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# Transient but not genetic loss of miR-451 is protective in the development of pulmonary arterial hypertension

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**Abstract:** MicroRNAs are small noncoding RNAs involved in the regulation of gene expression and have recently been implicated in the development of pulmonary arterial hypertension (PAH). Previous work has established that miR-451 is upregulated in rodent models of PAH. The role of miR-451 in the pulmonary circulation is unknown. We therefore sought to assess the involvement of miR-451 in the development of PAH. Silencing of miR-451 was performed in vivo using miR-451 knockout mice and an anti-miR targeting mature miR-451 in rats. Coupled with exposure to hypoxia, indices of PAH were assessed. The effect of modulating miR-451 on human pulmonary artery smooth muscle cell proliferation and migration was analyzed. We observed a reduction in systolic right ventricular pressure in hypoxic rats pretreated with anti-miR-451 compared with hypoxia alone ($47.7 \pm 1.36$ mmHg and $56.0 \pm 2.03$ mmHg, respectively; $P < .01$). In miR-451 knockout mice, compared with wild-type hypoxic mice, no significant differences were observed following exposure to chronic hypoxia. In vitro analysis demonstrated that overexpression of miR-451 in human pulmonary artery smooth muscle cells promoted migration under serum-free conditions. No effect on cellular proliferation was observed. In conclusion, transient inhibition of miR-451 attenuated the development of PAH in hypoxia-exposed rats. Genetic deletion of miR-451 had no beneficial effect on indices of PAH, potentially because of pathway redundancy compensating for the loss of miR-451.

**Keywords:** microRNA, pulmonary vascular disorder, hypoxia, smooth muscle cell, migration.

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## INTRODUCTION

Pulmonary arterial hypertension (PAH) is a complex disease characterized by narrowing of the pulmonary arteries that leads to an elevation in pulmonary artery pressure and right ventricular failure and can result in premature death.[1] Distinguishing features of this condition include endothelial cell proliferation and apoptosis resulting in the formation of plexiform lesions, fibroblast proliferation, production of matrix proteins, and muscularization of normally nonmuscular arteries culminating in remodeling of the vessel wall.[2] Current therapies for PAH aim to reverse the endothelial dysfunction and vasoconstriction observed.[3] However, these treatments do not prevent the aggressive progression of the disease, and mortality rates associated with PAH remain unacceptably high. Therefore, greater understanding of the pathways involved in PAH development and maintenance is required to more effectively manage PAH.

Recent work has highlighted the importance of small noncoding RNA molecules called microRNAs (miRNAs) in the development of PAH.[4,5] Mature miRNAs are approximately 22 nucleotides long and negatively regulate gene expression by entering the RNA-induced silencing complex (RISC). The miRNA then binds to the 3′ untranslated region of the messenger RNA (mRNA) leading to cleavage of target mRNA or translational repression.[6] miRNAs in general regulate numerous mRNAs; therefore, by targeting one specific miRNA, a range of cellular pathways can potentially be modulated. miRNAs are

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expressed at varying levels in a tissue-specific pattern throughout the body.[7,8] Earlier studies have shown that miRNAs play a role in the lung during the development of PAH. For example, downregulation of miR-204 has been observed in pulmonary artery smooth muscle cells (PASMCs) in both rodent and human PAH, and this downregulation is believed to play a role in stimulating proliferation and inhibiting apoptosis in PASMCs.[9] Conversely, miR-145 is found to be upregulated during the development of PAH, and knockdown of miR-145 expression in vivo has been shown to be protective against the development of PAH.[10]

A study performed by Caruso and colleagues[4] found that miR-451 was upregulated in the lungs of two rodent models of PAH. miR-451 is processed in a dicer-independent manner in which the precursor stem loop miRNA is short and can be cleaved directly by Ago2 to be incorporated into the RISC complex.[11,12] miR-451 is known to play a pivotal role in erythroid maturation. miR-451 levels are significantly increased in erythroid precursors and remain elevated throughout erythroid differentiation[13] with mice lacking miR-451 unable to effectively develop mature circulating red blood cells (RBCs) in response to stress[14,15] resulting in erythoid hyperplasia.[16] miR-451 has also been implicated in a variety of cancer-related pathways.[17-21] It has been reported that miR-451 is downregulated in tissue from non-small-cell lung carcinoma, and overexpression of miR-451 in vitro suppressed proliferation and colony formation of these cells by downregulating RAB14 protein.[19] It has also been found that miR-451 acts as a tumor suppressor in T cell acute lymphoblastic leukemia via targeting of Myc, a proto-oncogene which is essential for Notch-1-induced tumor formation.[20] Similarly, miR-451 was observed to reduce gastric and colorectal cancer cell proliferation by downregulating macrophage migration inhibitory factor,[21] thus providing additional evidence of miR-451 acting as a tumour suppressor. Recent work has highlighted the role of miR-451 in cardiac disease development with an upregulation of miR-451 observed to be cardioprotective against hypoxic stress in cardiomyocytes.[22] Likewise, knockdown of the miR-451 cluster prevents cardioprotection initiated by ischemic preconditioning in mice by upregulating RAC1 protein and thus activating oxidative stress pathways.[23]

Knowledge of the role of miR-451 in the development of PAH is largely unknown, and microRNAs appear to play an important role in the pathogenesis of PAH. We therefore sought to modulate miR-451 both in vivo and in vitro to assess the functional role of miR-451 in the development of PAH.

