Title of the article: Transient but not genetic loss of miR-451 attenuates the development of pulmonary arterial hypertension.

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Abstract

Rationale: MicroRNAs are small non-coding RNAs involved in the regulation of gene expression and have recently been implicated in the development of pulmonary arterial hypertension (PAH). Previous work established that miR-451 is up-regulated in rodent models of PAH.

Objectives: 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 pulmonary arterial hypertension.

Methods: Silencing of miR-451 was performed *in vivo* using miR-451 knockout mice and an antimiR targeting mature miR-451 in rats. Coupled with exposure to hypoxia, indices of pulmonary arterial hypertension were assessed. The effect of modulating miR-451 on human pulmonary artery smooth muscle cell proliferation and migration was analysed.

Measurements and Main Results: We observed a reduction in systolic right ventricular pressure in hypoxic rats pre-treated with antimiR-451 compared to hypoxia alone (47.7 ± 1.36mmHg and 56.0 ± 2.03mmHg respectively, $p<0.01$). In miR-451 knockout mice following exposure to chronic hypoxia, no significant differences were observed compared to wild type hypoxic mice. *In vitro* analysis demonstrated that over-expression of miR-451 in human pulmonary artery smooth muscle cells promoted migration under serum-free conditions. No effect on cellular proliferation was observed.

Conclusions: Transient inhibition of miR-451 attenuated the development of pulmonary arterial hypertension in hypoxia exposed rats. Genetic deletion of miR-451 had no beneficial effect on indices of pulmonary arterial hypertension, potentially due to pathway redundancy compensating for the loss of miR-451.

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

Key Messages: Over-expression of miR-451 in human pulmonary artery smooth muscle cells promotes smooth muscle cell migration in the absence of serum and *in vivo* data suggests that acute knockdown of miR-451 in male rats may be protective in the development of PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a complex disease characterised by narrowing of the pulmonary arteries leading to an elevation in pulmonary artery pressure, 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 muscularisation of normally non-muscular arteries culminating in remodelling 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 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 non-coding 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'-UTR of the 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 expressed at varying levels in a tissue-specific pattern throughout the body. [7-8] Previous studies have shown that miRNAs play a role in the lung during the development of PAH. For example, down regulation of miR-204 has been observed in

pulmonary artery smooth muscle cells (PASMCs) in both rodent and human PAH and this down-regulation is believed to play a role in stimulating proliferation and inhibiting apoptosis in PASMCs.[9] Conversely, miR-145 is found to be up-regulated during the development of PAH and knock down of miR-145 expression *in vivo* has been shown to be protective against the development of PAH.[10]

A study carried out by Caruso and colleagues [4] found that miR-451 was up-regulated in the lung of two rodent models of PAH. MiR-451 is processed in a dicer-independent manner where 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 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 down regulated in tissue from non-small cell lung carcinoma (NSCLC) and over-expression of miR-451 *in vitro* suppressed proliferation and colony formation of these cells by down-regulating RAB14 protein.[19] It has also been found that miR-451 acts as a tumour suppressor in T cell acute lymphoblastic leukemia via targeting of Myc, a proto-oncogene which is essential for Notch-1 induced tumour formation.[20] Similarly, miR-451 was observed to reduce gastric and colorectal cancer cell proliferation by down-regulating macrophage migration inhibitory factor [21] thus providing further evidence of miR-451 acting as a tumour suppressor. Recent work has highlighted the role of miR-451 in cardiac disease development with an up-regulation 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 up-regulating RAC1 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 pulmonary arterial hypertension.

Methods

MiR-451 overexpression in human pulmonary artery smooth muscle cells

Human pulmonary artery smooth muscle cells (hPASMCs, Lonza Group Ltd, 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 analysed 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 $4.5x10^5$cells/well using siPORT neoFX reverse transfection protocol. Once confluent, cells were quiesced in 0.1% serum media for 48h. Vertical scratches were drawn through the confluent monolayer, media replaced with 0.1% or 15% serum containing media and scratches analysed at 0, 6, 12 and 24h using ImageJ software. Independent experiments were performed three times, with two wells and four scratches per well for each condition. DNA synthesis of hPASMCs was assessed using a thymidine incorporation assay where cells were plated in a 24-well plate at a density of $2x10^4$cells/well and grown to ~50% confluency. Cells were quiesced in 0.1% serum for 48h and then transfected with 10 nM miR mimic. Differing serum concentrations (0.1%, 2.5% and 10% serum) were added to the cells for 72h with ^{3}H-thymidine added for the last 24h. Radioactivity was measured using a liquid scintillation counter and results expressed in counts per minute (cpm).

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 antimiR-451 (miRagen Therapeutics Inc, Colorado and consisting of LNA and DNA bases of the complementary reverse sequence bases 2-17 of miR-451) or a

non-targeted antimiR control (similar composition to the antimiR but directed against a miRNA in *C.elegans*) intravenously at a dose of 10mg/kg. After three days 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 as previous data had shown upregulation of miR-451 at this time point.[4] Pulmonary pressures were taken on day 7 and right ventricular hypertrophy was assessed by dissection of the heart (right ventricle (RV)/left ventricle + septum (LV+S)).

