Figure 4C and 4D). These data show that selective pharmacological manipulation of miR-145 but not miR-143 prevents the development of PAH in mice exposed to hypoxia. No changes in mean SAP were observed (Online Figure XIIIA). No changes across the groups were observed in the heart rate (Online Figure XIIIIB).

miR-145 in Human PAH

We next assessed whether miR-145 is expressed in lung tissue taken from patients with PAH and examined the expression levels in the lungs of patients with iPAH and hPAH and compared this with control lung tissue. We first extracted miRNA from paraffin-embedded lung tissue. Compared with controls, miR-145 was significantly elevated in both hPAH and iPAH samples (Figure 5A). We next used in situ hybridization to localize expression of miR-145 in human lungs selected from the above patient groups (Figure...
5B). In concordance with analysis of the mouse lungs, expression of miR-145 in control lung sections was confined to smooth muscle cells, including both vascular and bronchiolar lineages (Figure 5B). PAH is characterized by the development of both concentric and plexiform arterial lesions involving the pre-acinar and intra-acinar pulmonary arteries. In patients with iPAH and hPAH, miR-145 was expressed by arterial smooth muscle cells and observed within the muscular component of concentric lesions and plexiform vascular lesions wherever present (Figure 5B). miR-145–positive cells were also observed in pre-acinar and intra-acinar arteries where vessels had become muscularized (Figure 5B). In addition, newly muscularized arterioles at the level of alveolar ducts expressed abundant miR-145 (Figure 5B). Although in situ localization is not quantitative, we also observed that miR-145 expression was notably reduced in neointimal myofibroblasts compared with more differentiated SMCs resident within the medial layer (for example, see Online Figure XIV). Taken together, in human lung, miR-145 is expressed predominantly in the smooth muscle cell compartment and this was confirmed by colocalization studies (Online Figure XV), although we cannot rule out expression in other cells such as vascular endothelium. miR-145 expression is also observed within remodeled vessels of complex lesions in patients with hPAH and iPAH. In some vessels, levels of miR-145 appear reduced in neointimal cells.

Because the growth and characteristics of PASMCs isolated from patients with BMPR2 mutations is fundamentally different from those isolated from patients without germline mutations, we used human primary cells in culture to assess the effect of BMPR2 mutations on miR-145 regulation. We therefore cultured primary human PASMCs obtained from PAH patients and quantified miRNA levels at the pre-miRNA and mature miRNA levels. Both the preform of miR-145 as well as the mature form were significantly elevated in cells derived from patients with known BMPR2 mutations compared with nonmutated control PASMCs (Figure 6C and 6D). Therefore, basal levels of miR-145 are elevated in patients with germline BMPR2 mutations.

Effect of BMPR2 Downregulation on miR-143 and miR-145 Expression in Human PASMCs and Regulation of miR-145 in Cell Culture

Davis-Dusembery et al recently reported that BMP4 is able to induce the expression of the miR-143/145 cluster through the CArG box present in its promoter.17 We decided to evaluate
in vitro the effect of a siRNA-mediated knock-down of BMPR2 on miR-143 and miR-145 expression level in human WT PASMCs. Cells were therefore transfected with a short interfering (si) sequence, able to target and downregulate specifically BMPR2, or with an siScramble as negative control. RNA was extracted from these samples and from untreated cells after 72 hours for comparison. The downregulation of BMPR2 induced the significant upregulation of both miR-143 and miR-145, whereas no changes were observed in untreated or siScramble-treated cells (Online Figure XVI), suggesting that miR-145 acts downstream of BMP signaling in PASMC. Pathway analysis from our transcriptional profiling from in vivo samples resulted in some transcriptional changes in the BMP signaling pathway at the RNA level (Online Figures XVII and XVIII).

We also assessed the reverse to the above and asked whether miR-145 modulation in PASMC in vitro had an effect on BMPR2 expression. Anti-miR–driven downregulation or miR145 mimic-delivery miR-145 failed to influence expression of BMPR2 gene expression (Online Figure XVI), suggesting that miR-145 acts downstream of BMP signaling in PASMC. Pathway analysis from our transcriptional profiling from in vivo samples resulted in some transcriptional changes in the BMP signaling pathway at the RNA level (Online Figures XVII and XVIII).

We assessed the effect of hypoxia on miR-145 levels in both PASMC and PAECs but did not observe any effects at 3 different time points in either cell type (Online Figure XXII). Neither upregulation nor downregulation of miR-145 in PASMC influenced cell migration under our experimental conditions (Online Figure XXIII), although we observed a reduction in PASMC proliferation in the presence of miR-145 mimic delivery (Online Figure XXIV).

miR-145 Expression in BMPR2 R899X Mice
Considering the importance of miR-145 regulation in the mouse models of PAH and in PASMCs extracted from PAH patients with a mutation in the BMPR2 gene, we evaluated the effect of a truncating BMPR2 mutation on miR-145 expression in heterozygous R899X± mice. RNA was extracted from the whole lung of 6-month-old mice and analyzed by q-PCR and Northern blot for miR-145. This revealed a significant upregulation of miR-145 in the mutated mice compared with controls (Figure 7A through 7C). In situ localization of miR-145 in paraffin lung sections confirmed positive staining within the smooth muscle layer of pulmonary vessels and bronchi of both WT and BMPR2-mutated mice, with strong staining observed in mutated animals (Figure 7D).

Discussion
We describe the expression and regulation of miR-145 in the lung under normal conditions and in response to hypoxic injury. In the hypoxic mouse model, we show elevation of miR-145 levels in total lung and in the right ventricle compared with controls. Moreover, we demonstrate that genetic
Ablation of miR-145 prevents the development of PAH. This occurred in the absence of any modulation of miR-143, thus confirming the importance of miR-145 in the development of PAH. To confirm this protective effect of miR-145, we measured PAH development also in wt mice treated with an anti-miR specific for miR-145 and exposed to chronic hypoxia, observing again a significant reduction in the systolic RVP of the treated hypoxic animals in comparison with untreated mice exposed to the same environment. A substantial downregulation of miR-143 with a specific anti-miR did not show a similar effect suggesting a particular importance for miR-145 in PAH.

In human samples from patients with hPAH and iPAH, we show elevated miR-145 levels. Localization studies revealed miR-145 expression in SMCs of complex lesions. We further demonstrate a specific association between BMPR2 mutations and elevated levels of miR-145 in PASMCs extracted from PAH patients and in the lungs of mice mutated in this gene. Thus, we suggest an essential role for miR-145 in the development of PAH.