## METHODS

### miR-451 overexpression in human PASMCs (hPASMCs)

hPASMCs (Lonza Group; Basel, Switzerland) were transfected with a miR-451 mimic (Ambion; Carlsbad, CA) or control miR mimic using siPORT neoFX transfection reagent (Invitrogen; Carlsbad, CA). Migration of hPASMCs was analyzed using the scratch wound assay. Briefly, cells were simultaneously transfected with 10 nM miR mimic and plated in a 6-well plate at a density of $4.5 \times 10^5$ cells/well using siPORT neoFX reverse transfection protocol. Once confluent, cells were quiesced in 0.1% serum media for 48 hours. Vertical scratches were drawn through the confluent monolayer, media were replaced with 0.1% or 15% serum containing media, and scratches were analyzed at 0, 6, 12, and 24 hours using ImageJ software. Independent experiments were performed 3 times, with 2 wells and 4 scratches per well for each condition. DNA synthesis of hPASMCs was assessed using a thymidine incorporation assay in which cells were plated in a 24-well plate at a density of $2 \times 10^4$ cells/well and grown to approximately 50% confluency. Cells were quiesced in 0.1% serum for 48 hours and then transfected with 10 nM miR mimic. Differing serum concentrations (0.1%, 2.5%, and 10% serum) were added to the cells for 72 hours with $^3$H-thymidine added for the last 24 hours. Radioactivity was measured using a liquid scintillation counter, and results were expressed in counts per minute.

### Animal models

All protocols and surgical procedures were approved by the local animal care committee. Animal experiments were conducted in accordance with the Animals Scientific Procedures Act UK 1986. Male Wistar rats (aged 8 weeks) were administered anti-miR-451 (miRagen Therapeutics; consisting of locked nucleic acid [LNA] and DNA bases of the complementary reverse sequence bases 2–17 of miR-451) or a nontargeted anti-miR control (similar composition to the anti-miR but directed against an miRNA in *Caenorhabditis elegans*) intravenously at a dosage of 10 mg/kg. After 3 days of recovery, animals were exposed to normoxic (21% $O_2$) or hypoxic (10% $O_2$) conditions for 7 days. The 7-day exposure to hypoxia was chosen, because previous data had shown upregulation of miR-451 at this time point.[4] Pulmonary pressure measurements were taken on day 7, and right ventricular hypertrophy was assessed by dissection of the heart (right ventricle/left ventricle and septum).

Female miR-451 wild-type and knockout mice (kindly supplied by Eric Olson, University of Texas Southwestern

Medical Center) were exposed to normoxic (21% $O_2$) or hypoxic (10% $O_2$) conditions for 14 days with pulmonary pressure measurements taken on day 14 (at 10 weeks of age) along with right ventricular hypertrophy assessment. Female mice were chosen to study, because previous work from our laboratory has shown that PAH is more prominent in female transgenic mice than in male mice.[24-26]

For remodeling analysis, lung sections were stained with elastic Van Gieson stain, and the percentage of remodeled vessels was assessed. Pulmonary arteries of 80 μm or less in diameter were counted as remodeled if they consisted of a double elastic lamina for more than half of the diameter of the vessel. The percentage of remodeled vessels was calculated as the number of remodeled vessels/total number of vessels × 100. Lung sections from 4–6 animals were assessed per group.

### Gene targets for miR-451

A list of targets was obtained for miR-451 by searching the miRNA databases miRwalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk) and TargetScan (http://www.targetscan.org). Target genes for analysis were chosen on the basis of previous knowledge of the predicted target genes and their involvement in pathways thought to be important in the development of PAH. Target mRNA expression was assessed in the same samples used for miRNA analysis using quantitative real-time polymerase chain reaction (qRT-PCR) assay kits (Applied Biosystems; Carlsbad, CA) with results being normalized to β-2-microglobulin.

### miRNA expression

Expression levels of miR-451 and miR-144 were analyzed in the lung and other tissues by qRT-PCR with results being normalized to U87, U6, and RNU48 for rat, mouse, and human samples, respectively. Northern blot analysis was performed using hsa-miR-451 miRCURY LNA 5′-DIG labeled detection probe (Exiqon; Vedbaek, Denmark) and a hybridization temperature of 50°C, with band intensities normalized against U6 band intensity.

### Statistical analysis

All qRT-PCR results are expressed as fold change (±SEM) with all other results expressed as the mean (±SEM). A 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc test or 1-way ANOVA followed by Tukey post hoc test were used to analyze data as appropriate, with statistical significance accepted at $P$ less than 0.05.

## RESULTS

### Modulation of miR-451 in hPASMCs

The development of PAH is characterized by phenotypic changes in smooth muscle cells and endothelial cells within the medial and intimal layers.[27] Because of their role in the remodeling process observed during PAH, we focused on hPASMCs, and the effect of modulating miR-451 expression in hPASMCs on phenotypic characteristics of PAH was investigated. Therefore, miR-451 "mimics" were used to over-express miR-451 in vitro in hPASMCs. The miR mimic was tested over a variety of concentrations, and miR-451 expression levels were increased significantly at all concentrations compared with the control mimic and mock transfected cells (Fig. 1*A*). Because all concentrations produced efficient overexpression, 10 nM was used in all subsequent experiments and further evaluated by Northern blot analysis (Fig. 1*B*). The effect of miR-451 on proliferation of hPASMCs was assessed. Increasing concentrations of serum induced a consistent increase in DNA synthesis in control and mock transfected cells (Fig. 1*C*). This pattern was also observed in hPASMCs overexpressing miR-451, because proliferation was not altered in the absence or presence of serum, indicating that miR-451 does not affect proliferation in these cells under experimental conditions tested.