Female miR-451 wild type and knockout mice (kindly supplied by Eric Olson, UT Southwestern, USA) were exposed to normoxic (21% O_2) or hypoxic (10% O_2) conditions for 14 days with pulmonary pressures taken on day 14 (at 10 weeks of age) along with right ventricular hypertrophy assessment. Female mice were chosen to study as previous work from our laboratory has shown that PAH is more prominent in female transgenic mice compared to male mice.[24-26]

For remodelling analysis, lung sections were stained with elastic Van Gieson stain and the percentage of remodelled vessels assessed. Pulmonary arteries of d 80 microns in diameter were counted as remodelled if they consisted of a double elastic lamina for more than half of the diameter of the vessel. The percentage of remodelled vessels was calculated as the number of remodelled vessels/total number of vessels x 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 based on 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 normalised to beta-2-microglobulin (B2M).

miRNA expression

Expression levels of miR-451 and miR-144 were analysed 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 labelled detection probe (Exiqon, Vedbaek, Denmark) and a hybridization temperature of 50ºC, 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 ANOVA followed by Bonferroni post-hoc test or 1-way ANOVA followed by Tukey's post-hoc test were used to analyse data as appropriate, with statistical significance accepted at $p<0.05$.

Results

Modulation of miR-451 in hPASMCs

The development of PAH is characterised by phenotypic changes in smooth muscle cells (SMC) and endothelial cells (EC) within the medial and intimal layers.[27] Due to their role in the remodelling process observed during PAH, hPASMCs were focused on 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 to the control mimic and mock transfected cells (Figure 1A). Since all concentrations produced efficient over-expression, 10 nM was used in all subsequent experiments and further evaluated by northern blot analysis (Figure 1B). 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 (Figure 1C). This pattern was also observed in hPASMCs over-expressing miR-451 as 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 over-expressing miR-451 on the migration of hPASMCs was also examined as migration of SMCs into previously non-muscular 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 24h (Figure 1D, F). Similarly, cells transfected with miR-451 mimic and stimulated with 15% serum were no different to 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 those observed in the mock and control mimic cells at 24h (Figure 1D, E), thus suggesting that miR-451 promotes migration of hPASMCs in the absence of serum.

Modulation of miR-451 using antimiR-451 in vivo

We next assessed the contribution of miR-451 to hypoxic-induced vascular remodelling and PAH *in vivo* using a pharmacological inhibitor approach. To verify the degree of knock-down obtained using an antimiR targeting mature miR-451 *in vivo*, tissues were harvested from rat following 7 days hypoxic exposure and miR-451 expression levels analysed. MiR-451 expression was extremely low in all tissues treated with antimiR-451 compared to the control treated tissues (Figure 2A, C, D) demonstrating that the antimiR 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 two separate mature miRNAs.[28] Expression levels for miR-144 were also analysed by qRT-PCR (Figure 2B). MiR-144 expression increased significantly in RBCs when exposed to hypoxia however, there were no significant differences between control and antimiR-451 treated animals in either normoxic or hypoxic

conditions in any of the tissues analysed, hence indicating that antimiR-451 selectively down-regulates miR-451 *in vivo*.

The effect of silencing miR-451 on PAH development was then investigated. Heart rate was unchanged between groups (Figure 3A). Rats exposed to hypoxia and control antimiR had a significant increase in systolic right ventricular pressure (RVP) compared to normoxic animals, while pre-treatment with antimiR-451 prior to hypoxic exposure lowered the RVP compared to control antimiR treated animals (Figure 3B). There was no significant difference between right ventricular hypertrophy (RVH) measurements between groups (Figure 3C). There was an increase in remodelling (Figure 3D, E) in all hypoxic animal groups compared with normoxic controls. Administration of antimiR-451 did not significantly reduce vessel remodelling.

Analysis of miR-451 targets

The reduction in RVP in hypoxic conditions when animals were pre-treated with antimiR-451 (Figure 3B) is most likely due 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 including Akt1 and Bcl2,[19, 29] Rac1,[23] Tbx1 [30] and Ywhaz. [31-32] These genes were therefore chosen to analyse using mRNA extracted from the whole lung of antimiR-451 and control treated animals (Figure 4A-E). Three of the chosen genes; Akt1, Rac1 and Ywhaz showed a significant down-regulation when miR-451 was knocked down, while none of the genes investigated showed derepression in antimiR-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 (Figure 5A, C, D). MiR-144 levels were also analysed and were unchanged between wild type and knockout mice (Figure 5B), although interestingly this miRNA was decreased in response to hypoxia. Assessment of PAH indices were performed along with heart rate measurements. No difference in heart rate was observed between groups (Figure 5E). RVP, RVH and remodelling showed the expected increase in wild type animals in response to hypoxia (Figure 5F-H). Knockout animals exposed to hypoxia demonstrated comparable RVP values to hypoxic wild type animals (Figure 5F) with similar results in RVH and remodelling analysis (Figure 5G, H). Target gene analysis was performed on mRNA extracted from whole lung. No significant up-regulation was observed in the knockout mice compared to wild type mice although we did observe that a number of these targets were modulated by hypoxia (Figure 6A-F).

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 over-expression of miR-451 in hPASMCs promotes migration in the absence of serum but has no effect on proliferation. *In vivo* data suggests that acute knockdown of miR-451 in male rats diminishes aspects of the PAH phenotype while genetic ablation of miR-451 has no beneficial effect.