Recently, several studies pointed out not only the specific smooth muscle localization of this miRNA but also its fundamental role in the differentiation of this cell type. In particular, miR-145 has been identified as the most abundant miRNA in normal arteries and in differentiated VSMCs. Considering the central role of PASMCs in remodeling in the small peripheral, normally nonmuscular PAs during the development of PAH, we decided to evaluate the effect of the ablation of this miRNA in mice exposed to hypoxia. In particular, we used knockout mice deleted for the sequence coding for miR-145 but able to express miR-143, transcribed in the same precursor. This suggested a predominant role of miR-145 in SMCs differentiation, leading us to use a single miR-145−/− in this study to evaluate its role in the development of PAH. Cell culture

**Figure 6. Analysis of Human BMPR2-mutated PASMCs.** A and B, q-PCR analysis of miR-145 expression in PASMCs. Total RNA was extracted from PASMCs of hPAH patients with a mutation in the BMPR2 gene. Passage 4 primary cells were used. cDNA was analyzed for A, premature, and B, mature miR-145 expression in comparison with unaffected controls. Results were normalized to GAPDH for the premature and Rnu-48 for the mature miR-145 and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analyzed using an unpaired t test (**P<0.005, ***P<0.001 versus control samples). C and D, The same total RNA extracted from WT and BMPR2-mutated cells was also used for Northern blot analysis to confirm the q-PCR result. Blot quantification (D) was performed with Scion Image software (www.scioncorp.com): band intensities of the miRNA of interest were established and normalized to the relative U6 signal. Data were analyzed using an unpaired t test (**P<0.001 versus control samples).

**Figure 7. miR-145 expression in WT and BMPR2 R899X mice.** RNA was extracted from the total lung of 4 WT or BMPR2-mutated mice and assessed for miR-145 expression by q-PCR (A) and Northern blot (B and C). D, In situ hybridization showing miR-145 localization in the lung of the same mice. Paraffin sections were rehydrated and incubated with an anti-miR-145 or scramble probe as negative control. For colocalization, α-actin was detected in the same samples using an immunohistochemistry assay, with nonimmune isotype-IgG antibody as negative control. Images ×200 magnification, scale bars=50 µm.
studies in both PASMC and PAEC failed to show an effect of hypoxia per se on expression levels of miR-145. This suggests that miR-145 is expressed in both compartments, but the regulation cannot simply be induced in isolated cell compartments in vitro. This is consistent with recent data demonstrating the important interactions in vivo between vascular cells through miR-145–mediated cellular communication. Our cell culture studies in isolated primary PASMC failed to show an effect of miR-145 modulation on cell migration, although we did observe an inhibition of cell proliferation by miR-145 mimic delivery. Further studies are required to detail such phenotypes in detail, but it is likely that complex cell:cell interplay in vivo is relevant to the regulation of cellular changes that lead to vascular remodeling in the development of PAH, as has been observed in other vascular pathological settings. A functional analysis of the vasculature of miR-145−/− mice revealed a reduced pulmonary vascular response to chronic hypoxia and reduced right ventricular hypertrophy. In addition, the percentage of remodeled vessels was significantly reduced in the knockout animals, strongly suggesting a protective role for miR-145 ablation in PAH development. The similar protective effect was observed in WT mice treated with an anti-miR able to reduce miR-145 expression substantially. Interestingly, we did not observe an effect of miR-145 anti-miR on RVH in the setting of anti-miR delivery to wild-type mice. This may be due to inefficient target derepression using our dosing regimen or the possibility that residual miR-145 is able to negate this effect compared with knockout animals. This will require further analysis to assess these possibilities. Moreover, we demonstrated significant upregulation of this miRNA in both hPAH and iPAH and in particular in BMPR2-mutated PASMCs extracted from hPAH patients. A similar level of miR-145 upregulation was also identified in mice carrying a mutation in this gene. This conserved dysregulation suggests the presence of a link between the regulation of miR-145 and the TGF-β superfamily, already known to be involved in the maturation process of other miRNAs, including miR-21, through the interaction of SMAD proteins with DROSHA during the second step of miRNA biogenesis. Very recently, this link has been proved with the observation that both TGF-β and BMP4 are able to activate transcription of the miR-143/145 cluster through the CArG box present in its promoter, though via a different mechanism. In fact, MRTF-A was shown to be essential for BMP4-dependent induction of the cluster, whereas TGF-β enhances miR-143/145 expression via SRF and MYOCOD expression. Interestingly, despite the upregulation of miR-145 in the lung tissues of the analyzed PAH patients, the in situ localization of this miRNA in the vessels of iPAH and hPAH patients showed abundant miR-145 expression in hypertrophied arteries, pulmonary vascular lesions, and newly muscularized arterioles. The lack of neointimal expression of miR-145 is consistent with the presence of a dedifferentiated mesenchymal cell in the neointima. However, lack of miR-145 profoundly impedes the development of a neointima in the mouse carotid injury model due to diminished migratory activity of SMCs. The mouse hypoxic model is a model of distal muscularization of the pulmonary circulation rather than neointima formation. Nevertheless, taken together, our findings and results in the systemic circulation suggest that inhibition of miR-145 may perturb both the process of muscularization and neointima formation. It is also interesting that upregulation of miR-145 in the PASMCs obtained from iPAH patients has been recently noted in another study, confirming our findings. However, in that study, the focus was on the role of miR-204 in PAH, without further analysis of miR-145 expression and regulation. We did not observe an effect on the prevention of PAH in WT mice treated with an anti-miR to miR-143, suggesting a predominant role for miR-145 in the development of PAH. There are previous studies showing that miR-145 and miR-143 KO do not always have the same effect on phenotype. In vitro, antago-miR–mediated inhibition of miR-145 expression blocked the ability of myocardin to convert fibroblasts into VSMCs, with a less substantial effect by miR-143 knock-down. Moreover, the upregulation of miR-145 (but not miR-143) in neural crest stem cells was sufficient to induce the conversion of ≈75% of cells into VSMCs. In vivo, abnormalities in the vessel wall of the aorta and other arteries, noticeably thinner than those of WT animals, were observed in miR-145 and miR-143/145 double KO mice but not in miR-143 mice. Further studies are warranted to confirm the predominance of miR-145 over miR-143 in this setting. 

