AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension

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Aims	Dysregulation of the bone morphogenetic protein receptor type 2 (BMPR2) is a hallmark feature that has been described in several forms of pulmonary hypertension. We recently identified the microRNA miR-20a within a highly conserved pathway as a regulator of the expression of BMPR2. To address the pathophysiological relevance of this pathway *in vivo*, we employed antagomiR-20a and investigated whether specific inhibition of miR-20a could restore functional levels of BMPR2 and, in turn, might prevent pulmonary arterial vascular remodelling.
Methods and results	For specific inhibition of miR-20a, cholesterol-modified RNA oligonucleotides (antagomiR-20a) were synthesized. The experiments in mice were performed by using the hypoxia-induced mouse model for pulmonary hypertension and animal tissues were analysed for right ventricular hypertrophy and pulmonary arterial vascular remodelling. Treatment with antagomiR-20a enhanced the expression levels of BMPR2 in lung tissues; moreover, antagomiR-20a significantly reduced wall thickness and luminal occlusion of small pulmonary arteries and reduced right ventricular hypertrophy. To assess BMPR2 signalling and proliferation, we performed *in vitro* experiments with human pulmonary arterial smooth muscle cells (HPASMCs). Transfection of HPASMCs with antagomiR-20a resulted in activation of downstream targets of BMPR2 showing increased activation of Id-1 and Id-2. Proliferation of HPASMCs was found to be reduced upon transfection with antagomiR-20a.
Conclusion	This is the first report showing that miR-20a can be specifically targeted in an *in vivo* model for pulmonary hypertension. Our data emphasize that treatment with antagomiR-20a restores functional levels of BMPR2 in pulmonary arteries and prevents the development of vascular remodelling.
Keywords	BMPR2 • Pulmonary hypertension • MicroRNA • AntagomiR • Vascular remodelling

Introduction

Vascular remodelling of small pulmonary arteries is, together with vasoconstriction and microthrombosis, one of the pathogenetic hallmarks of pulmonary arterial hypertension (PAH). However, it is largely unknown what causes vascular remodelling or how it can be treated. Genetic mutations of the bone morphogenetic protein receptor type II (BMPR2), a member of the transforming growth factor (TGF-) β family expressed on the surface of endothelial and vascular smooth muscle cells of the pulmonary arterial circulation, have been described in familial and idiopathic PAH.[1,2] Moreover, reduced expression of BMPR2 without concomitant

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mutations has also been found in other forms of pulmonary hypertension including established animal models of the disease.[3–5] It has been debated controversially whether the altered expression of BMPR2 due to mutations or down-regulation might have a functional readout and, thus, an impact on the pathogenesis of PAH. Two recent studies, however, have underscored the potential role of BMPR2-mediated signalling for vascular remodelling.[6,7] Long *et al.*[6] observed that dysfunctional BMPR2/TGF-β signal transduction results in altered expression of transcription factors, including Smads and Id (inhibitor of DNA binding), leading to migration of vascular smooth muscle cells. Similar findings have been reported by another study showing that heterogeneous mutations of BMPR2 are associated with disrupted Smad signalling resulting in a pro-proliferative and anti-apoptotic phenotype of pulmonary arterial smooth muscle cells.[7]

MicroRNAs (miRNAs) comprise a class of small, non-coding RNA molecules that regulate gene expression on the post-transcriptional level;[8] miRNAs preferentially bind to conserved regions in the 3′ untranslated region (UTR) of their target genes and either suppress the translation or the mRNA stability of these genes.[9] There is a growing body of evidence that the abnormal expression of miRNAs modulates human diseases by regulating key molecules of apoptosis, proliferation, or cell signalling.[10] Conversely, efficient and non-toxic inhibition of miRNAs *in vivo* is of particular interest since such methods would offer a therapeutic approach to suppress altered miRNA expression in human disease. Krutzfeldt *et al.*[11] recently described the inhibition of miRNAs by the application of small anti-sense RNA molecules conjugated with cholesterol called antagomiRs in mice.

We have recently proposed that miRNAs might regulate the expression of BMPR2 and identified a phylogenetically conserved pathway involving the action of the signal transducer and activator of transcription (STAT)3 and the microRNA cluster miR-17/92, mainly of miR-20a.[12] Others have emphasized this hypothesis by showing that miRNAs derived from miR-17/92 are overexpressed in experimental pulmonary hypertension *in vivo*[13] making these miRNAs interesting targets for further investigations. With respect to pathogenesis and therapy, this model offers the potential to restore functional expression levels of BMPR2 by antagonizing distinct miRNAs.

In the present study, we addressed feasibility and functional readout of specific antagonization of miR20-a by using antagomiR in an animal model of hypoxia-induced pulmonary hypertension *in vivo* and *in vitro*.

Methods

Animal experiments

Male mice (BL6) were obtained by the Institute for Veterinary Physiology at the University of Zurich. The study design of the *in vivo* experiments is provided in *Figure 1A*. Four animal groups were used ($n = 8$ each): three groups were employed in hypoxic conditions. The fraction of oxygen was decreased gradually from 21 to 10% over 60 min. Hypoxic conditions were provided by chambers connected to a gas mixer (Ruskinn Life Science). Animals were treated with antagomiR-20a (25 mg/kg), antagomiR negative control (antagomiR_MM), or phosphate buffered saline (PBS), respectively. Injections

were performed at baseline (Day 0) with boosters at Day 5 and 12. Blood analysis, morphometry, and tissue preparation for later analysis were performed on Day 21. The experiments were approved by Zurich Canton's Veterinary office. To assess morphometry, hearts and lungs were removed, formalin-fixed without prior perfusion, paraffin-embedded and stained with haematoxylin and eosin. Heart hypertrophy, cell wall thickness, and vessel occlusion were determined by using the MCID analysis program 7.0 (InterFocus Imaging, Cambridge, UK).