The effect of overexpressing miR-451 on the migration of hPASMCs was also examined, because migration of smooth muscle cells into previously nonmuscular arteries plays a major role in PAH development.[2] In cells transfected with control mimic stimulated with 15% serum, the wound was completely closed after 24 hours (Fig. 1*D*, 1*F*). Similarly, cells transfected with miR-451 mimic and stimulated with 15% serum were no different from control mimic transfected cells, indicating that miR-451 does not inhibit serum-induced migration of hPASMCs. In the presence of 0.1% serum, cells transfected with miR-451 mimic showed significantly smaller wounds than did those observed in the mock and control mimic cells at 24 hours (Fig. 1*D*, 1*E*), thus suggesting that miR-451 promotes migration of hPASMCs in the absence of serum.

### Modulation of miR-451 using anti-miR-451 in vivo

We next assessed the contribution of miR-451 to hypoxic-induced vascular remodeling and PAH in vivo using a pharmacological inhibitor approach. To verify the degree of knockdown obtained using an anti-miR targeting mature miR-451 in vivo, tissues were harvested from rat after 7 days of hypoxic exposure, and miR-451 expression levels were analyzed. miR-451 expression was extremely low in all tissues treated with anti-miR-451 compared

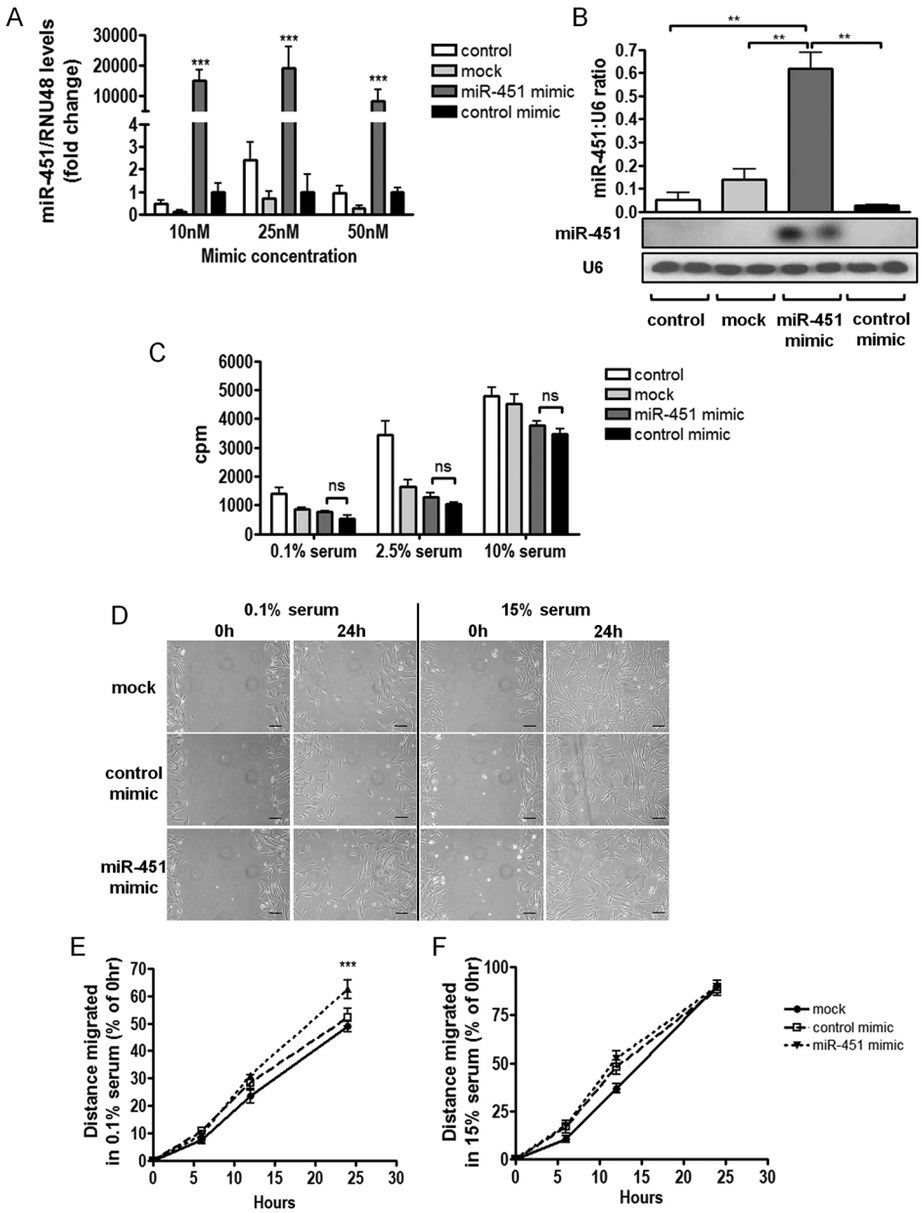


Figure 1. Effect of overexpressing miR-451 on human pulmonary artery smooth muscle cell (hPASMC) proliferation and migration. *A*, miR-451 expression in hPASMCs after transfection with miR mimic in 15% serum for 72 hours, as detected by quantitative real-time polymerase chain reaction. An arbitrary value of 1 was assigned to the control mimic. Representative graph of 2 independent experiments with technical triplicates. $***P < 0.001$ versus control mimic. *B*, Northern blot analysis of hPASMCs, as in Figure 1*A*, using a concentration of 10 nM of miR mimic, quantified by normalizing the band intensity of mature miR-451 to the relative U6 signal ($n = 2$ per group). $**P < 0.01$. *C*, hPASMC thymidine incorporation assay. Representative graph of 2 independent experiments with 4 technical repeats per condition. cpm: counts per minute; ns: non-significant. *D*, Representative images of hPASMC scratch wound at 0 hours and 24 hours. Scale bar = 100 μm. *E*, *F*, Quantification of hPASMC migration assay in 0.1% serum and 15% serum, respectively. Representative graphs from 3 independent experiments with technical duplicates, $***P < 0.001$ versus control mimic in 0.1% serum. Data analyzed by 1-way analysis of variance followed by Tukey post hoc test.

with the control treated tissues (Fig. 2*A*, 2*C*, 2*D*), which demonstrated that the anti-miR reduced miR-451 expression globally. miR-451 is located on chromosome 10 in rats and is transcribed together with miR-144 as a bicistronic primary transcript, which is processed to generate the 2 separate mature miRNAs.[28] Expression levels for miR-144 were also analyzed by qRT-PCR (Fig. 2*B*). miR-144 expression increased significantly in RBCs when exposed to hypoxia. However, there were no significant differences between control and anti-miR-451-treated animals in either normoxic or hypoxic conditions in any of the tissues analyzed, hence indicating that anti-miR-451 selectively downregulates miR-451 in vivo.