Our studies suggest that miR-145 is acting downstream of BMP signaling because modulation of miR-145 in vitro and in vivo failed to influence BMPR2 levels or components of the BMP signaling axis. In contrast, downregulation of BMPR2 using siRNA in PASMC elevated miR-145 levels. Dysregulation of several miRNAs in pathological samples (animal or human) in PAH is now clear, with each miRNA having many hundreds of potential gene targets. This defines a complex transcriptional situation with multiple pathways and cell types potentially involved. Several studies recently showed the important role of KLF4 and KLF5 in SMC proliferation and differentiation. In particular, Cheng et al studied the capacity of miR-145 to control neointima lesion formation through the regulation of KLF5. Cordes et al showed that miR-143 and miR-145 can repress several factors (including KLF4 and 5) that normally promote the less differentiated, more proliferative smooth muscle phenotype. In the present study, we show that KLF4 and 5 expression correlate with miR-145 regulation, with a significant upregulation of both these targets in hypoxic miR-145 knockout mice. However, it is not possible to evaluate this process in the hypoxic mouse because of the lack of neointima formation in this model. In our study, we evaluated global gene expression in response to genetic depletion of miR-145 to clarify the mechanism behind the protective effect of this depletion against the development of PAH. The resistance of miR-145−/− mice to hypoxia induced PAH seems likely to be due to differential regulation of multiple direct and indirect miR-145 targets. However, the validation of direct miR-145 targets and direct or indirect miR-145 targets points to the modulation of specific pathways. Most notably, 3 validated targets from the WT hypoxic versus miR145−/− hypoxic comparison inhibit canonical
Wnt/β-catenin signaling. WIF1 and FRZB bind to Wnt proteins in the extracellular space and prevent ligand-receptor interactions,\textsuperscript{31,32} whereas DAB2 stabilizes phosphorylated axin and attenuates Wnt/β-catenin signaling by increasing proteasomal degradation of β-catenin.\textsuperscript{33} These factors inhibit Wnt signaling leading to decreased expression of Wnt target genes, such as cyclin D1 (CCND1) and E cadherin (CDH), which were also found downregulated in the miR145\textsuperscript{−/−} hypoxic group (Online Table II). Downregulation of CDH and CCND1 can cause cytoskeletal reorganization and decreased proliferation, respectively.\textsuperscript{34,35} It was previously reported that Wnt signaling can drive a program of smooth muscle differentiation in the lung, including the pulmonary vasculature.\textsuperscript{36} In agreement with analysis of in vivo tissues, we also observed regulation of targets at the mRNA level in PASMC with miR-145 manipulation via both anti-miR and pre-miR overexpression strategies. Therefore, inhibition of Wnt/β-catenin signaling may contribute to the phenotype observed in hypoxia in the miR145\textsuperscript{−/−} mice and is worthy of further detailed investigations.

Despite the complexity, the manipulation of individual miRNAs, however, such as miR-145 in the present study, or miR-204,\textsuperscript{29} appears to exert a substantial impact on the development of PAH. For this reason, our study also highlights the potential for miR-145 downregulation as a novel therapeutic approach for PAH. Strategies to achieve this may require selective delivery to the pulmonary system to avoid potentially deleterious effects of downregulation of miR-145 in the peripheral vasculature. Our in situ studies in mouse and human show expression of miR-145 in the smooth muscle cells of the lung and within vascular lesions associated with PAH. Hence, strategies to deliver selectively within the lung are attractive. Because miR-145 is also expressed in bronchial smooth muscle cells, the effect on bronchial tone remains to be evaluated. Taken together, the results of this study have demonstrated an essential role for miR-145 in the development of PAH through remodeling associated with the SMC compartment and association of elevated miR-145 levels in patients and mice with mutations in the BMPR2 receptor.

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Disclosures

E.V.R. is a co-founder and employee of MiRagen Therapeutics; E.O. is a co-founder of MiRagen Therapeutics; A.H.B., M.M., and N.M. have filed a patent on the role of miR145 in this disease setting.

References

miR-145 is upregulated specifically in the lung and right heart of mice. In patients with pulmonary arterial hypertension, miR-145 is upregulated in the pulmonary vasculature. This miRNA is expressed in vascular smooth muscle and can regulate the differentiation and proliferation of these cells. Expression of certain miRNAs are known to be altered in samples from patients with pulmonary arterial hypertension.

**What New Information Does This Article Contribute?**

- miR-145 is upregulated in the pulmonary vasculature of patients with different forms of pulmonary arterial hypertension.
- miR-143 and miR-145 are involved in the onset of several pathologies.
- Expression of certain miRNAs is known to be altered in samples from patients with pulmonary arterial hypertension.
- miR-145 is expressed in vascular smooth muscle and can regulate the differentiation and proliferation of these cells.
A Role for miR-145 in Pulmonary Arterial Hypertension: Evidence From Mouse Models and Patient Samples

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SUPPLEMENTAL MATERIAL

Detailed Methods

Ethical Information

All animal procedures conform with the United Kingdom Animal Procedures Act (1986) and with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Animal approval was granted by the University Committee Board. Experimental procedures using human PASMCs conform to the principles outlined in the Declaration of Helsinki.

Cell culture and transfection

Single donor HPASMCs and hPAECs were obtained from PromoCell (GmbH, Heidelberg, Germany). PASMCs were explanted and cultured as described previously. Smooth muscle cell lines were obtained from 4 patients with PAH known to harbor a mutation in BMPR2. These included: 1 patient with a mutation in the kinase domain of BMPR2 in which arginine is substituted for cysteine at position 347 (C347R); 1 patient with a missense mutation in the cytoplasmic tail of BMPR2, leading to a serine in place of asparagine at position 903 (N903S); 1 patient with a truncating mutation at amino acid position 9 (W9X) and 1 patient with a truncating mutation at amino acid position 899 (R899X). HPASMCs from a 41 year old female patient with IPAH (without mutations in BMPRII) and three further PASMC preparations were obtained from unaffected subjects. Cells were cultured as previously described. Cells were used between passages 1 and 4. For hypoxic exposure hPASMCs and hPAECs were grown
to 80% confluency in 6 well plates. Cells were then quiesced in 0.2% FBS for 24 hours before being placed in a hypoxic chamber (5% O\textsubscript{2}, 5% CO\textsubscript{2}, balance N\textsubscript{2}) for 4-72 hours.