One of the main characteristics of PAH is muscularisation of formally non-muscular arteries and remodelling 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 remodelling observed in PAH. In this study, over-expression of miR-451 promoted hPASMC migration. Hence this may be one of the factors leading to increased muscularisation of the vessel during

the early development of PAH. Cell culture studies, however, showed no effect of over-expressing 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 over-expression 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 antimiR-451 treated rats and the miR-451 knockout mice. MiR-451 has relatively few validated or predicted targets and highlighting the genes which 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 Rac1 in the heart to mediate the cardioprotection observed in ischemic preconditioning. In this system, miR-451 represses target gene Rac1 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 (14-3-3¶) 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 pulmonary arterial hypertension.[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. Further work is required to give a more comprehensive understanding 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 antimiR-451. MiR-451 is known to play an essential role in normal erythroid differentiation. [15-16, 31, 37] AntimiR treated animals still had very

high miR-451 expression levels in the RBC compartment allowing 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 antimiR decreased right ventricular pressure compared to controls. This effect was not observed in the RVH or remodelling data from these animals however this may be due to the relatively short period of hypoxic exposure chosen as outlined earlier and further studies in chronic hypoxia should be repeated. This data indicates that transiently reducing miR-451 attenuates the development of PAH due to a modest reduction in RVP with exposure to hypoxia. Further studies are clearly warranted to study the effects of this approach in a more chronic exposure to hypoxia, or indeed 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 haematocrit level and it is known that miR-451 plays in important role in erythropoiesis and therefore has an impact on haematocrit. Hence, the monocrotaline model of PAH would allow assessment of knocking down miR-451 on PAH phenotype without the additional complication of haematocrit regulation by hypoxia.

We did also perform studies assessing chronic knock down of miR-451 using knockout mice. The knockout mice displayed high right ventricular pressure, RVH and remodelling in hypoxia similar to wild type hypoxic mice. Therefore genetic knock down of miR-451 in this setting appears to have no beneficial effect on the development of PAH under the experimental conditions tested. It is difficult to ascertain the differences that lead to these conflicting datasets. 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 due to the genetic deletion, however, in the antimiR experiments miR-451 remained at high levels in RBCs. In other tissues we observed very high levels of miR-451 knockdown. Due to the important role of miR-451 in erythropoiesis, we cannot rule out interplay of the different modulatory systems utilised on lung pathophysiology in the development of PAH. Further studies are warranted to fully unpick these important issues.

In vitro data shows 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 due to differences in the degree of hypoxic pulmonary vasoconstriction obtained. It has been shown recently that physiologically, rats and mice respond differently to hypoxic insult. Hypoxic exposure causes sustained rho-kinase dependent vasoconstriction in the rat,[39] while although vasoconstriction is an important mechanism involved in mouse hypoxic PAH development, structural narrowing of the lumen also plays a critical role.[40] In addition to this, pulmonary vascular remodelling is more pronounced in the hypoxic rat compared to 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 knock-out mice, an important tool in studying the relevance of particular genes, however, the moderate PAH phenotype observed in the hypoxic mouse (compared to the hypoxic rat) is something that must be taken into consideration when analysing this 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 relatively low number of known and predicted targets,[44] 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 antimiR knock down of a miRNA (reviewed by van Rooij [45]) and this may be down to the compensatory pathways mentioned above.

Another reason which may account for the differences between the two *in vivo* models is gender, and PAH is a condition with a gender bias at the clinical level.[46] A microarray carried out by Caruso and colleagues [4] studied male rats (both hypoxic and monocrotaline models of PAH) and found that miR-451 was up-regulated in both disease models. Using hypoxia in the current study, we have observed beneficial effects of knocking down miR-451 in male rats with antimiR-451, while no effect was found in female knockout mice. Taking this data together, it indicates that miR-451 expression may be gender-dependent and the differences in miR-451 expression between the genders 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. Further work is needed to understand the genes targeted by miR-451 in the lung in this disease model.

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Figure Legends

Figure 1: Effect of over-expressing miR-451 on hPASMC proliferation and migration. (A) MiR-451 expression in hPASMCs after transfection with miR mimic in 15% serum for 72h, as detected by qRT-PCR. Arbitrary value of 1 assigned to control mimic. Representative graph of two independent experiments with technical triplicates, ***p<0.001 vs control mimic. (B) Northern blot analysis of hPASMCs as in Figure 1A using a concentration of 10 nM miR mimic, quantified by normalising 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 two independent experiments with four technical repeats per condition, ns = non-significant. (D) Representative images of hPASMC scratch wound at 0h and 24h. Scale bar = 100μm. Quantification of hPASMC migration assay in 0.1% serum (E) and 15 % serum (F). Representative graphs from three independent experiments with technical duplicates, ***p<0.001 vs control mimic in 0.1% serum. Data analysed by one-way ANOVA followed by Tukey's post-hoc test.

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 pre-treatment with control or antimiR-451 and exposure to normoxic or hypoxic conditions for 7 days, as detected by qRT-PCR. Arbitrary value of 1 assigned to the normoxic control group for each tissue (n=9 per group). Northern blot (C) was carried out 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 analysed using a two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05, **p<0.01 and ***p<0.001 vs normoxic control. PA = pulmonary artery, RBC = red blood cells.

Figure 3: Quantification of PAH indices in antimiR-451 treated rats. Quantification of heart rate (A), systolic right ventricular pressure (B) and right ventricular hypertrophy (C) in male rats (n=7 per group). Pulmonary arterial remodelling quantification (D, n=4-6 per group) and representative pictures stained

with elastin Van Gieson (E), magnification X40, scale bar = 25μm. Pressures and tissue taken after 7 days in normoxic or hypoxic conditions. Data analysed using a two-way ANOVA followed by Bonferroni post-hoc test, **p<0.01, ***p<0.001.