The effect of silencing miR-451 on PAH development was then investigated. Heart rate was unchanged between groups (Fig. 3*A*). Rats exposed to hypoxia and control anti-miR had a significant increase in systolic right ventricular pressure (RVP) compared with normoxic animals, whereas pretreatment with anti-miR-451 before hypoxic exposure lowered the RVP compared with control anti-miR-treated animals (Fig. 3*B*). There was no significant difference between right ventricular hypertrophy (RVH)

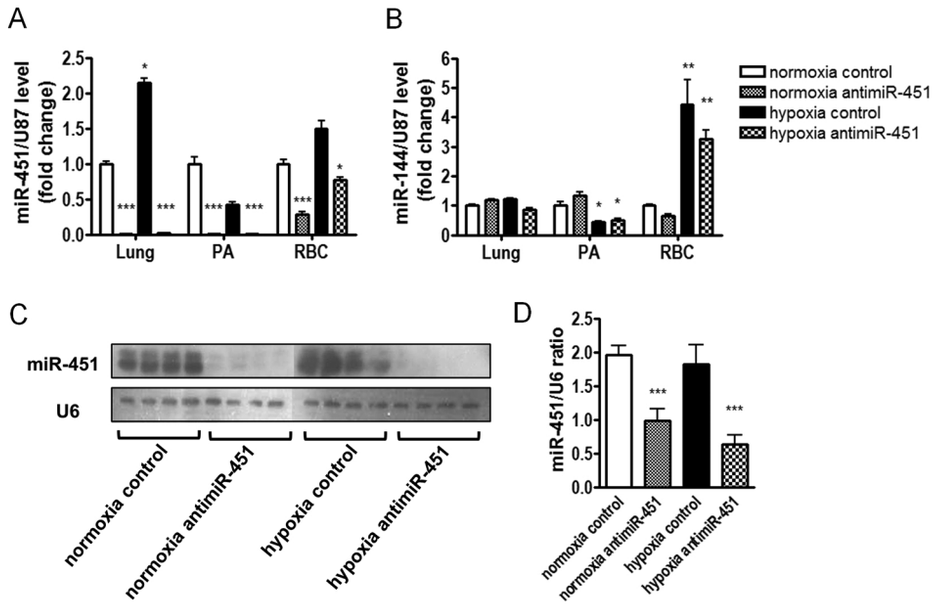


Figure 2. miR-451 and miR-144 expression in rat tissue. Expression of miR-451 (*A*) and miR-144 (*B*) in rat tissue harvested after pretreatment with control or anti-miR-451 and exposure to normoxic or hypoxic conditions for 7 days, as detected by quantitative real-time polymerase chain reaction. Arbitrary value of 1 assigned to the normoxic control group for each tissue ($n = 9$ per group). Northern blot (*C*) was performed on RNA extracted from whole lung and quantified (*D*) by normalizing the band intensity of mature miR-451 to the relative U6 signal ($n = 4$ per group). Data analyzed using a 2-way analysis of variance followed by Bonferroni post hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus normoxic control. PA: pulmonary artery; RBC:red blood cells.

measurements between groups (Fig. 3*C*). There was an increase in remodeling (Fig. 3*D*, 3*E*) in all hypoxic animal groups compared with normoxic controls. Administration of anti-miR-451 did not significantly reduce vessel remodeling.

### Analysis of miR-451 targets

The reduction in RVP in hypoxic conditions when animals were pretreated with anti-miR-451 (Fig. 3*B*) is most likely attributable to derepression of target genes regulated by miR-451. Target prediction algorithms miRWalk and TargetScan were used along with searching the literature to select target genes for miR-451. Previous studies have shown, using different tissues along with target data analysis software, that miR-451 targets genes that include *Akt*1 and *Bcl*2,[19,29] *Rac*1,[23] *Tbx*1,[30] and *Ywhaz*.[31,32] These genes were therefore chosen for analysis using mRNA extracted from the whole lung of anti-miR-451 and control treated animals (Fig. 4). Three of the chosen genes (*Akt*1, *Rac*1, and *Ywhaz*) showed a significant downregulation when miR-451 was knocked down, whereas none of the genes investigated showed derepression in anti-miR-451–treated animals. This suggests that miR-451 modulation in PAH affects alternate pathways.

### Effect of miR-451 knockout mice on PAH development

We then assessed the effect of chronic knockdown of miR-451 on the development of PAH with the use of miR-451 knockout mice. Mice were exposed to hypoxic conditions for 14 days at 8 weeks of age (with age-matched wild-type control mice), after which in vivo measurements were taken. qRT-PCR and Northern blot analysis of tissue taken from these animals confirmed the absence of miR-451 in lung tissue (Fig. 5*A*, 5*C*, 5*D*). miR-144 levels were also analyzed and were unchanged between wild-type and knockout mice (Fig. 5*B*), although interestingly this miRNA was decreased in response to hypoxia. Assessment of PAH indices was performed along with heart rate measurements. No difference in heart rate was observed between groups (Fig. 5*E*). RVP, RVH, and remodeling showed the expected increase in wild-type animals in response to hypoxia (Fig. 5*F*–5*H*). Knockout animals exposed to hypoxia demonstrated RVP values comparable to those of hypoxic wild-type animals (Fig. 5*F*) with similar results in RVH and remodeling analysis (Fig. 5*G*, 5*H*). Target gene analysis was performed on mRNA extracted from whole lung. No significant upregulation was observed in the knockout mice compared with wild-type mice, although we did observe that a number of these targets were modulated by hypoxia (Fig. 6).