The synthetic miR-145 mimic (pre-miR-145 precursor molecule) and the cy3 scrambled pre-miR were obtained from Ambion. Anti-miR to miR-145 (miR-145 anti-miR) and negative control (a scrambled oligonucleotide, scrambled-antimiR) were obtained from Miragen Therapeutics (Boulder, CO 80301). A random sequence anti-miR molecule (scrambled anti-miR, Miragen) and random sequence pre-miR (scrambled pre-miR) served as negative controls. The miR-145 antimiR, Scrambled-antimiR, pre-miR-145 and Scrambled-pre-miR were introduced into the HPASMCs using the transfection agent Lipofectamine 2000 (Invitrogen) the standard protocol for transfection small RNAs was followed. In brief, 50nM pre-miR or anti-miR were transfected per 1x10\textsuperscript{4} cells in 24 well plate format or 5x10\textsuperscript{4} cells in 6 well plate format. Cell culture media was changed after 6 hours to remove the transfection reagent in an attempt to avoid toxicity which may be caused by Lipofectamine 2000. BMP4 was used for 48 hours at a concentration of 1ng/µl. Gene or miRNA expression was analyzed 48 hours after transfection. During the course of the 48 hour incubation period, cells were viewed microscopically and consistently found to be adherent and viable. The \textit{in vitro} experiments were performed in triplicate, and the data shown as Average ±SEM.

\textit{Haemodynamic Measurements}

For all the experiments, systolic right ventricular systolic pressure (sRVP) was measured in mice under isoflurane (1.5% in O\textsubscript{2}) anesthesia via a needle advanced into the right ventricle trans-diaphragmatically. Systemic arterial pressure (SAP) was
recorded via a cannula placed in the carotid artery as previously described \(^2\). Right ventricular hypertrophy (RVH) was determined as ratio of the right ventricular wall (RV) weight to the left ventricle plus septum (LV + S) weight. Lung sections were stained with Elastic-Van Gieson (EVG) stain and the percentage of remodelled vessels assessed in a blinded fashion as previously described \(^2\). Lung sections of 8 animals per group were stained with Elastin-Van Gieson and microscopically assessed in a blinded fashion. Pulmonary arteries (≤ 80 microns of external diameter) were considered remodelled if they possessed a distinct double elastic lamina for at least half of the diameter of the vessel cross section. The % of remodelled vessels was calculated as the number of vessels possessing a double elastic lamina / total number of vessels × 100. Approximately 150 arteries from each lung section were assessed.

**RNA extraction from frozen lungs, PASMCs and reverse transcription**

Total RNA from tissues and cells was obtained using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions, treated with the DNAse 1 (amplification grade; Sigma, St. Louis, MO, USA) in order to eliminate genomic DNA contamination and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA for miRNA analysis was synthesized from total RNA using stem-loop reverse transcription primers according to the TaqMan MiRNA Assay protocol (Applied Biosystems, Foster City, CA, USA). Each reaction contained 50 ng of extracted total RNA, 50 nM stem-looped RT primer, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 U/ml Multiscribe reverse transcriptase and 0.25 U/ml RNase inhibitor. The 15 ml reactions were incubated in a 96-well plate for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C and then held at 4°C. Total cDNA for pre-
miRNA analysis was obtained from total RNA using the SuperScript II Reverse Transcriptase (Invitrogen, Paisley, UK). Each reaction contained 1 mg of total RNA, 1 × SuperScript II buffer, 10 U/ml SuperScript II RT, 0.15 mg/ml of random hexamer primers (Invitrogen, Paisley, UK), 1 U/ml of RNase inhibitor (Promega, Madison, WI, USA) and 0.25 mM each of dNTPs. Cycling conditions were the following: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C. cDNA was stored at -20°C. GAPDH was selected as housekeeping gene due to its stability across all in vivo groups.

miRNA extraction from paraffin-embedded human lungs

Formalin fixed paraffin wax embedded tissues blocks were obtained from the Papworth NHS Foundation Trust Hospital Tissue Bank adhering to LREC and HTA guidelines. Lung samples were used from informed and consenting patients undergoing transplantation at Papworth Hospital for end stage pulmonary hypertension. Tissue was obtained from a range of conditions including hPAH associated with mutant BMPR2 (n=5), iPAH (n=6; without mutations in BMPRII), and controls (n=6). Controls comprised of tissue taken from lobes of lung clear of tumour from patients undergoing pneumonectomy for lung carcinoma and reported free of tumour by a pathologist. The RecoverAll total RNA Isolation kit (Ambion, Streetsville, Canada) was used to extract total RNA (including miRNA) from Formalin-Fixed, Paraffin-Embedded FFPE) samples. Three 10 mm slices were dewaxed with xylene for 3 minutes at 50°C, washed twice with ethanol, and digested with protease at 50°C for 15 minutes, then for 15 minutes at 80°C. The lysate was passed through a filter cartridge and DNAse digested, then RNA was eluted in 30 ml of RNase free water and quantified using the NanoDrop ND-1000 Spectrophotometer.
**TaqMan q-PCR Analysis of Mature miRNAs and mRNAs**

For quantitative PCR (q-PCR), 10 ml reactions were incubated in a 386-well optical plate at 95°C for 10 minutes, following by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Results were normalized to U6 or Rnu-48 values for mouse and human miRNAs respectively and to GAPDH for pre-miR-145 expression. The fold change for every miRNA expression was obtained using the 2-ΔΔCt method\(^3\). The q-PCRs for each miRNA were run in triplicate and results are presented as the mean ± standard error of samples.

**Microarray analysis of WT and miR-145-/- pulmonary arteries**

Main branch pulmonary arteries were dissected from 6 mice from each group at 10 weeks of age and stored frozen at -80°C prior to RNA isolation. RNA quantity and quality were assessed by NanoDrop® Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA integrity was assessed with the Agilent 2100 bioanalyser using the RNA 6000 Nano Kit (Santa Clara, CA). The Illumina TotalPrep RNA amplification kit (Ambion) was used to generate biotinylated, amplified RNA, from 500 ng input RNA, for hybridization with the Illumina arrays (Applied biosystems Carlsbad, California). The Illumina mouseWG-6 v2.0 Expression beadchips were hybridised following the manufacturers protocol, scanned with the Illumina BeadArray Reader and read into Illumina GenomeStudio® software (version 1.1.1). For microarray data analysis and validation, quantile normalised and background subtracted intensity values were exported from GenomeStudio® software for data processing and analysis in R (http://www.R-project.org) in which rank product statistical analysis was carried out.
Adjacent comparisons were carried out between the 4 groups. Probes with a false discovery rate less than 0.05 were considered significant. Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) were used for target validation on cDNA derived from the 24 samples used for the microarray (n=6 per group).