AntagomiR design

Cholesterol-modified RNA oligonucleotides (antagomiRs) directed against human and murine miR-20a (MIMAT0000529) were designed as described[11] and synthesized by Microsynth (Microsynth, Balgach, Switzerland). As a negative control, twelve point mutations were introduced into the miR-20a mature sequence (antagomiR_MM) creating an RNA sequence that is not encoded in the murine or human genome.
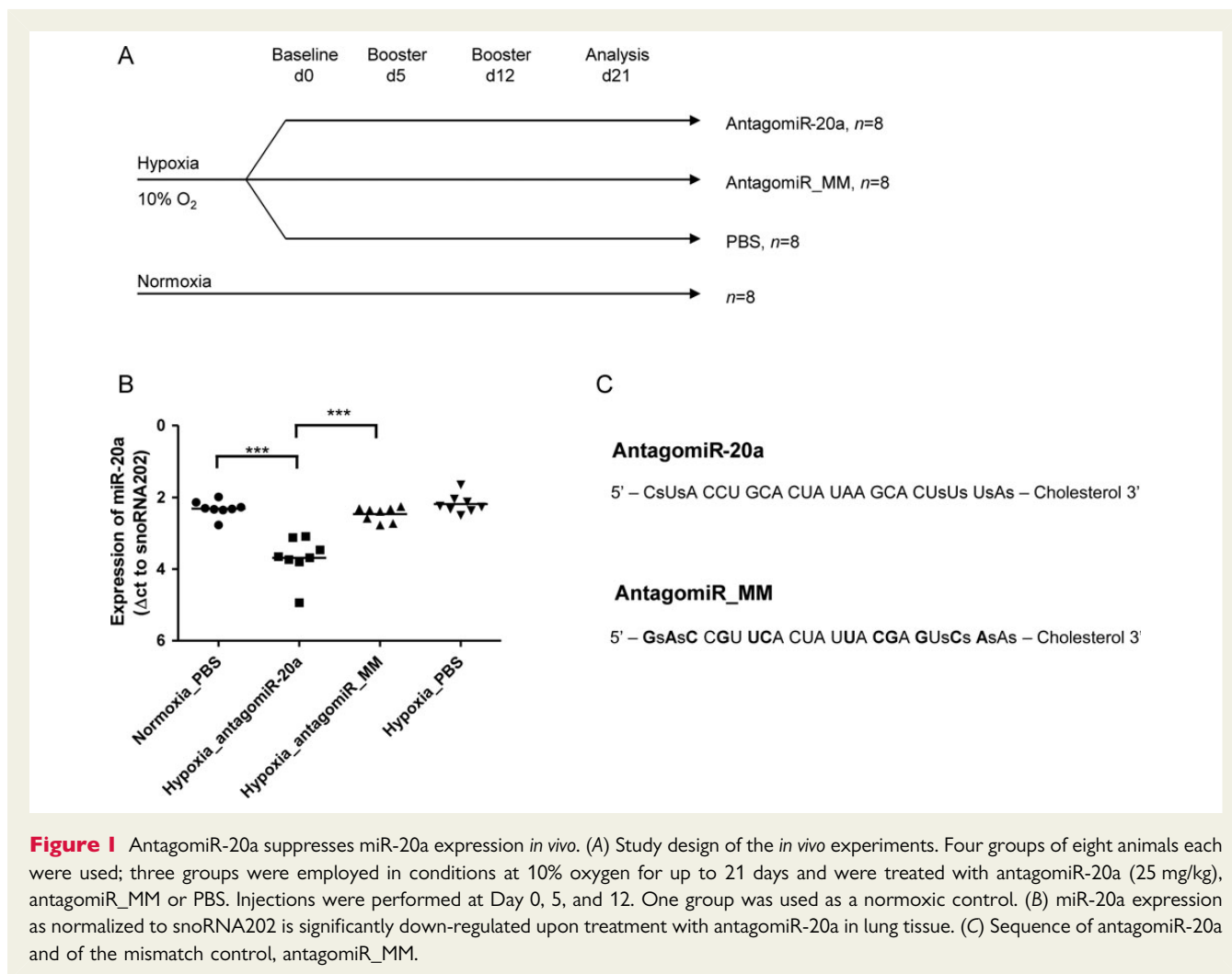
Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples with parametric distribution, the paired or unpaired *t*-test was applied and a *P*-value <0.05 was considered to be statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). For statistical analysis between more than two groups one-way ANOVA (Tukey post-test) was used. In all statistical analyses, two-sided tests were applied. A table showing all statistical analyses is provided (Supplementary material online, *Table S1*). All data are shown as mean $\pm$ SD.

Extensive materials and methods are provided in the Supplementary material online.

Results

Application of antagomiR-20a suppresses miR-20a expression in target tissues

We recently identified a novel signalling pathway that regulates the expression of BMPR2 through the action of miR-20a.[12] Here, we addressed the implications of this pathway in an animal model of hypoxia-induced pulmonary hypertension. The *in vivo* experiments were performed as described in the section 'Methods' (*Figure 1A*). As shown in *Figure 1B*, repeated i.p. injections of antagomiR-20a significantly down-regulated the expression levels of miR-20a in lung tissues when assessed by qPCR at Day 21. AntagomiR-20a-treated animals showed an expression level of miR-20a of ΔCt 3.68 ± 0.58 when compared with ΔCt 2.31 ± 0.23 in normoxic control animals thus resulting in a reduction in 59.3% ($P < 0.001$). On the other hand, no significant changes were observed between normoxic control mice and antagomiR_mismatch (antagomiR_MM, ΔCt 2.46 ± 0.2) or PBS (ΔCt 2.18 ± 0.26) treated animals. Similar results were observed for other tissues analysed, in the particular heart and liver (data not shown). These data indicate that intraperitoneally injected antagomiRs are not subjected to a significant hepatic first-pass effect and, thus, are a feasible approach to inhibit the expression of distinct miRNAs in target tissues including the lungs. The sequences of antagomiR-20a and antagomiR_MM are provided in *Figure 1C*.