## DISCUSSION

The role of miR-451 in the lung is largely unknown. The involvement of miR-451 in the development of PAH was assessed in this study using both in vivo and in vitro studies. We show that overexpression of miR-451 in hPASMCs promotes migration in the absence of serum

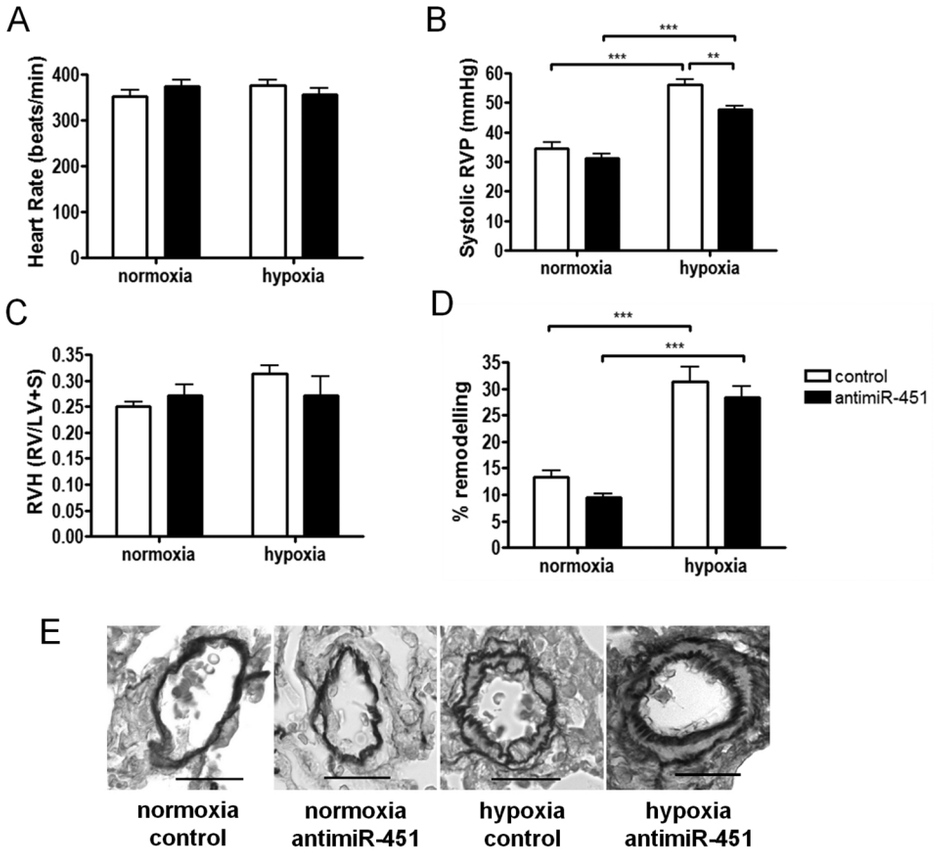


Figure 3. Quantification of PAH indices in anti-miR-451 treated rats. Quantification of heart rate (*A*), systolic right ventricular pressure (RVP; *B*) and right ventricular hypertrophy (RVH; *C*) in male rats ($n = 7$ per group). Pulmonary arterial remodeling quantification (*D*; $n = 4-6$ per group) and representative pictures stained with elastin Van Gieson (*E*), magnification ×40, scale bar: 25 μm. Pressures and tissue taken after 7 days in normoxic or hypoxic conditions. Data analyzed using a 2-way ANOVA followed by Bonferroni post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$. LV+S: left ventricle and septum; RV: right ventricle.

but has no effect on proliferation. In vivo data suggest that acute knockdown of miR-451 in male rats diminishes aspects of the PAH phenotype, whereas genetic ablation of miR-451 has no beneficial effect.

One of the main characteristics of PAH is muscularization of formally nonmuscular arteries and remodeling of the pulmonary vessels.[33] Smooth muscle cells are one of the principle cell types involved in this process, and phenotypic dysregulation of PASMC proliferation and migration contributes to the complex remodeling observed in PAH. In this study, overexpression of miR-451 promoted hPASMC migration. Hence this may be one of the factors leading to increased muscularization of the vessel during the early development of PAH. Cell culture studies, however, showed no effect of overexpressing miR-451 on hPASMC proliferation. Of course, it is clear that a number of divergent pathways can lead to dysregulation of PASMC proliferation.[34,35] Certainly, under the experimental conditions studied here, including overexpression to very high levels using a mimic-based approach, we observed no effect of miR-451 manipulation on hPASMC proliferation.