Figure 4: Target gene analysis of antimiR-451 treated rats. Target gene expression in frozen lung tissue by qRT-PCR for Akt1 (A), Bcl2 (B), Rac1 (C), Tbx1 (D) and Ywhaz (E). Arbitrary value of 1 assigned to the normoxic control group for each gene (n=9 per group). Data analysed using a two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05, **p<0.01.

Figure 5: Quantification of miR-451 expression and PAH indices in miR-451 knockout mice. Expression of miR-451 (A) and miR-144 (B) in whole lung tissue from female miR-451 wild type and knockout mice, as detected by 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 (F), right ventricular hypertrophy (G) (n=7-14 per group) and remodelling analysis (H) (n=4-6 per group). Pressures and tissue taken after 14 days in normoxic or hypoxic conditions. Data analysed by two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05 and ***p<0.001. WT = wild type, KO = knockout.

Figure 6: Target gene analysis of miR-451 knockout mice. Target gene expression in frozen lung tissue by qRT-PCR for Akt1 (A), Bcl2 (B), Rac1 (C), Rab14 (D), Tbx1 (E) and Ywhaz (F). Arbitrary value of 1 assigned to the normoxic miR-451 WT group (n=5-7 per group). Data analysed using a two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05, **p<0.01 and ***p<0.001. WT = wild type, KO = knockout.

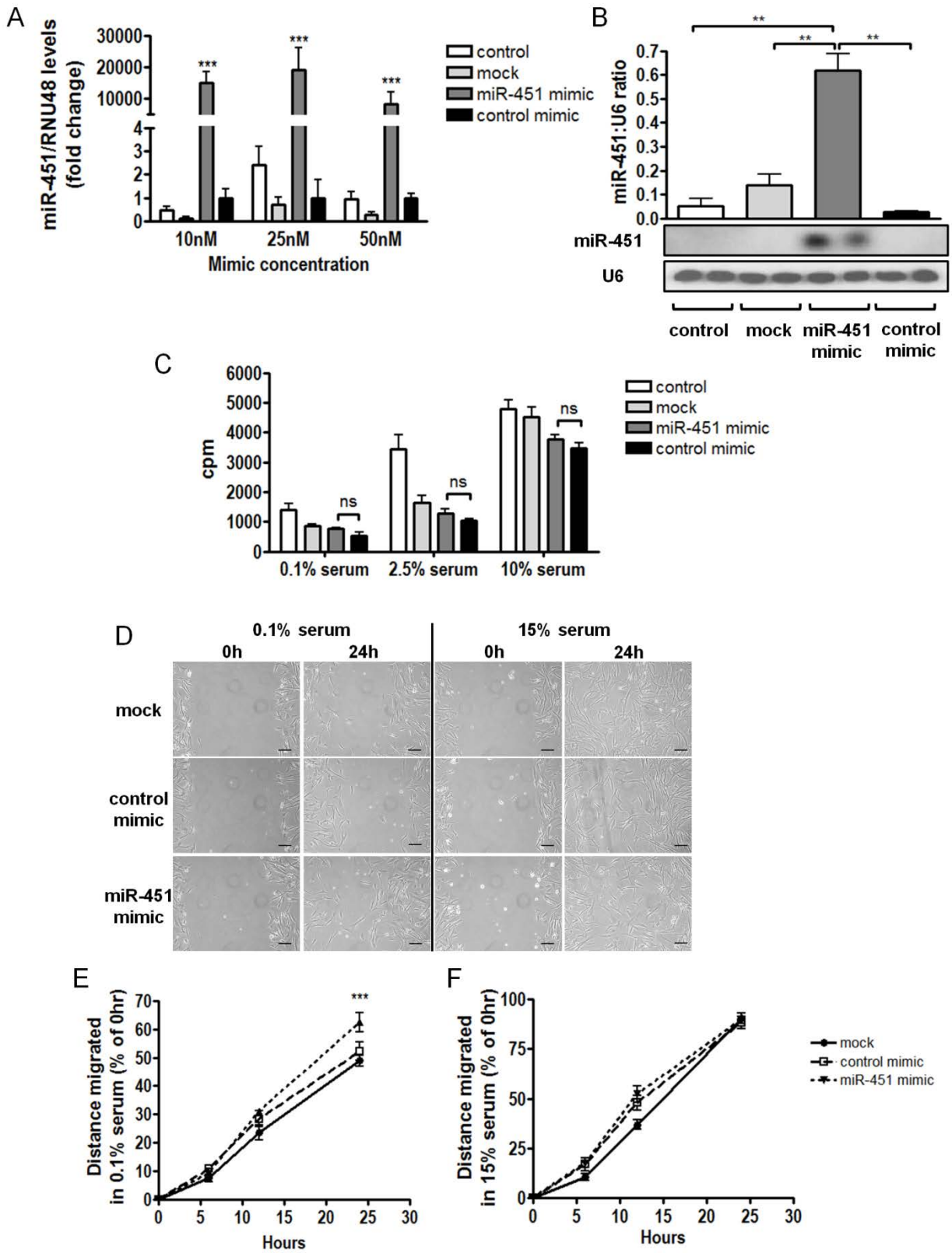
A
miR-451/RNU48 levels (fold change)
30000
20000
10000
4
3
2
1
0
***
***
***
10nM
25nM
50nM
Mimic concentration
control
mock
miR-451 mimic
control mimic
B
miR-451:U6 ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
**
**
miR-451
U6
control
mock
miR-451 mimic
control mimic
C
cpm
6000
5000
4000
3000
2000
1000
0
ns
ns
ns
0.1% serum
2.5% serum
10% serum
control
mock
miR-451 mimic
control mimic
D
0.1% serum
15% serum
0h
24h
0h
24h
mock
control mimic
miR-451 mimic
E
Distance migrated in 0.1% serum (% of 0hr)
70
60
50
40
30
20
10
0
***
0
5
10
15
20
25
30
Hours
F
Distance migrated in 15% serum (% of 0hr)
100
75
50
25
0
0
5
10
15
20
25
30
Hours
mock
control mimic
miR-451 mimic