Figure 1 AntagomiR-20a suppresses miR-20a expression *in vivo*. (*A*) Study design of the *in vivo* experiments. Four groups of eight animals each were used; three groups were employed in conditions at 10% oxygen for up to 21 days and were treated with antagomiR-20a (25 mg/kg), antagomiR_MM or PBS. Injections were performed at Day 0, 5, and 12. One group was used as a normoxic control. (*B*) miR-20a expression as normalized to snoRNA202 is significantly down-regulated upon treatment with antagomiR-20a in lung tissue. (*C*) Sequence of antagomiR-20a and of the mismatch control, antagomiR_MM.

AntagomiR-20a prevents the development of hypoxia-induced morphological changes within the cardiopulmonary circulation

Morphometric analyses were performed to assess the functional and haemodynamic consequences of reduced expression levels of miR-20a achieved by the application of antagomiR-20a. Representative images of pulmonary arterioles stained for α-smooth muscle actin are shown in *Figure 2A*; treatment with antagomiR-20a significantly inhibited the vascular remodelling observed in PBS- and antagomiR_MM-treated hypoxic animals. Computer-based analysis further quantified the occlusion of these vessels to be reduced from 13.47 ± 0.73 and 13.33 ± 0.61 (antagomiR_MM- and PBS-treated animals, respectively) to 7.72 ± 0.93 in antagomiR-20a-treated mice or to 1.73 ± 0.48 in normoxic controls (*Figure 2B*). Similar effects were observed for the thickness of the vessel wall showing a reduction from 1.18 ± 0.06 and $1.28 \pm 0.15 \mu M$ in antagomiR_MM- and PBS-treated animals to $0.96 \pm 0.05 \mu M$ in antagomiR-20a-treated animals ($0.71 \pm 0.04 \mu M$ in normoxic controls; *Figure 2C*).

Right ventricular hypertrophy, assessed in *Figure 2D*, after 10% O_2 for 21 days was strongly reduced in animals receiving

antagomiR- when compared with hypoxic mice treated with antagomiR_MM or PBS (representative images are shown in *Figure 2D*). These results were confirmed by calculating heart weight relative to body weight (antagomiR_MM: 0.0061 ± 0.0006 vs. antagomiR-20a: 0.0056 ± 0.0007; *Figure 2E*) and by the relation of the right ventricular to the left ventricular volume (RV/LV + S; antagomiR_MM: 0.25 ± 0.01; antagomiR-20a: 0.21 ± 0.01; *Figure 2F*), indicating that the right ventricular afterload was reduced in antagomiR-20a-treated mice when compared with mock-treated hypoxic animals. Blood analysis performed at Day 21 revealed that hypoxia resulted in erythrocytosis, which was most prominent in hypoxic PBS-treated animals (*Figure 2G*). Whereas significant differences were observed between normoxic controls (11.63 ± 0.91 g/dL) and all hypoxic groups (20.23 ± 2.04 g/dL for antagomiR-20a, 21.75 ± 1.28 g/dL for antagomiR_MM, and 23.27 ± 1.31 g/dL for PBS, respectively), differences between antagomiR-20a- and antagomiR_MM-treated animals did not reach statistical significance.