Analysis of miR-451 target genes did not show derepression of any of the genes of interest in both the anti-miR-451-treated rats and the miR-451 knockout mice. miR-451 has relatively few validated or predicted targets, and highlighting the genes that are genuine targets presents a challenge. In this study, target prediction algorithms were used along with searching the literature for targets of miR-451. Wang and colleagues[23] demonstrated that miR-451 targets *Rac*1 in the heart to mediate the cardioprotection observed in ischemic preconditioning. In this system, miR-451 represses target gene *Rac*1, therefore inhibiting the production of reactive oxygen species and causing damage to the heart tissue. Similarly, other studies[31,32] have found that miR-451 targets *Ywhaz* (14–3–3ζ) in erythroblasts. Repression of *Ywhaz* by miR-451 releases the inhibitory effect of *Ywhaz* on the transcription factor FoxO3, which regulates anti-oxidant genes. Both of these miR-451 target genes are involved in the regulation of reactive oxygen species production, which is known to be upregulated in the lung during hypoxia and PAH.[36] However, these target genes have been identified in different tissues and different disease models, indicating that miR-451 may not directly target these genes in the lung during the development of PAH. Additional work is required to give a more comprehensive understanding

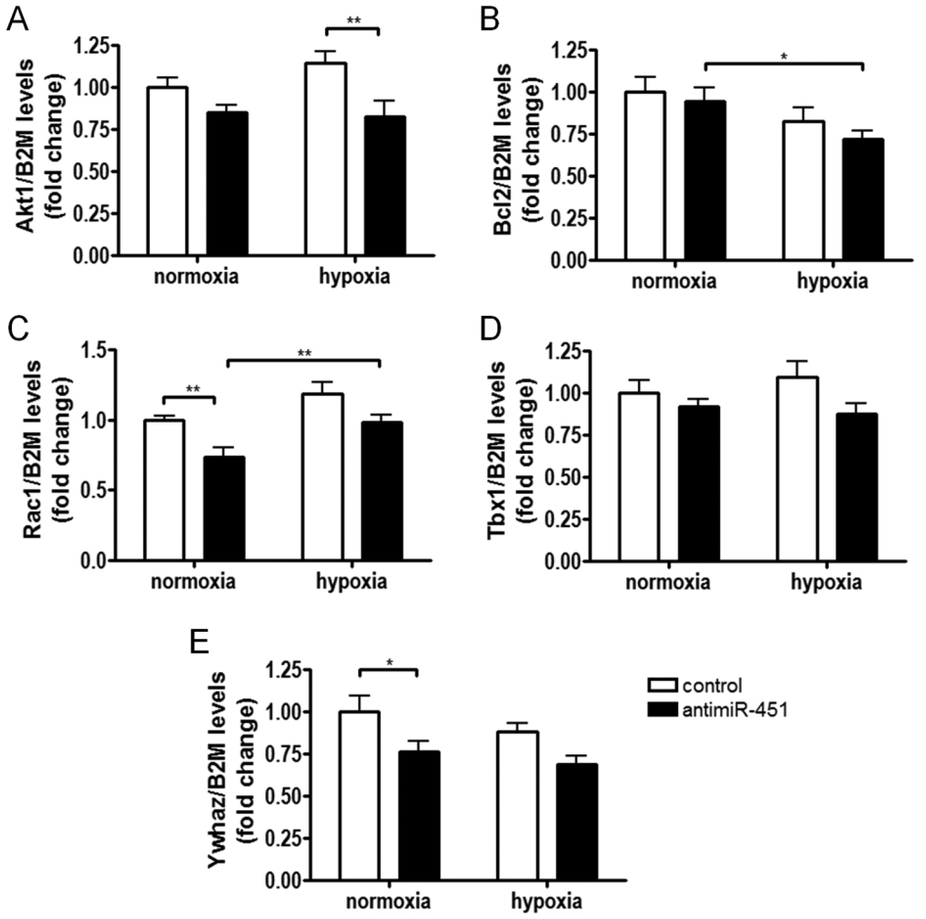


Figure 4. Target gene analysis of anti-miR-451–treated rats. Target gene expression in frozen lung tissue by quantitative real-time polymerase chain reaction for *Akt*1 (*A*), *Bcl*2 (*B*), *Rac*1 (*C*), *Tbx*1 (*D*), and *Ywhaz* (*E*). Arbitrary value of 1 assigned to the normoxic control group for each gene ($n = 9$ per group). Data analyzed using a 2-way ANOVA followed by Bonferroni post hoc test. $*P < 0.05$, $**P < 0.01$.

of the pathways involved in miR-451 modulation during PAH development, such as microRNA microarrays or a proteomics-based approach.

Global and selective knock-down of miR-451 was achieved using an anti-miR-451. miR-451 is known to play an essential role in normal erythroid differentiation.[15,16,31,37] Anti-miR-treated animals still had very high miR-451 expression levels in the RBC compartment, which allowed us to assess the potential role of miR-451 in hypoxia-induced PAH in a relatively selective manner.

In male rats exposed to hypoxia, silencing of miR-451 by anti-miR decreased right ventricular pressure compared with controls. This effect was not observed in the RVH or remodeling data from these animals. However, this may be attributable to the relatively short period of hypoxic exposure chosen, as outlined earlier, and additional studies in chronic hypoxia should be performed. These data indicate that transiently reducing miR-451 attenuates the development of PAH because of a modest reduction in RVP with exposure to hypoxia. Additional studies are clearly warranted to study the effects of this approach in rodents with more chronic exposure to hypoxia or, indeed, in alternate rodent models of PAH, such as the hypoxia/sugen model.[38] The original study in which miR-451 expression was increased in experimental PAH[4] used the monocrotaline model of PAH, and it would be interesting to investigate whether transient knock down of miR-451 in the monocrotaline rat model of PAH showed a more pronounced reduction in PAH phenotype. In addition, hypoxic exposure elevates the hematocrit level, and it is known that miR-451 plays an important role in erythropoiesis and therefore has an impact on hematocrit level. Hence, the monocrotaline model of PAH would allow assessment of knocking down miR-451 on PAH phenotype without the additional complication of hematocrit regulation by hypoxia.