**Northern Blot Analysis**

Total RNA was separated on a 15% TBE-Urea gel (Invitrogen, Paisley, UK), transferred to an uncharged nylon membrane, Hybond-NX (Amersham Bioscience UK Ltd, Buckingham, UK) using a trans-blot semi-dry system (Bio-Rad Laboratories, Hemel Hempstead, UK), and UV cross-linked. Pre-hybridization was carried out at 55°C for 30 minutes with hybridization buffer (50% de-ionized formamide, 5X SSPE, 5X Denhardts solution, 0.1% SDS, and 2 µg of heat-denatured herring sperm DNA). Then 25 pmol of miR-145 or U6 miRCURY LNA™ Detection probe, 5`-Digoxigenin (DIG)-labeled (Exiqon, Denmark), were added over night at the same pre-hybridization temperature. Following hybridization, the membrane was washed for 30 minutes at 50°C with the low stringency wash solution (invitrogen, Paisley, UK) followed by a 30 minutes wash with the high stringency wash solution (invitrogen, Paisley, UK). After that, the membrane was blocked for 30 minutes in blocking solution (1% Blocking reagent in maleic acid) and incubated for 30 minutes with an anti-DIG antibody (Roche Applied Science, Indianapolis, IN, USA) 1:5000 at room temperature. The CDP Star Chemiluminescent Substrate (Sigma-Aldrich, Poole, UK) was used to detect the presence of the miRNA of interest on the membrane. miRNA quantification was performed with the Scion Image software (www.scioncorp.com): band intensities of the miRNA of interest were established and normalized to the relative U6 signal.
**Immunohistochemistry**

Human and mouse lungs were fixed in 4% paraformaldehyde solution at 40°C for 18 hours and embedded in paraffin. After deparaffinization with graded concentrations of xylene and ethanol, slides were immersed in 3% H2O2 in phosphate buffered saline (PBS) for 30 minutes at room temperature to block endogenous peroxidase activity. Then, they were incubated with 20% normal goat serum for 30 minutes to reduce non-specific background staining. The sections were then incubated with mouse monoclonal antibody against α-smooth muscle actin (Dako, Clone 1A4, High Wycombe, UK), in 1% (w/v) bovine serum albumin (BSA) in PBS or isotype matched mouse IgG nonimmune control (Dako, High Wycombe, UK). Sections were then incubated with appropriate biotinylated secondary antibody (Dako, High Wycombe, UK) diluted 1:200 in 1% (w/v) BSA in PBS, and then horseradish peroxidase-labeled Extravidin™ (Sigma, St. Louis, MO, USA) diluted 1:400 in 1% (w/v) BSA in PBS. Staining was visualized using 3.3 diaminobenzidine and the nuclei were counterstained with Mayer’s haematoxylin.

**In situ hybridization for detected of miRNA localization**

For the detection of miR-145 in mouse and human lung, sections were rehydrated with histoclear and graded concentrations of ethanol. Slides were then boiled for 10 minutes within 10 mM sodium citrate pH 6.0, cooled to room temperature (RT), incubated with 10 μg/ml proteinase K at 37°C for 15 minutes and finally fixed in 4% PFA for 10 minutes at room temperature in order to allow antigen retrieval. Following antigen retrieval, slides were incubated with hybridisation buffer (50% formamide, 4X SSC, 2.5X Denhardt’s solution, 2.5 mg/ml salmon DNA, 0.6 mg/ml yeast tRNA, 0.025% SDS and
0.1% blocking reagent) at 60°C for 1 hour followed by a 60°C overnight incubation with
40 nM miR-145 or scramble miRCURY LNA™ Detection probe, 5’-DIG labeled (Exiqon, Denmark) in the same buffer. Melting temperatures were 79 °C and 78 °C, respectively. Immunodetection was performed blocking the sections in 1% blocking reagent in PBS and 10% FCS for 1 hour at RT followed by a 4°C overnight incubation with an anti-DIG antibody (Roche Applied Science, Indianapolis, IN, USA) diluted 1:1000. Slides were then incubated with 0.1M Tris pH 9.0 for 5 minutes. In order to stain miR-145, BM purple solution (Roche Applied Science, Mannheim, Germany) or NBT/BCIP solution (Roche Applied Science, Mannheim, Germany) was added to each human or mouse section respectively and left at room temperature for 3 days. We assessed the specificity of the above as detailed in the Supplementary Methods section.

**Colocalisation of SMA and miR145**

MicroRNA in situ hybridisation was performed on routinely fixed paraffin-embedded 6um human lung sections. The slides were deparaffinized with histoclear, and treated with 10ug/ml proteinase K (Sigma) at 37 degree for 20 minutes, then fixed with 4% paraformaldehyde for 10 minutes. After washing with phosphate buffered saline (PBS), slides were incubated with hybridisation buffer at 60 degree for 1 hr. Then slides were hybridised with 40nM DIG-labelled miR145 or scramble probe (Exiqon) at 60 degree overnight. After washing and blocking, slides were incubated with anti-DIG_AP Fab fragments (Roche)in blocking buffer at 4 degree overnight, and washed with PBST (PBS plus 0.1% Tween 20) and 0.1M Tris-HCL (pH9.5). miR-145 was visualized with BM purple solution (Roche) for 1-2 days at room temperature until the staining was visible under microscope.
After washing with PBS, slides were quenched with 0.3% H2O2 in PBS for 10min. To block non-specific background, 10% rabbit normal serum was applied on slides for 1hr, then slides were incubated with monoclonal anti-human alpha smooth muscle actin (DAKO) in 10% normal serum at 4 degree overnight. The slides were visualized with 3,3'-diaminobenzidine as chromogen for 5 minutes.