These data imply that the pathomorphological sequelae of hypoxia on the pulmonary vasculature, which lead to the development of pulmonary hypertension and, subsequently, to right ventricular hypertrophy, can be prevented by the application of antagomiR-20a.
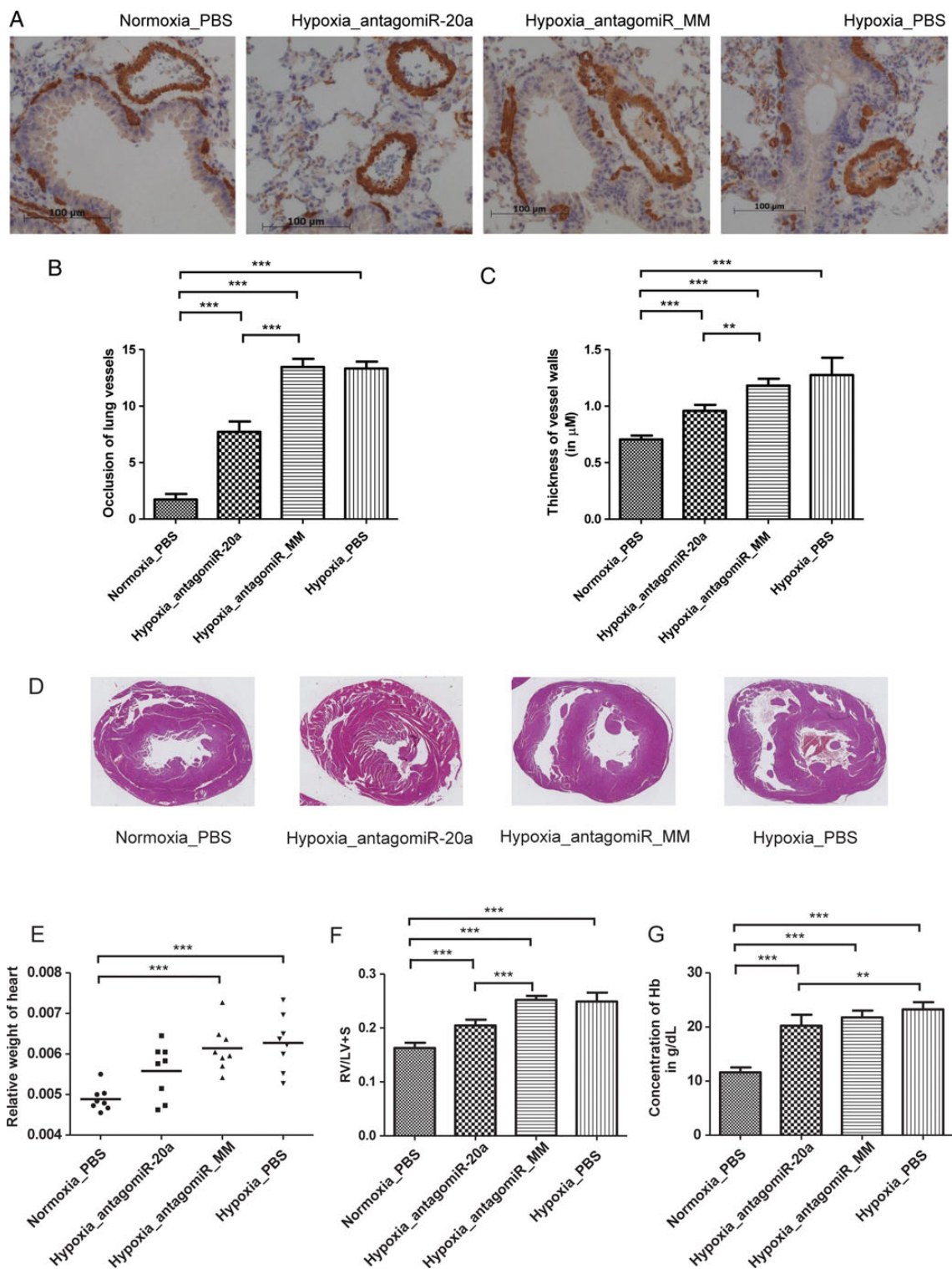
Figure 2 AntagomiR-20a prevents pulmonary arterial vascular remodelling. (*A*) Representative images of small pulmonary arteries stained against α-smooth muscle actin showing reduced hypoxia-induced remodelling of intimal and medial vessel layer upon treatment with antagomiR-20a (second from left), quantified by the analysis of lung vessel occlusion (*B*) and thickness of the vessel wall (*C*). Similar findings were observed for heart morphometry as shown by representative images revealing reduced right ventricular hypertrophy by treatment with antagomiR-20a (*D*, second from left), heart weight relative to body weight (*E*) and heart hypertrophy (*F*). Hypoxia-induced erythrocytosis was not affected significantly by treatment with antagomiR-20a (*G*).
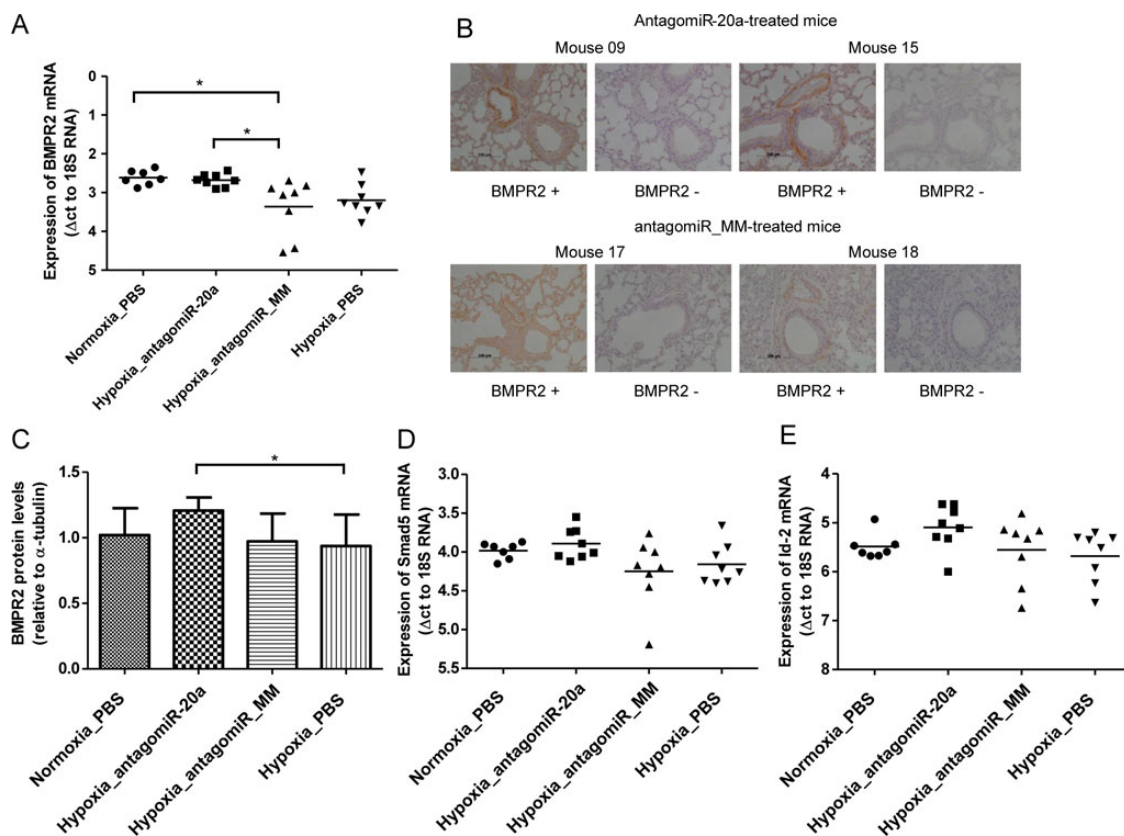
Figure 3 Restoration of functional expression levels of bone morphogenetic protein receptor type 2 by antagomiR-20a in mice. Treatment with antagomiR-20a increased the expression of bone morphogenetic protein receptor type 2 in lung tissue as shown by qPCR (*A*), immunohistochemistry (*B*) (antagomiR-20a-treated animals shown in upper panel, antagomiR_MM in lower panel, magnification 200-fold), and (*C*) western blotting. Similarly, expression of Smad 5 (*D*) and Id-2 (*E*) was increased in lung tissue by treatment with antagomiR-20a.