We also performed studies that assessed chronic knock down of miR-451 using knockout mice. The knockout mice displayed high right ventricular pressure, RVH, and remodeling in hypoxia similar to wild-type hypoxic mice. Therefore, genetic knockdown of miR-451 in this setting appears to have no beneficial effect on the de-

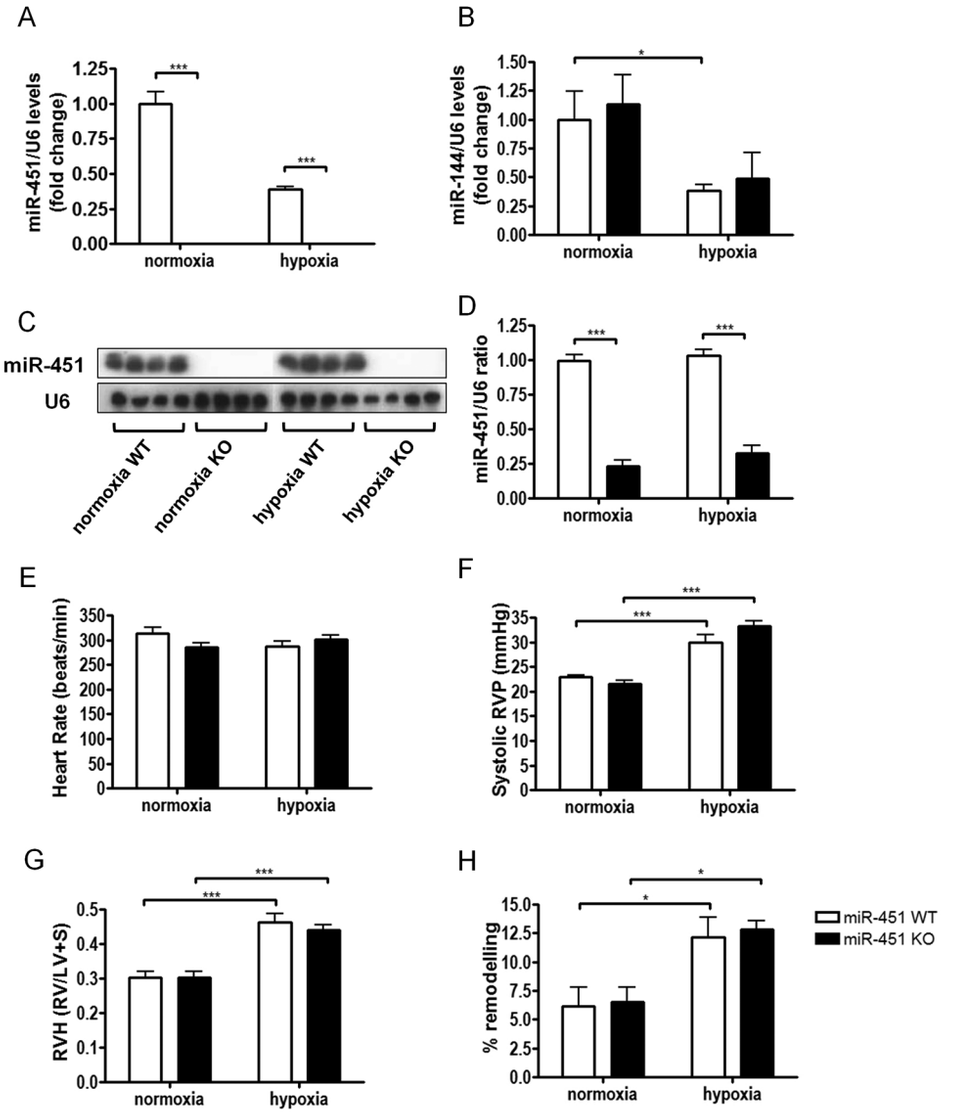


Figure 5. Quantification of miR-451 expression and pulmonary arterial hypertension indices in miR-451 knockout (KO) mice. Expression of miR-451 (*A*) and miR-144 (*B*) in whole-lung tissue from female miR-451 wild-type (WT) and KO mice, as detected by quantitative real-time polymerase chain reaction (qRT-PCR; $n = 6$ per group) and Northern blot for miR-451 (*C, D*; $n = 4$ per group). Arbitrary value of 1 assigned to the normoxic control group for the qRT-PCR data. Quantification of heart rate (*E*), systolic right ventricular pressure (RVP; *F*), right ventricular hypertrophy (RVH; *G*; $n = 7-14$ per group) and remodeling analysis (*H*; $n = 4-6$ per group). Pressures and tissue taken after 14 days in normoxic or hypoxic conditions. Data analyzed by 2-way analysis of variance followed by Bonferroni post hoc test. $^{*}P < 0.05$, $^{***}P < 0.001$. LV+S: left ventricle and septum; RV: right ventricle.

velopment of PAH under the experimental conditions tested. It is difficult to ascertain the differences that lead to these conflicting data sets. The finding that we did not observe target derepression in lung tissue from either model suggests that alternate targets to the ones tested are responsible for the phenotype observed in the rat hypoxia experiment. It is clear also that the rat and mouse experiment differ substantially in terms of cell compartments where loss of miR-451 is observed. In the mouse, this is global because of the genetic deletion. However, in the anti-miR experiments, miR-451 remained at high levels in RBCs. In other tissues, we observed very high levels of miR-451 knockdown. Because of the important role of miR-451 in erythropoiesis, we cannot rule out interplay of the different modulatory systems used on lung pathophysiology in the development of PAH. Additional studies are warranted to fully explore these important issues.