Figure 1

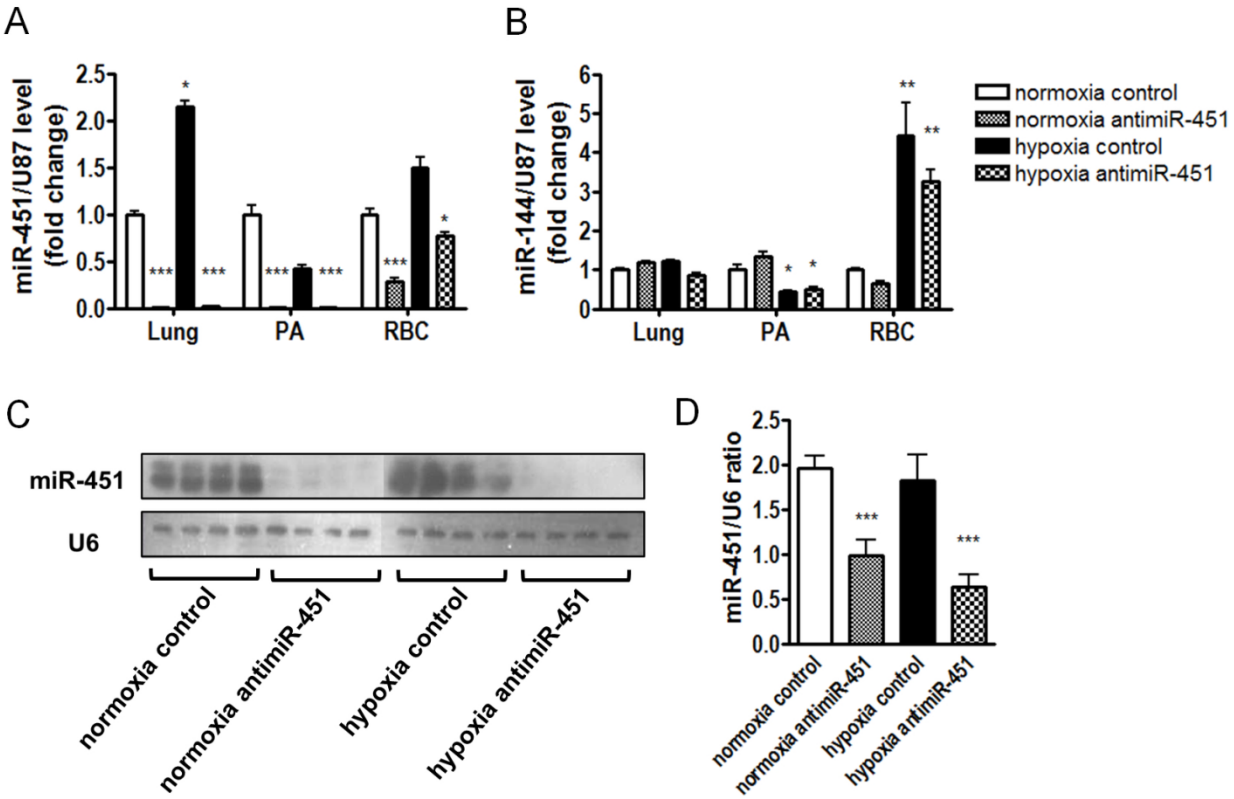
A
miR-451/U87 level (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Lung
PA
RBC
B
miR-144/U87 level (fold change)
6
5
4
3
2
1
0
normoxia control
normoxia antimiR-451
hypoxia control
hypoxia antimiR-451
C
miR-451
U6
normoxia control
normoxia antimiR-451
hypoxia control
hypoxia antimiR-451
D
miR-451/U6 ratio