AntagomiR-20a restores functional expression levels of bone morphogenetic protein receptor type 2 in mice

Since we hypothesized that the reduced expression levels of BMPR2 observed in pulmonary hypertension might be due to targeted inhibition by miR-20a,[12] we next investigated whether the application of specific antagomiRs to mice increases BMPR2 expression levels, and whether restoration of this receptor might have functional implications. As shown in *Figure 3A*, the mRNA expression levels of BMPR2 in lung tissue were found to be reduced in both hypoxic control groups (antagomiR_MM: ΔCt 3.36 $\pm$ 0.73; PBS: ΔCt 3.2 $\pm$ 0.41) when compared with normoxic mice (ΔCt 2.61 $\pm$ 0.19). Interestingly, mRNA levels of BMPR2 under hypoxic conditions could be normalized to the levels observed in normoxic control animals by the application of antagomiR-20a (ΔCt 2.68 $\pm$ 0.16, *Figure 3A*). These findings are further illustrated by immunohistochemical and western blot analysis using antibodies directed against BMPR2 showing that the application of antagomiR-20a increased the expression of BMPR2 on endothelial and smooth muscle cells of pulmonary arterioles when compared with antagomiR_MM-injected mice

(*Figure 3B*) and PBS-treated hypoxic mice (*Figure 3C*, western blots provided as Supplementary material online, *Figure S1*).

Similarly, Smad5 and Id-2, two validated signalling elements downstream of the BMPR2 pathway[14] showed enhanced expression levels in lung tissues of animals treated with antagomiR-20a when compared with hypoxic controls (Smad5: antagomiR-20a ΔCt 3.89 $\pm$ 0.2 vs. hypoxia PBS ΔCt 4.16 $\pm$ 0.26, *Figure 3D*; Id-2: antagomiR-20a ΔCt 5.09 $\pm$ 0.46 vs. hypoxia PBS ΔCt 5.68 $\pm$ 0.52, *Figure 3E*). Expression levels for Id-1, however, were not found to be changed in normoxic or hypoxic animals (Supplementary material online, *Figure S2*). Protein analysis by western blotting for Id-2 further confirmed these findings and is provided as supplementary information (Supplementary material online, *Figure S3*). These data suggest that treatment with antagomiR-20a increases expression levels of BMPR2 within the pulmonary vasculature and, most important, restored functional BMPR2 signalling.

AntagomiR-20a enhances BMP-2-mediated signalling *in vitro*

Upon binding of the ligand BMP-2 to BMPR2, Smad5 is activated by phosphorylation, which, in turn, induces the expression of the
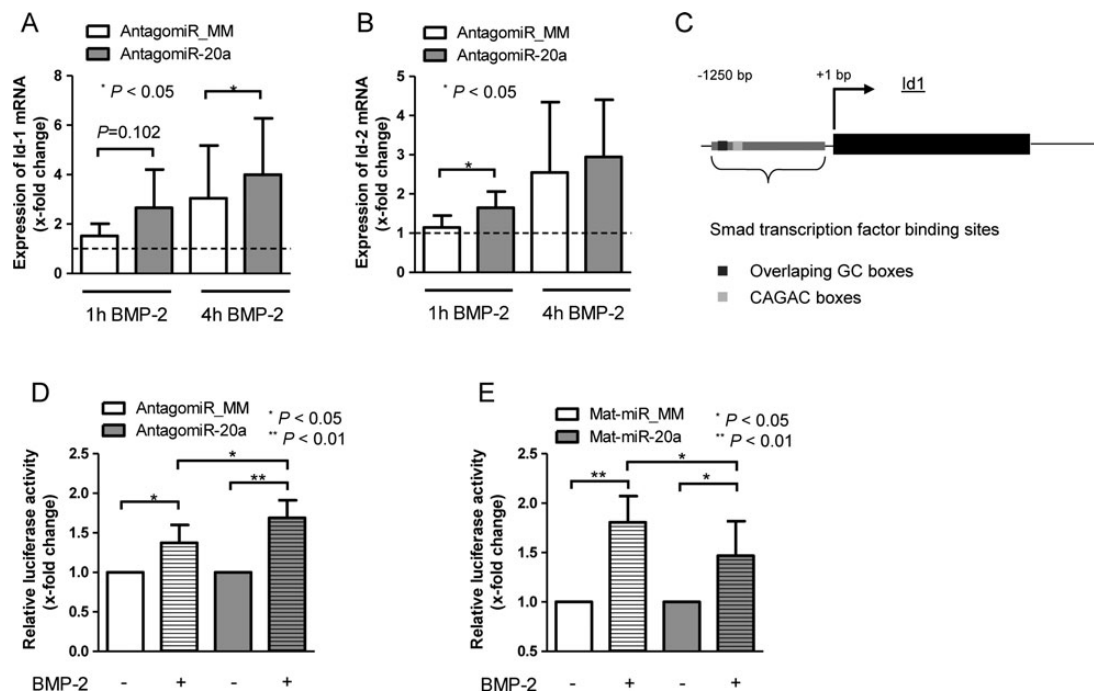
Figure 4 AntagomiR-20a enhances BMP-2 mediated signalling *in vitro*. Expression of Id-1 (*A*) and Id-2 (*B*) is significantly increased after stimulation with BMP-2 when transfected with antagomiR-20a (assessed at 1 and 4 h). Dashed line indicates unstimulated human pulmonary arterial smooth muscle cells. (*C*) For promoter studies, the promoter of human Id-1 was cloned containing multiple Smad transcription factor binding sites (as described in Lopez-Rovira *et al.*[15]). (*D*) The reporter gene assay in HepG2 cells: co-transfection of pGL3basic-Id-1 and antagomiR_MM or antagomiR-20a. Stimulation with BMP-2 for 4 h increased response in antagomiR-20a-treated cells. (*E*) Conversely, the response to BMP-2 was reduced when miR-20a was overexpressed.