**Down-regulation of BMPR2 expression with a si sequence**

PASMCs were seeded in 6-well plates (1.5 x 10^5 cells/well) and grown for two days in DMEM/10%FBS. Prior to transfection, PASMCs were incubated in Optimem I for 3h. PASMCs were transfected on Day 0 with a final concentration of 10nM siRNA [Dharmacon™ On-TARGETplus siRNA for BMPR-II or Smad4, or siControl Non-targeting Pool (siCP)(Perbio Science UK Ltd)] in complex with DharmaFECT1™ (4ml/well) diluted in Optimem I. The Dharmafect was incubated in half the final volume (200ml for 1 well) of Optimem I for 5 minutes followed by addition of Optimem I (2ml for 1 well) containing 10X final concentration of the relevant siRNA, making the siRNA at 5X final concentration. The mix was incubated for 20 minutes at room temperature to allow lipoplexes to form. The Transfection mix (4ml/well) was dropped onto cells in fresh Optimem I (1.6ml/well), ensuring full coverage of the well. Cells were incubated with the complexes for 4h at 37oC, followed by replacement with DMEM/10% FBS for 24h Day 1). RNA was extracted after 4 days.

**Migration assay**

The migration assay was based on a previously published protocol. In brief, 5x10^4 cells in 6 well plate format cells were quieced in media containing 0.2% serum for
48 hours, the cells were then scratched with a p200 pipette tip and containing 0.2% serum was added to all wells except the serum control to which media containing 15% serum was added. Scratch closure was measured at 0 hour, 12 hour and 24 hour time points. The data was analysed using Image Pro analyser 7.0, percentage scratch closure and ±SEM were calculated for each timepoint.

**Proliferation assay**

To measure DNA synthesis, hPASMCs were seeded in 24-well plates at a density of 20,000 per/well and grown to 60% confluency before quiescence in 0.2% FBS for 24 h. Cells were then transfected as described above, exposed to 0.2, 2.5 or 10% serum, and allowed to proliferate for 72 hours. Twenty four hours prior to the end of each experiment, 0.5 µCi [3H]thymidine was added to each well. hPASMCs were then rinsed with phospho-buffered saline and 5% trichloroacetic acid, then lysed with 0.3 mol/L NaOH. Radioactivity was measured using a liquid scintillation counter, and data expressed as % change compared with control.

**Western Blot Analysis**

Total protein concentration was determined using the bicinchoninic acid method (Pierce BCA). Protein samples were denatured by boiling for 10 minutes and equal amounts (20 µg) resolved in SDS/PAGE 10-12% gels. Proteins were transferred onto Hybond-P nitrocellulose membranes (Amersham Bioscience U.K. Ltd), assessed for equal loading/transference by Ponceau S Solution (Sigma) staining and immunoblotted with rabbit anti-KLF4 or KLF5 (Abcam, Cambridge, UK 1:500), mouse anti-BMPR2 (BD Transduction Laboratories 1:300) or Rabbit polyclonal antibody against phospho-Smad1 (Ser463/Ser465) / Smad5 (Ser463/Ser465) / Smad8 (Ser426/Ser428) before incubation.
with rabbit anti-mouse IgG-HRP or goat anti-rabbit respectively (Dako, 1:1000). Proteins were visualized using the ECL Plus Western blotting detection kit (Amersham Biosciences U.K. Ltd).
Suppl. Figure I. miR-143 in normoxic versus hypoxic mice. q-PCR analysis of normoxic and 14 days hypoxic wt mice. Total RNA was extracted from the total lung (A) or the right ventricle (B) of normoxic (white bars) or hypoxic (black bars) 10 week old mice. 6 mice/group were analyzed. Each sample was tested in triplicate. Results were normalized to U6 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analyzed using an unpaired t-test (*p<0.05, ***p<0.001 vs normoxic samples).
Suppl. Figure II. miR-145 expression in the brain, kidney and spleen of hypoxic wt mice. (A, B, C) Total RNA was extracted in quadruplicate from each organ and analyzed by q-PCR. Data were analyzed using a t-test but no significance was identified.
Suppl. Figure III. Analysis of miR-145 expression in miR-145 -/- mice in comparison with wt mice. miR-145 ablation in ko mice was confirmed by q-PCR (A) and in situ analysis (B). Images all x100 magnification, scale bars = 100 µm.
Suppl. Figure IV. miR-143 expression in wt and miR-145 -/- mice in response to hypoxia. q-PCR analysis of normoxic and 14 days hypoxic wt and ko mice. Total RNA was extracted from normoxic (white bars) or hypoxic (black bars) 10 week old mice. 6 mice/group were analyzed. Results were normalized to U6 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analyzed using a two-way ANOVA followed by Bonferroni’s post-hoc test (***p<0.001 vs control samples).
Suppl. Figure V. Assessment of (A) systemic arterial pressure (SAP, n = 6–9) and (B) heart rate (HR, n=6-10) in wt and miR-145-/- mice, normoxic and hypoxic. Data were analyzed using a two-way ANOVA followed by Bonferroni’s post-hoc test (**p<0.005).
Suppl. Figure VI. Analysis of miR-145 selected targets. The expression level of SMAD5 (A), SMAD4 (B), KLF4 (C) and KLF5 (D) was assessed by q-PCR in the total lung of wt and miR-145 -/- mice, normoxic and hypoxic. 6 mice/group were analyzed. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test (*p<0.05, ***p<0.001).
KLF4 in normoxic conditions

**B** KLF5 in normoxic conditions

- WT
- miR-145 -/-

**C** KLF4 in normoxic conditions

- WT
- miR-145 -/-

KLF5 in hypoxic conditions

**D** KLF5 in hypoxic conditions

- WT
- miR-145 -/-

**E** KLF4 in hypoxic conditions

- WT
- miR-145 -/-

**F** KLF4 in hypoxic conditions

- WT
- miR-145 -/-
Suppl. Figure VII. Analysis of KLF4 and KLF5 protein expression level in WT and miR-145 -/- hypoxic female mice. (A, D) The expression level of KLF4 and KLF5 was assessed by western blot in the total lung of WT and miR-145 -/- mice exposed to chronic hypoxia for 14 days. 4 mice/group were analyzed, and the intensity of the western blot bands was measured using a specific software (Scion Image software). The resulting quantification bars are represented in graphs in B, C, E and F. Results were normalized to GAPDH values and expressed as fold increase, with an arbitrary value of 1 assigned to the WT group. Data were analyzed using an unpaired t-test. **p<0.005 cf. wt mice.
Suppl. Figure VIII. Validation of microarray data.
The expression level of *WIF1* (A), *TGFB2* (B), *FRZB* (C) and *DAB2* (D) was assessed by q-PCR in pulmonary artery of wt and miR-145 -/- mice, normoxic and hypoxic. 6 mice/group were analyzed. Data were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc test (*p<0.05, ***p<0.001).
Suppl. Figure VIII (cont). Validation of microarray data; validated targets