Figure 2

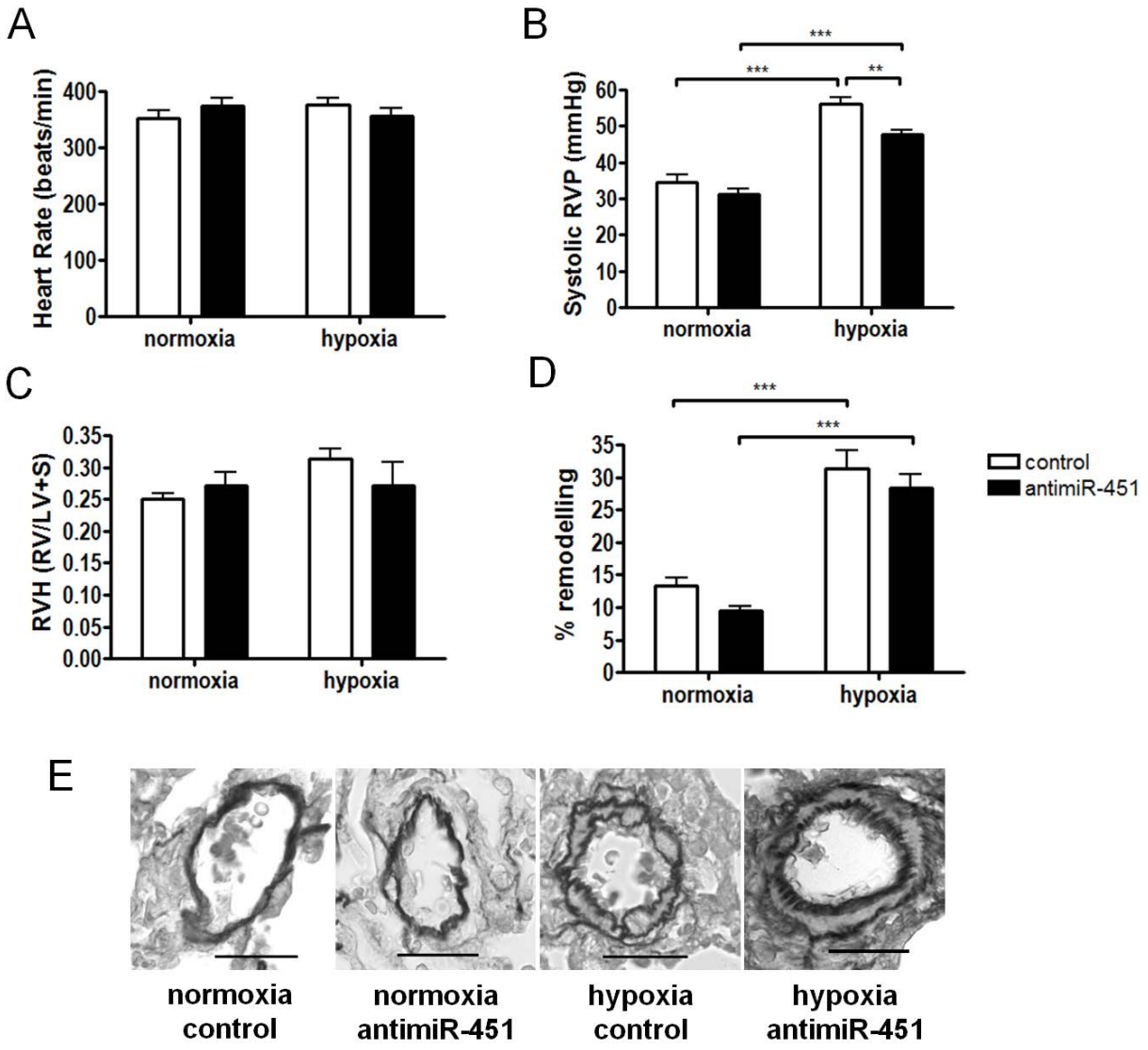
A
Heart Rate (beats/min)
400
300
200
100
0
normoxia
hypoxia
B
Systolic RVP (mmHg)
60
50
40
30
20
10
0
***
***
**
normoxia
hypoxia
C
RVH (RV/LV+S)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
normoxia
hypoxia
D
% remodelling
35
30
25
20
15
10
5
0
***
***
normoxia
hypoxia
control
antimiR-451
E
normoxia
control
normoxia
antimiR-451
hypoxia
control
hypoxia
antimiR-451

Figure 3

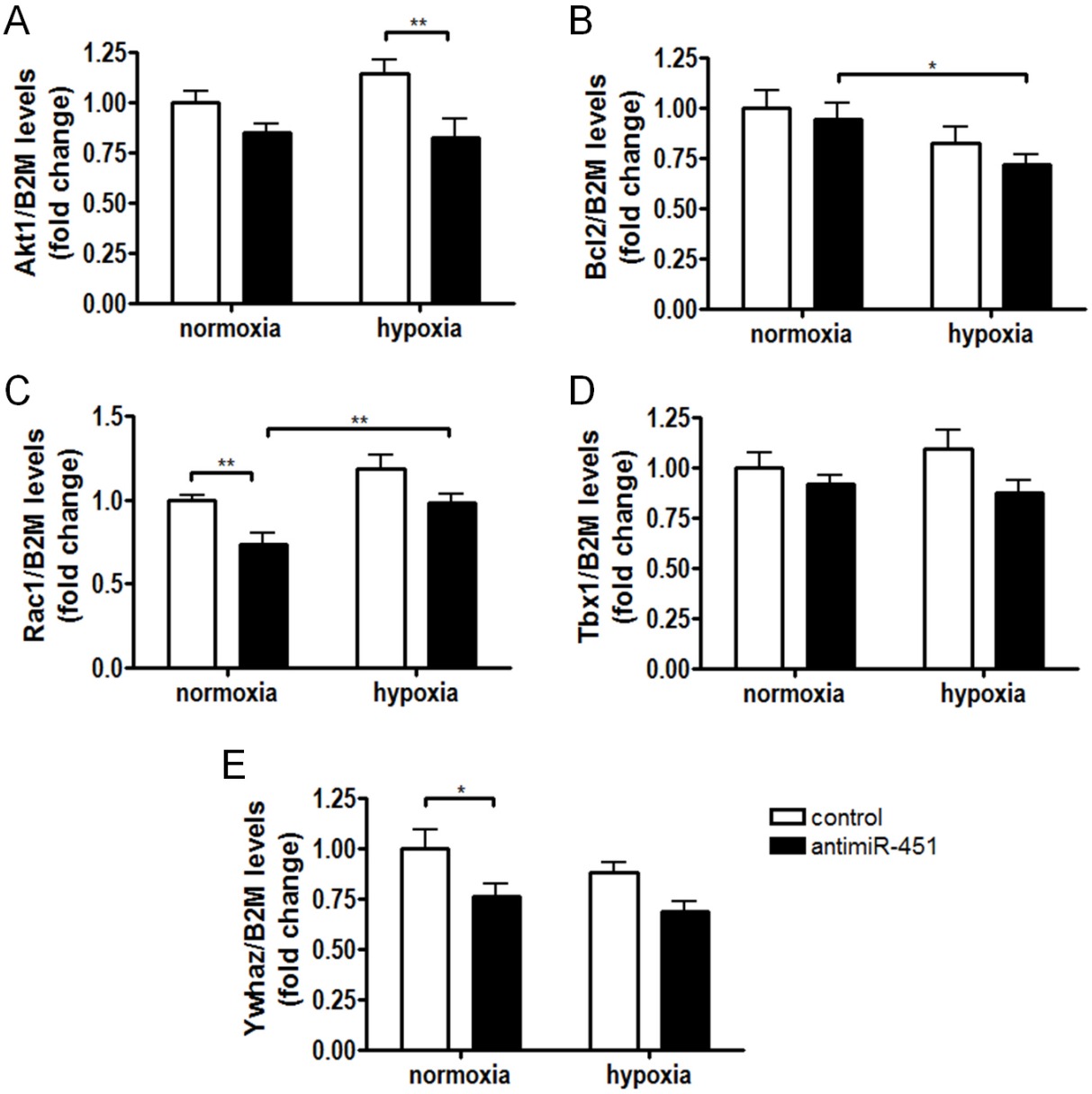
A
Akt1/B2M levels (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
**
normoxia
hypoxia
B
Bcl2/B2M levels (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
*
normoxia
hypoxia
C
Rac1/B2M levels (fold change)
1.5
1.0
0.5
0.0
**
**
normoxia
hypoxia
D
Tbx1/B2M levels (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
normoxia
hypoxia
E
Ywhaz/B2M levels (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
*
normoxia
hypoxia
control
antimiR-451