transcription factors Id-1 and -2. Since treatment with antagomiR-20a up-regulated the expression levels of BMPR2 *in vivo*, we next investigated whether antagonization of miR-20a might also enhance the BMP-2-mediated signalling *in vitro*. We thus utilized human pulmonary arterial smooth muscle cells (HPASMCs) that were stimulated with BMP-2 for different time periods and found that treatment with antagomiR-20a enhanced BMPR2-induced signalling activity, i.e. the expression levels of Id-1 and -2 showed an augmentation of the BMP-2-mediated induction of gene expression by transfection of antagomiR-20a. As shown in *Figure 4A*, 1 h of BMP-2 stimulation enhanced the mRNA levels of Id-1 in antagomiR-20a-treated cells by 2.65 ± 1.55-fold (when compared with 1.52 ± 0.5-fold in antagomiR_MM-transfected cells, $P = 0.102$). After 4 h of BMP-2 stimulation Id-1 was significantly more induced in HPASMCs treated with antagomiR-20a (4 ± 2.28) when compared with control cells (3 ± 2.14, $P = 0.035$). The mRNA levels of Id-2, on the other hand, were found to be significantly more elevated by BMP-2 stimulation in antagomiR-20a-treated HPASMCs after 1 h only (antagomiR_MM: 1.14 ± 0.31-fold, antagomiR-20a: 1.65 ± 0.41-fold, $P = 0.02$, *Figure 4B*).

Finally, promoter studies were performed to illustrate an enhancement of BMP-2 signalling by antagonizing miR-20a. The promoter of Id-1 containing multiple Smad transcription factor binding sites[15] was cloned into a luciferase-based reporter gene vector. As

shown in *Figure 4D*, 4 h of BMP-2 stimulation induced the relative promoter activity of Id-1 by 1.38 ± 0.22-fold in antagomiR_MM transfected cells. Interestingly, the increase in promoter activity upon stimulation with BMP-2 was significantly more enhanced when cells were transfected with antagomiR-20a (1.69 ± 0.22-fold, $P = 0.016$). Conversely, the overexpression of miR-20a by transfection of small RNA oligonucleotides (mat-miR-20a) reduced the BMP-2-mediated induction of the promoter activity of Id-1 (mat-miR_MM: 1.81 ± 0.27-fold; mat-miR-20a: 1.47 ± 0.35-fold, $P = 0.013$; *Figure 4E*).

Note that, Smad5 was predicted to be directly targeted by miR-20a (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org,[16]). Accordingly, the expression of Smad5 after silencing of miR-20a in HPASMCs was analysed. As shown in Supplementary material online, *Figure S4*, transfection of antagomiR-20a significantly increased mRNA levels of Smad5 after 72 h, but failed to enhance protein levels. Moreover, a reporter gene assay comprising the predicted seed match of miR-20a in the 3′ UTR of Smad5 could not show a direct miRNA–mRNA interaction. Phosphorylation of Smad5 (normalized to Smad5 protein levels), however, showed a trend towards enhanced phosphorylation activity in stimulated as well as in unstimulated conditions when compared with antagomiR_MM controls, but these data did not reach statistical significance (Supplementary material online, *Figure S5*).
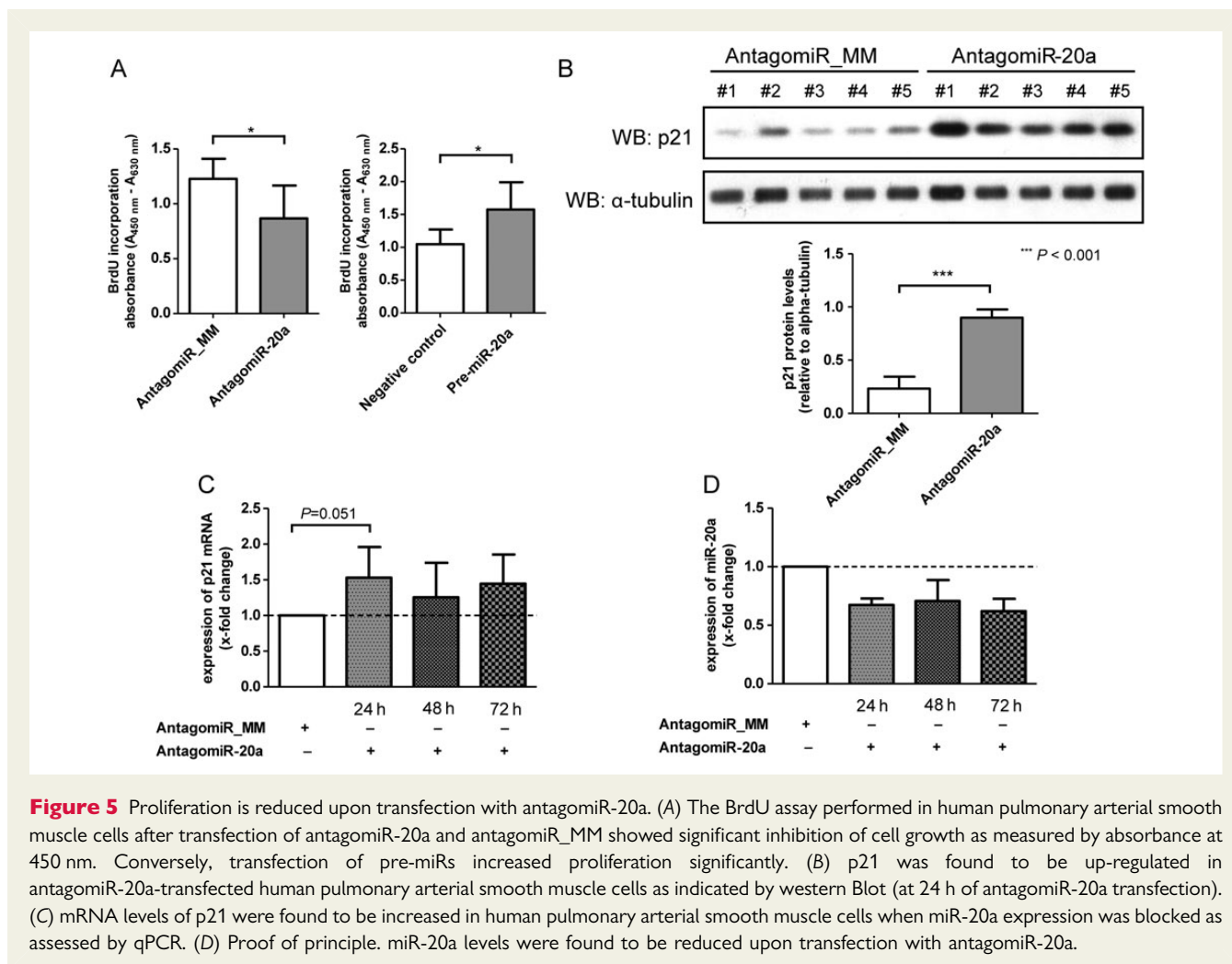
Figure 5 Proliferation is reduced upon transfection with antagomiR-20a. (*A*) The BrdU assay performed in human pulmonary arterial smooth muscle cells after transfection of antagomiR-20a and antagomiR_MM showed significant inhibition of cell growth as measured by absorbance at 450 nm. Conversely, transfection of pre-miRs increased proliferation significantly. (*B*) p21 was found to be up-regulated in antagomiR-20a-transfected human pulmonary arterial smooth muscle cells as indicated by western Blot (at 24 h of antagomiR-20a transfection). (*C*) mRNA levels of p21 were found to be increased in human pulmonary arterial smooth muscle cells when miR-20a expression was blocked as assessed by qPCR. (*D*) Proof of principle. miR-20a levels were found to be reduced upon transfection with antagomiR-20a.