The expression level of ACE (E), FSCN1 (F), was assessed by q-PCR in pulmonary artery of wt and miR-145 −/− mice, normoxic and hypoxic. 6 mice/group were analyzed. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test (*p<0.05, ***p<0.001).
Suppl. Figure VIII (cont). Validation of microarray data. The expression level of CTGF (G), Angptl4 (H), AP2B1 (I) and ITGBL1 (J) was assessed by q-PCR in pulmonary Artery of wt and miR-145 +/- mice, normoxic and hypoxic. 6 mice/group were analyzed. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test (*p<0.05, **p<0.005 ***p<0.001).
Figure IX. miR-145 Target expression in human PASMCs transfected with an antimiR specific for miR-145. The expression level of (A) miR-145 (B) ACE (C) ANGPTL4 (D) CTGF (E) DAB2 (F) FRZB (H) FSCN1 (I) ITGBL1 and (J) AP2B1 was assessed in human pulmonary artery smooth muscle cells transfected with antimiR-scramble or anti-miR-145 (n=12 per group). Results were normalized to RNU48 (A) or GAPDH (B-L) values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analysed using T-test (*p<0.05, *** p<0.001 n=12).
Figure X. miR-145 Target expression in human PASMCs transfected with a premiR specific for miR-145. The expression level of (A) miR-145 (B) ACE (C) ANGPTL4 (D) CTGF (E) DAB2 (F) FRZB (G) FSCN1 (H) ITGBL1 (I) TGFB2 and (J) AP2B1 was assessed in human pulmonary artery smooth muscle cells, transfected with pre-miR-scramble or pre-miR-145 (n=12 per group). Results were normalized to GAPDH values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analysed using T-test (*p<0.05, *** p<0.001 n=12).
Figure XI. Assessment of p53 levels in wt and miR-145 -/- mice, normoxic and hypoxic. The expression level of p53 was assessed by q-PCR in human proximal pulmonary artery smooth muscle cells, transfected with anti-miR-scramble, anti-miR-145 pre-mir-scramble or pre-miR-145 (n=9 per group). Results were normalized to B2M values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analyzed using a one-way ANOVA followed by Bonferroni's post-hoc test (*p<0.05 ***p<0.001).
Figure XII. Selective knock-down of miR-145 obtained after the injection of an antimiR miRNA specific. 8-week old female mice were injected subcutaneously with an antimiR perfectly complementary to the 5’ region of either the mature miR-143 or miR-145 sequence and subjected to chronic hypoxic for 14 days. Mice received a second injection after 8 days of hypoxia. After 14 days, mice were sacrificed and total RNA extracted from the right ventricle (A) or the left ventricle plus septum (B). miR-145 expression was assessed in normoxic vehicle treated animals, hypoxic vehicle-treated animals, and hypoxic animals injected with a scramble or a miR-145 antimiR. At least 6 mice/group were analyzed. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test (*p<0.05, ***p<0.001. miR-145 down-regulation is statistically significant versus all the other groups.).
Suppl. Figure XIII. Assessment of (A) systemic arterial pressure (SAP, n = 6–8) and (B) heart rate (HR, n = 7–13) in vehicle–treated normoxic mice and hypoxic mice injected with vehicle, scrambled, miR-145 or miR-143 antiMir. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test.
Suppl. Figure XIV. miR-145 detection in the neointimal lesion of hPAH patients. Images all x100 magnification, scale bars = 50 μm.
Suppl. Figure XV. Co-localisation of the miR145 probe to SMC. Normal human lung sections were processed for miR145 and SMA staining as detailed in the Supplementary methods. (A) miR145 probe and SM actin, (B) Control probe and SM actin. Mag x 10. (C) Analysis in normal human lung and lung from patients with iPAH and fPAH.
Suppl. Figure XVI. miR-143 and miR-145 up-regulation in human PASMCs downregulated for BMPR2 expression via a siRNA. Primary human PASMCs were transfected with a siRNA able to target and repress specifically BMPR2 expression or with a siScramble as a negative control. Total RNA was extracted after 72 h from these samples and untreated cells for comparison. The efficiency of BMPR2 down-regulation was evaluated by TaqMan Real-Time PCR (A). miR-143 (B) and miR-145 (C) expression were also assessed in the same samples. Results were normalized to GAPDH for BMPR2 and Rnu-48 for miR-143 and miR-145 and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analyzed using a one-way ANOVA followed by Bonferroni’s post-hoc test. **p<0.005, ***p<0.001 cf. CTR or siScramble group as indicated.
Supplementary Figure XVII. BMP signaling KO hypoxic versus WT hypoxic comparison
The diagram was created using Ingenuity Pathway Analysis Software (http://www.ingenuity.com/; Redwood, CA). Significantly differentially expressed genes (P-value < 0.05) were loaded into and viewed with the Ingenuity pathway analysis software. Green represents genes with decreased expression and red represents genes with increased expression in the KO hypoxic group compared to WT hypoxic.
Supplementary Figure XVIII. BMP signaling WT hypoxic versus WT normoxic comparison