In vitro data show that miR-451 promotes hPASMC migration, hence suggesting that knocking down miR-451 would reduce PASMC migration in vivo, and this could contribute to the attenuated development of PAH in the rat hypoxic model. This effect may not have been observed in the hypoxic knockout mouse model because of differences in the degree of hypoxic pulmonary vasoconstriction obtained. It has recently been shown that, physiologically, rats and mice respond differently to hypoxic insult. Hypoxic exposure causes sustained ρ-kinase dependent vasoconstriction in the rat,[39] whereas, although vasoconstriction is an important mechanism in-

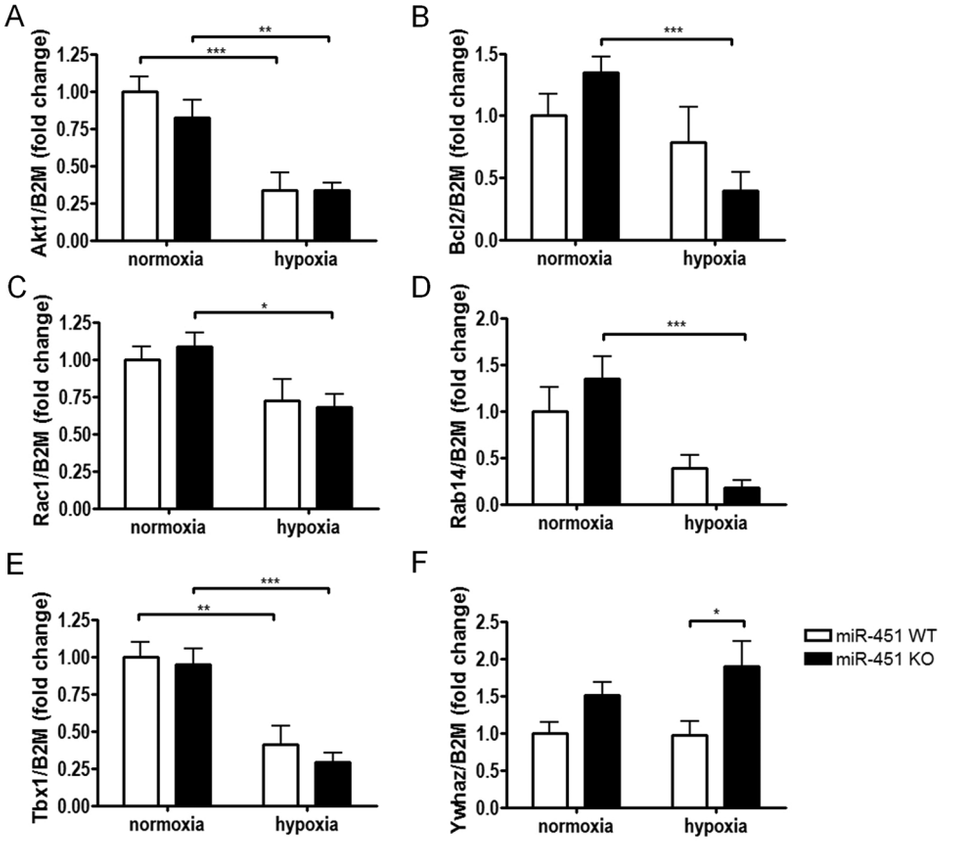


Figure 6. Target gene analysis of miR-451 knockout (KO) mice. Target gene expression in frozen lung tissue by quantitative real-time polymerase chain reaction for *Akt*1 (*A*), *Bcl*2 (*B*), *Rac*1 (*C*), *Rab*14 (*D*), *Tbx*1 (*E*) and *Ywhaz* (*F*). An arbitrary value of 1 was assigned to the normoxic miR-451 wild-type (WT) group ($n = 5-7$ per group). Data were analyzed using a 2-way analysis of variance followed by Bonferroni post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

volved in mouse hypoxic PAH development, structural narrowing of the lumen also plays a critical role.[40] In addition to this, pulmonary vascular remodeling is more pronounced in the hypoxic rat than in the mouse.[41] Therefore, the hypoxic mouse model of PAH used in this study may not have induced sufficient stimuli (i.e., resulting in PASMC migration) as in the rat hypoxic model, and hence knocking out miR-451 showed no beneficial effect. This highlights the importance in choice of animal model for each study. Of course, the benefit of using mice is the ability to generate knockout mice, an important tool in studying the relevance of particular genes. However, the moderate PAH phenotype observed in the hypoxic mouse (compared with the hypoxic rat) is something that must be taken into consideration when analyzing these data.

The observed variation in different model systems may also be due to other pathways/miRNAs compensating for the chronic genetic loss of miR-451 in knockout animals. miRNAs can target hundreds of genes,[42] and in turn, each specific gene can be modulated by many different miRNAs.[43] However, miR-451 has a relatively low number of known and predicted targets[44] and thus is limited to the pathways it can potentially modulate. As a result, other miRNAs may have been modulated in the lung that can subsequently activate these genes under conditions of prolonged absence of miR-451 (i.e., act in a compensatory manner for loss of miR-451). There are other cases in which there is disparity between genetic ablation and anti-miR knockdown of a miRNA (reviewed by van Rooij and Olson[45]), and this may be attributable to the compensatory pathways mentioned above.

Another reason that may account for the differences between the two in vivo models is sex, and PAH is a condition with a sex bias at the clinical level.[46] A microarray performed by Caruso and colleagues[4] studied male rats (both hypoxic and monocrotaline models of PAH) and found that miR-451 was upregulated in both disease models. Using hypoxia in the current study, we have observed beneficial effects of knocking down miR-451 in male rats with anti-miR-451, whereas no effect was found in female knockout mice. Taking these data together, it indicates that miR-451 expression may be sex-dependent, and the differences in miR-451 expression between the sexes requires further investigation.

Taken together, the results of this study demonstrate that transient knockdown of miR-451 expression reduces

the development of PAH induced by transient exposure to hypoxia. However, genetic deletion of miR-451 has no advantageous effect on PAH. Additional work is needed to understand the genes targeted by miR-451 in the lung in this disease model.

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**Conflict of Interest:** None declared.

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