Proliferation of vascular smooth muscle cells is reduced upon transfection with antagomiR-20a

Proliferation of pulmonary arterial smooth muscle cells is an essential part of the vascular remodelling in pulmonary hypertension; we thus addressed whether transfection with antagomiR-20a affects proliferation assessed by the BrdU assay. As shown in *Figure 5A*, proliferation was significantly reduced in antagomiR-20a-transfected HPASMCs (A_{450nm}: from 1.229 ± 0.182 to 0.867 ± 0.3) resulting in a reduction by 29.5% when compared with antagomiR_MM-transfected controls ($P = 0.018$); conversely, when cells were transfected with precursor molecules of miR-20a (pre-miR-20a), proliferation was significantly increased (A_{450nm}: from 1.049 ± 0.223 to 1.576 ± 0.418, or by 50.2%, respectively. $P = 0.012$). On the other hand, no significant changes were seen when apoptosis was analysed (Supplementary material online, *Figure S6*). AntagomiR-20a transfection of HPASMCs resulted after 24 h in up-regulation of the cell cycle inhibitor p21 on the protein level (antagomiR_MM: 0.232 ± 0.112 vs. antagomiR-20a: 0.804 ± 0.077, $P < 0.001$ *Figure 5B*) as well as on the mRNA level (1.53 ± 0.43-fold, $P = 0.051$ *Figure 5C*). The expression of miR-20a was analysed in the same samples showing

down-regulation of miR-20a in HPASMCs by antagomiR-20a treatment (*Figure 5D*).

Discussion

We have recently identified a microRNA-mediated signalling pathway that regulates the expression of BMPR2 on human pulmonary arterial endothelial cells.[12] While this pathway might explain, at least *in vitro*, the reduced expression of BMPR2 as observed in several forms of pulmonary hypertension, the functional relevance of this pathway was unclear so far. A very recent study has confirmed the pathogenetic importance of the microRNA cluster 17/92 by inhibition of miR-17 in experimental pulmonary hypertension;[17] based on our previous work that identified two functionally related miRs as regulators of BMPR2 expression, i.e. miR-17 and miR-20a but the latter one being more potent,[12] we focused here on the action of miR-20a.

In the present study, we employed an established animal model of pulmonary hypertension and found that (i) i.p. injection of antagomiR in general is a feasible approach to efficiently down-regulate distinct miRNAs in target tissues; (ii) antagonization of miR-20a reduces the hypoxia-induced remodelling of pulmonary arterioles

and, subsequently, reduces right heart hypertrophy in hypoxic animals; and, (iii) that treatment with antagomiR-20a restores functional expression levels of BMPR2 both *in vivo* and *in vitro*.