The diagram was created using Ingenuity Pathway Analysis Software (http://www.ingenuity.com/; Redwood, CA). Significantly differentially expressed genes (P-value < 0.05) were loaded into and viewed with the Ingenuity pathway analysis software. Green represents genes with decreased expression and pink represents genes with increased expression in the KO hypoxic group compared to WT hypoxic.
Supplementary Figure XIX. miR-145, BMPR2 and ID1 expression in hPASMCs treated with an antimir or a pre-miR-145. The expression level of miR-145 (A, B), BMPR2 (C, D) and ID1 (E) was assessed in human pulmonary artery smooth muscle cells, transfected for 48 hours with anti-miR-scramble, anti-miR-145, pre-mir-scramble or pre-miR-145. An n of 9 was used per group. (E) PASMCs were stimulated for 48 hours with 1 ng/ml of BMP4. Data were analysed using Students t-test (A-D) or one-way ANOVA (E) (**p<0.001 n=12).
Supplementary Figure XX. BMPR2 and ID1 expression in PASMCs isolated from a IPAH patients and treated with an antimiR specific for miR-145. PASMCs were isolated from an IPAH patient and transfected with a miR-145 antimiR or a scramble sequence for comparison. Total RNA was extracted after 48h from these samples and the expression of BMPR2 (A) and ID1(B) was evaluated by TaqMan Real-Time PCR. Results were normalized to GAPDH and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. Data were analysed using a t-test. (C) Total lung protein expression of BMPR2 was analyzed by Western blot. Alpha tubulin was used as an endogenous control.
Supplementary Figure XXI. Analysis of the BMP signalling pathway in WT and miR-145 KO mice. The expression level of BMPR2 was assessed by q-PCR in the total lung (A) and pulmonary artery (B) of normoxic and hypoxic WT and miR-145 -/- female mice. (C) Total lung protein expression of phospho Smad 1-5-8 was analyzed by Western blot. Alpha tubulin was used as an endogenous control. (D) ID1 gene expression in total lung samples was assessed by q-PCR. For all q-PCR experiments, samples from n=6 mice per group were tested in triplicate. Results were normalized to β2-microglobulin values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. All data were analyzed using a two-way ANOVA.
Supplementary Figure XXII. miRNA expression in hypoxic human cells. q-PCR analysis of human PAECs (A) and PASMCs (B) showing the expression level of miR-145 after 4, 24 and 72h of hypoxic (5% O$_2$) stimulation in comparison with normoxic cells. Samples (n=5 per group) were tested in triplicate. Results were normalized to Rnu-48 values and expressed as relative fold change, with an arbitrary value of 1 assigned to the control group. For statistical analysis, a one way ANOVA was conducted.
Supplementary Figure XXIII. MiR-145 modulation does not affect hPASMC migration in 0.2% serum.

(A) hPASMC cells were seeded in 6 well plate format at a density of $5 \times 10^4$ per well and
(B) quieced in media containing 0.2% serum for 48 hours. The cells were then scratched with
(C) a p200 pipette tip and media containing 0.2% serum was added to all wells except the
(D) serum control to which media containing 15% serum was added. Scratch closure was
(E) measured at 0 hour, 6 hour, 12 hour and 24 hour time points after the “wound”.
(F)(B) Graphical representation of the effect of miR-145 modulation on wound healing.
(G) The data was analysed using Image Pro analyser 7.0, percentage scratch closure and
(H) ±SEM were calculated for each timepoint.
Supplementary Figure XXIV. Effects of pre-miR-145 on proliferation of hPASMCs. hPASMCs were seeded in 24-well plates at a density of 20 000 per/well and quiesced in 0.2% FBS for 24 h. Cells were then transfected with a pre-miR-145 or a scramble sequence for comparison, and exposed to 0.2, 2.5 or 10% serum, and allowed to proliferate for 72 hours. Twenty four hours prior to the end of each experiment, 0.5μCi [³H] thymidine was added to each well. For each experiment n=4 wells were assessed per treatment group. Experiments were performed in triplicate. Data were analysed by two-way ANOVA. ***P<0.001.
<table>
<thead>
<tr>
<th>Group</th>
<th>RV (mg)</th>
<th>LV+S (mg)</th>
<th>RV/LV+S</th>
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<tbody>
<tr>
<td>WT Normoxic</td>
<td>22.06 ± 1.13</td>
<td>85.12 ± 1.24</td>
<td>0.26 ± 0.015</td>
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<td>WT Hypoxic</td>
<td>25.71 ± 0.84</td>
<td>73.69 ± 1.77</td>
<td>0.35 ± 0.012***</td>
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<td>miR-145 -/- Normoxic</td>
<td>18.60 ± 1.25</td>
<td>69.74 ± 3.08</td>
<td>0.27 ± 0.014†††</td>
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<tr>
<td>miR-145 -/- Hypoxic</td>
<td>19.21 ± 0.85</td>
<td>68.63 ± 1.92</td>
<td>0.28 ± 0.014†††</td>
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</table>

Suppl. Table I. Ventricle weight in WT and miR-145 -/- mice. Right ventricle (RV) weight, left ventricle plus septum (LV+S) weight and RV/LV+S ratio. 
***P<0.001 cf. WT normoxic mice; †††P<0.001 cf. WT hypoxic mice; Data expressed as mean ± SEM. n=9-10.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Normoxic</th>
<th>WT hypoxic</th>
<th>KO Normoxic</th>
<th>KO Hypoxic</th>
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<td>sRVP, mmHg</td>
<td>20.00±0.80</td>
<td>29.71±1.16***</td>
<td>21.21±0.61</td>
<td>24.07±1.38†††</td>
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<tr>
<td>SAP, mmHg</td>
<td>83.22±1.55</td>
<td>94.01±2.91</td>
<td>71.34±8.14***</td>
<td>70.92±5.59***</td>
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<tr>
<td>Heart rate, bpm</td>
<td>329.42±8.92</td>
<td>329.18±12.66</td>
<td>325.88±6.67</td>
<td>342.08±14.78</td>
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Suppl. Table II. Haemodynamics in normoxic and hypoxic WT and miR-145 -/- mice. Systolic right ventricular pressure(sRVP), systemic mean arterial pressure (SAP) and heart rate measurements in normoxic and chronically hypoxic female WT and miR-145 -/- mice. ***P<0.001 cf. WT normoxic mice; †††P<0.001 cf. WT hypoxic mice; Data expressed as mean ± SEM. n=6-10.
<table>
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<tr>
<th>PROBE_ID</th>
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<th>Fold change</th>
<th>miR-145 target?</th>
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Suppl. Table III. Microarray data for targets selected for validation. 16 targets were selected using the selection criteria outlined in the results section.
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<th>PROBE_ID</th>
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<th>Fold Change</th>
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</tr>
</tbody>
</table>

Suppl. Table IV. Significant targets from the microarray that are components of the wnt pathway.
Supplemental References


2. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR. Contribution of the 5-HT(1B) receptor to hypoxia-induced pulmonary hypertension: converging evidence using 5-HT(1B)-receptor knockout mice and the 5-HT(1B/1D)-receptor antagonist GR127935. Circ Res. 2001;89:1231-1239.