Figure 4

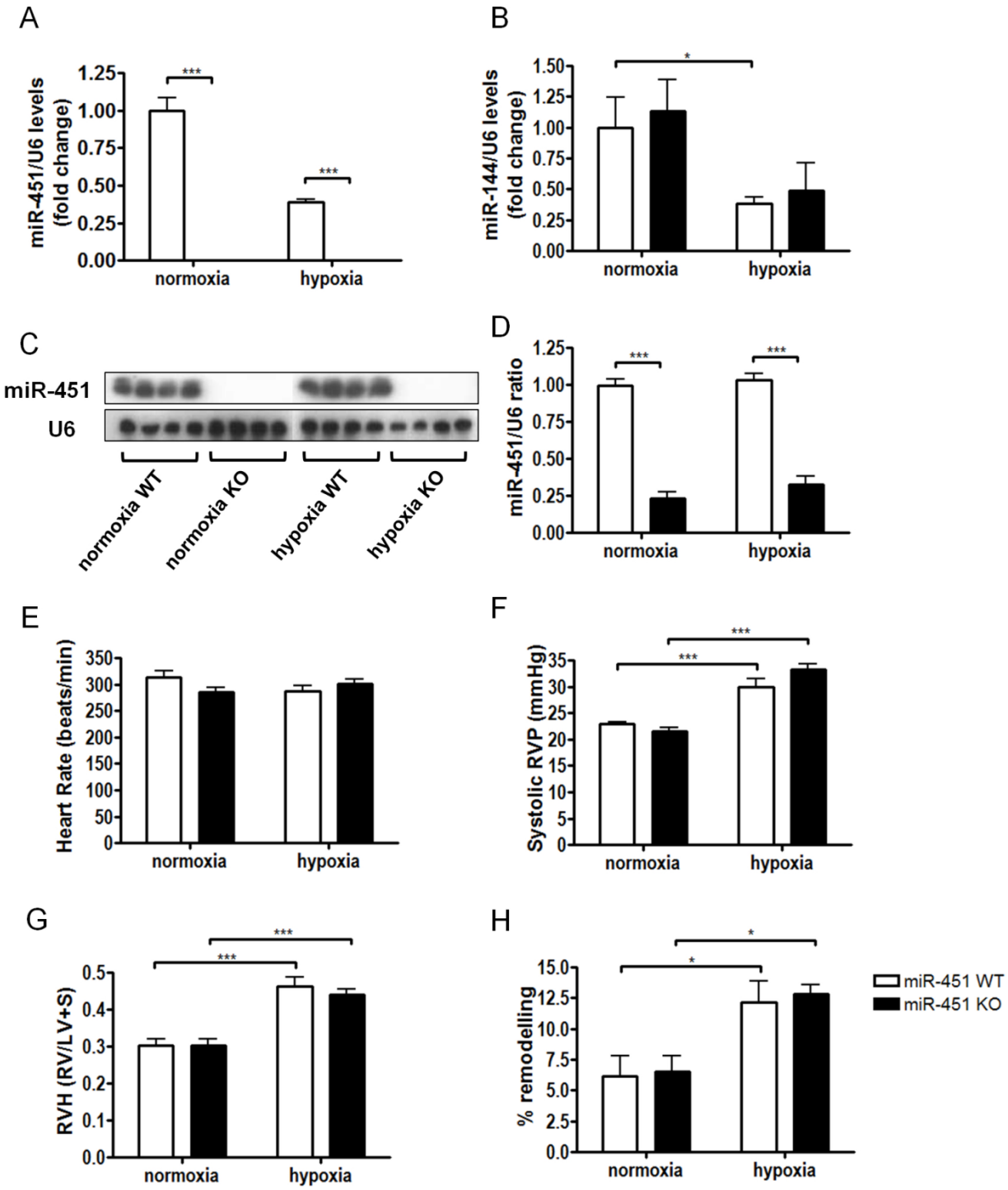
A
miR-451/U6 levels (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
normoxia
hypoxia
B
miR-144/U6 levels (fold change)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
normoxia
hypoxia
C
miR-451
U6
normoxia WT
normoxia KO
hypoxia WT
hypoxia KO
D
miR-451/U6 ratio
1.25
1.00
0.75
0.50
0.25
0.00
***
***
normoxia
hypoxia
E
Heart Rate (beats/min)
350
300
250
200
150
100
50
0
normoxia
hypoxia
F
Systolic RVP (mmHg)
35
30
25
20
15
10
5
0
***
***
normoxia
hypoxia
G
RVH (RV/LV+S)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
normoxia
hypoxia
H
% remodelling
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
normoxia
hypoxia
miR-451 WT
miR-451 KO