In these experiments, antagonization of miR-20a significantly reduced the vascular remodelling in hypoxic animals as assessed by wall thickness and luminal occlusion of small pulmonary arteries. Thickness of the vessel wall was less prominent than in hypoxic control animals, probably due to a lesser grade of muscularization upon reduced proliferation of vascular smooth muscle cells in the medial layer. The vascular occlusion of these vessels that is commonly observed in pulmonary hypertension and might result from vasoconstriction, proliferation of the intimal and medial layer and the development of plexiform lesions, has also been found to be reduced significantly in antagomiR-20a-treated animals. Vascular occlusion and narrowing of the pulmonary arterial lumen increase pulmonary vascular resistance and right ventricular afterload; these alterations result in cardiac hypertrophy and, ultimately, right heart failure. In our experiments, antagomiR-20a-treated animals revealed significantly less right ventricular hypertrophy than their mock-treated hypoxic controls, indicating improved pulmonary arterial haemodynamics in antagomiR-20a-treated mice. However, since the expression of miR-20a has been antagonized not only in the lungs, it remains unclear whether the observed reduction in right ventricular hypertrophy is an indirect effect of reduced pulmonary arterial pressure or, alternatively, whether it might be due to concomitant inhibition of miR-20a in cardiac myocytes. In contrast to other miRNAs, including miR-133,[18] miR-21,[19] or miR-27b[20] that also have been antagonized experimentally, miR-20a has not been described to be overexpressed in response to cardiac stress. With regard to other cell types, treatment with antagomiR-20a did not significantly alter the hypoxia-induced polycythaemia of erythrocytes, indicating that, at least, no such pleiotropic effects by antagonizing miR-20a have occurred on haematopoietic cells.

We further showed that treatment with antagomiR-20a increased the expression levels of BMPR2 in lung tissue, thus confirming that miR-20a targets the mRNA of BMPR2 as it was already suggested previously by our *in vitro* data on human pulmonary arterial endothelial cells. Moreover, in the current study transfection of smooth muscle cells with antagomiR-20a could rescue expression and downstream signalling events of BMPR2 that was silenced by siRNA (Supplementary material online, *Figure S7*). It has been discussed controversially, whether the observed dysregulation of BMPR2 in several forms of pulmonary hypertension might be of pathogenetic relevance. However, genetic studies and recent *in vitro* data suggest that BMPR2 and its downstream signalling play an important role in cell proliferation, vascular remodelling and, thus, in the development of pulmonary hypertension. For instance, Wong *et al.*[21] showed that the activation of the BMPR2 signalling pathway in HPASMCs leads to the inhibition of proliferation probably due to the induction of the cell cycle repressor p21. Our findings are along the line of these data and further provide evidence both *in vivo* and *in vitro* that a functional BMPR2 signalling prevents major vascular remodelling within small pulmonary arteries. In particular, we showed that the restoration of BMPR2 expression in lung tissue leads to activation of the BMP-2 target Id-2. Similarly, *in vitro*, stimulation of HPASMCs with BMP-2 and transfection

with antagomiR-20a revealed increased BMP-2 signalling as indicated by enhanced promoter activity of Id-1 and up-regulated expression levels of Id-1 and -2. These transcription factors have been associated with cell proliferation;[22] activated Id-1/-2 thus would reduce the proliferation rate of cells. Here, levels of Id-1 and -2 have been found to be increased in antagomiR-20a-treated HPASMCs, and, in subsequent experiments, the proliferation of HPASMCs was reduced by transfection of antagomiR-20a, whereas apoptosis was not affected significantly. These data strongly indicate that the restoration of BMPR2 by treatment with antagomiR-20a is functional and, thus, reconstitutes the downstream signalling of BMPR2 in pulmonary arterial smooth muscle cells and in lung tissue. The reduced proliferation of smooth muscle cells was further shown to be associated with enhanced expression of the cell cycle repressor p21. We suggest that the observed increased expression of p21 by antagomiR-20a is caused by two ally acting mechanisms. On the one hand it was shown that miR-20a directly regulates the expression of p21,[23] and on the other hand Wong *et al.*[21] demonstrated that BMP-2 signalling enhances protein levels of p21. Therefore, antagomiR-20a treatment increases p21 levels by the inhibition of miR-20a and by enhancing BMP-2 signalling, which might result in growth arrest of smooth muscle cells. In summary, functional restoration of BMPR2 appears to inhibit the development of hypoxia-induced vascular remodelling by enhanced intracellular signalling activity and reduced proliferation.

Finally, antagonization of miR-20a, one of the key microRNAs in pulmonary hypertension, was successfully achieved in hypoxic mice by repeated i.p. injections. Since antagomiRs are employed to a growing extent for antagonization of distinct microRNAs for pathogenetic investigations and therapeutic purposes,[11] these findings make antagomiRs to interesting tools for several reasons: down-regulation of miR-20a was observed in target organs behind the liver, a finding that mutually excludes a significant hepatic first-pass effect; the effects have been longstanding, thus minimizing the need for frequent applications; toxic effects have not been observed in terms of well-being of the animals or interferon-gamma expression in liver tissue (data not shown). Moreover, to our knowledge, this is the first report on i.p. application of antagomiRs.

Our data might be limited by the fact that the increase in miR-20a observed under hypoxic conditions did not reach statistical significance. However, miRs are considered to act as fine tuners of gene regulation, and, thus, even small and non-significant elevations might have functional relevance. Accordingly, the increase of miR-20a and other members of the cluster miR-17/92 was rather small in other *in vivo* studies performed in experimental models of pulmonary hypertension.[13] Together with these data, the experiments from our study underscore the major importance of a completely intact BMPR2 signalling pathway to prevent vascular remodelling; here, this could be achieved by specific inhibition of miR-20a, an endogenously expressed repressor of BMPR2, resulting in normalization of BMPR2 mRNA levels or overexpression of protein levels, respectively.

Taken together, we show here for the first time that the development of hypoxia-induced changes of small pulmonary arteries can be prevented by the application of antagomiR-20a, though it

remains speculative whether this treatment would also reverse the once established disease. Our *in vivo* data, however, suggest that antagonization of miR-20a inhibits vascular remodelling of pulmonary arteries in patients with pulmonary hypertension and, thus, might provide a major impact on our understanding and treatment of this disease.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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