Figure 5

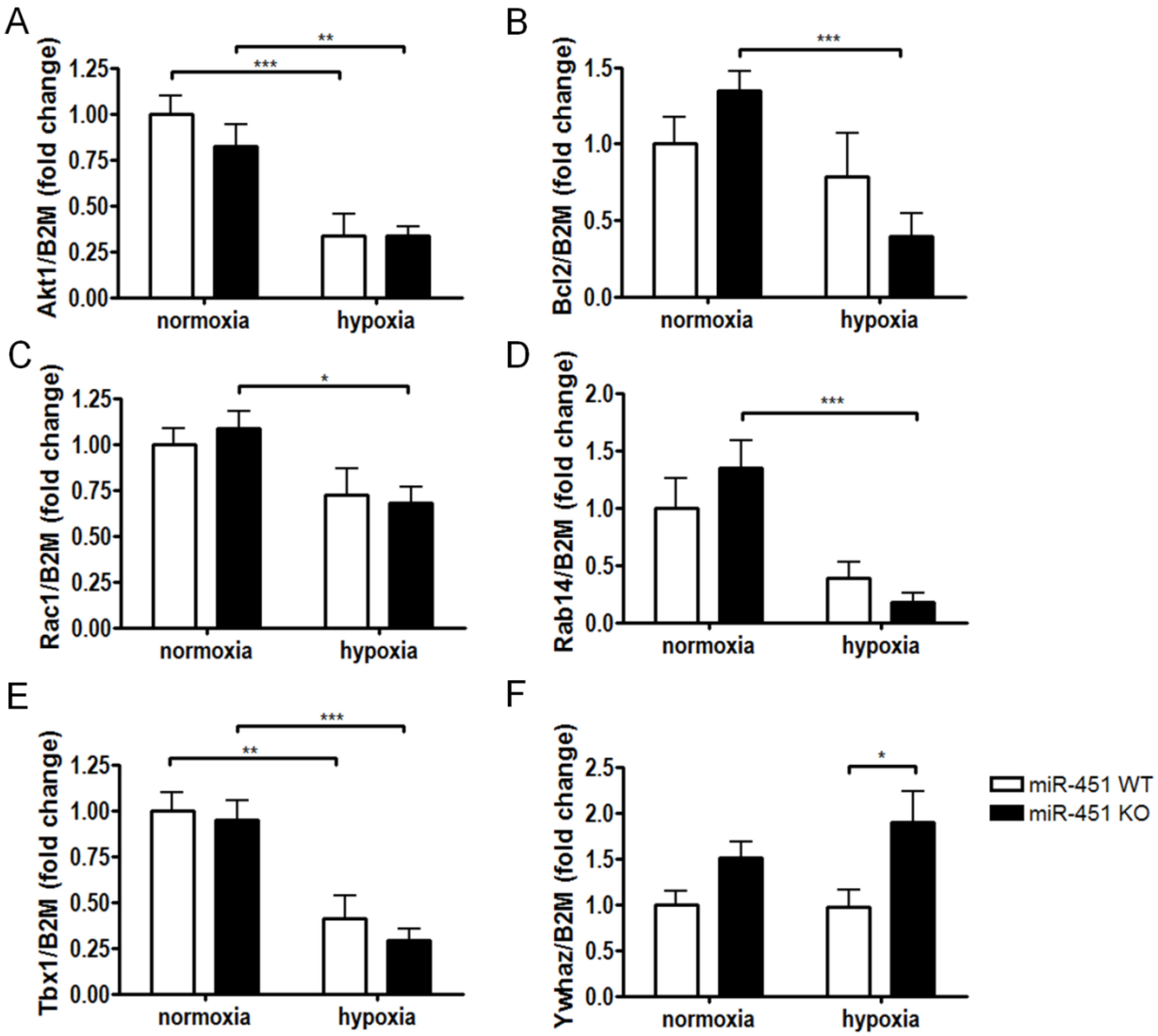
A
Akt1/B2M (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
**
***
normoxia
hypoxia
B
Bcl2/B2M (fold change)
1.5
1.0
0.5
0.0
***
normoxia
hypoxia
C
Rac1/B2M (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
*
normoxia
hypoxia
D
Rab14/B2M (fold change)
2.0
1.5
1.0
0.5
0.0
***
normoxia
hypoxia
E
Tbx1/B2M (fold change)
1.25
1.00
0.75
0.50
0.25
0.00
***
**
normoxia
hypoxia
F
Ywhaz/B2M (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
normoxia
hypoxia
miR-451 WT
miR-451 KO

Figure 6