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ORIGINAL ARTICLE

# miR-1 induces endothelial dysfunction in rat pulmonary arteries

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## Abstract

Endothelial dysfunction plays a central role in the pathophysiology of pulmonary arterial hypertension (PAH). MicroRNAs (miRNAs) are small single-strand and non-coding RNAs that negatively regulate gene function by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs. microRNA-1 (miR-1) is upregulated in plasma from idiopathic PAH patients and in lungs from an experimental model of PAH. However, the role of miRNA-1 on endothelial dysfunction is unknown. The aim of this study was to analyze the effects of miR-1 on endothelial function in rat pulmonary arteries (PA). Endothelial function was studied in PA from PAH or healthy animals and mounted in a wire myograph. Some PA from control animals were transfected with miR-1 or scramble miR. Superoxide anion production by miR-1 was quantified by dihydroethidium (DHE) fluorescence in rat PA smooth muscle cells (PASMC). Bioinformatic analysis identified superoxide dismutase-1 (SOD1), connexin-43 (Cx43), caveolin 2 (CAV2) and Krüppel-like factor 4 (KLF4) as potential targets of miR-1. The expression of SOD1, Cx43, CAV2, and KLF4 was determined by qRT-PCR and western blot in PASMC. PA incubated with miR-1 presented decreased endothelium-dependent relaxation to acetylcholine. We also found an increase in the production of $O_2^-$ and decreased expression of SOD1, Cx43, CAV2, and KLF4 in PASMC induced by miR-1, which may contribute to endothelial dysfunction. In conclusion, these data indicate that miR-1 induces endothelial dysfunction, suggesting a pathophysiological role in PAH.

**Keywords** Posttranscriptional regulation · miRNA-1 · Endothelial dysfunction · Superoxide dismutase · Pulmonary arterial hypertension

## Introduction

Pulmonary arterial hypertension (PAH) is a rare and progressive disease characterized by an elevation of the mean pulmonary arterial pressure. It is a disorder with a significant burden in terms of both severity and prevalence. PAH affects young and middle-aged persons, preferentially women [9]. The disease progression involves an increase in pulmonary vascular resistance (PVR) leading to right heart failure and eventually death. PAH is characterized by an imbalance in endothelial-derived vasoactive factors, inflammation, and structural remodelling of pulmonary arteries (PA) [28].

The endothelium is a single layer of cells that lines the lumen of blood vessels and plays an important physiological role in vascular homeostasis [13]. Endothelial dysfunction is defined as a chronically impaired production of vasodilators such as nitric oxide (NO) leading to a proliferative, pro-oxidant, pro-inflammatory, and pro-thrombotic status. Various pathological conditions including diabetes, hyperlipidemia, erectile dysfunction [33], hypertension [5], and PAH [12] may impact on endothelial function by disrupting the molecular mechanisms regulating NO bioavailability, which is an independent predictor of poor prognosis of these diseases. Thus, decreased NO production and/or increased NO degradation by reactive oxygen species (ROS), mainly superoxide anions, are considered early markers of endothelial

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dysfunction [17, 42]. Several studies have highlighted the close relationship between mitochondrial ROS production, endothelial dysfunction, and excess cardiovascular risk. Superoxide dismutases (SOD) are a family of enzymes that catalyze the conversion of superoxide anion to hydrogen peroxide ($H_2O_2$). In particular, SOD1 plays a key role in scavenging superoxide anions in the pulmonary vasculature [8].

MicroRNAs (miRNAs) are single-strand and non-coding RNAs of 18–24 nucleotides that negatively regulate gene function by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs. The interaction between the miRNA and the 3′UTR sequence of the specific mRNA leads to translational repression and/or degradation of the mRNA, resulting in a decrease of the target protein. Mature miRNAs play a key role in different cellular processes, such as proliferation, apoptosis, differentiation, and organogenesis [2]. Aberrant regulation of miRNA expression has been implicated in a large number of clinically important cardiovascular diseases. In the last decade, multiple studies have proposed the analysis of miRNA as a potential diagnostic and prognostic tool [19, 32]. Therapeutic strategies based on the upregulation or downregulation of different miRNAs have recently emerged [29].

Upregulated miRNAs can be antagonized using antagomirs, while downregulated miRNAs can be restored with miRNA mimetics. miRNA mimetics are synthetic RNA duplexes mimicking the endogenous miRNA, while antagomirs are modified antisense oligonucleotides that harbor the complete or partial complementary sequence of a mature miRNA.

Recent studies suggest that different miRNAs are dysregulated in patients with PAH and in PAH experimental models, particularly in pulmonary artery smooth muscle cells (PASMCs) and in pulmonary artery endothelial cells (PAECs) [43]. Among the miRNAs that are increased in PAH, miR-1 was the one with the highest upregulation (8-fold increase in microarray experiments and a 12-fold increase in the PCR analysis) in plasma from 12 idiopathic PAH patients [31]. miR-1 is also upregulated in peripheral blood mononuclear cells in essential hypertensive patients [16] and in preeclampsia [11]. Moreover, we have recently demonstrated that lungs from rats with PAH induced by Su5416, a vascular endothelial growth factor type 2 receptor (VEGFR) inhibitor, plus 3 weeks under hypoxia (10%, Hyp/Su5416) showed a marked upregulation of miR-1 (≈4-fold increase) [20]. We also found an increased expression of miR-1 in pulmonary arteries from healthy animals incubated for 48 h under a hypoxic environment (3% $O_2$) in the presence of Su5416, mimicking the condition of human and animal models of PAH. Our study also showed that miR-1 downregulates the voltage-dependent potassium channel Kv1.5 in PASMCs, which plays an important role in the control of pulmonary vascular tone and whose activity is impaired in patients and in different animal models of PAH.

Herein, we hypothesized that miR-1 could also be involved in an additional key pathophysiological feature of pulmonary vascular diseases, such as endothelial dysfunction. Therefore, the aim of the present study was to analyze the effects of miR-1 on pulmonary endothelial function.

## Material and methods

### Ethical approval

All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-251/15). All investigators understand the ethical principles.

### Animals and in vitro models of PAH

Male Wistar rats of 220–250 g ($n = 22$) were obtained from Envigo (Barcelona, Spain). All animals were kept with free access to standard rat chow and water in an enriched environment throughout the whole experiment period and maintained at 24 °C under a 12 h light/12 h dark cycle. Rats were euthanized using $CO_2$.

For the in vivo model of Su5416 plus hypoxia-induced PAH (Hyp/Su5416), rats were injected subcutaneously with a single dose of the VEGFR type 2 inhibitor Su5416 (20 mg/Kg) or vehicle [10, 37]. Then, SU5416-treated animals were introduced in glass cages and ventilated with 10% $O_2$ (hypoxia, $n = 5$) for 3 weeks. $CO_2$ and water vapor produced by the animals were captured with soda lime and silica gel, respectively. Control rats (normoxia, $n = 3$) were kept in the same room. Oxygen was monitored using an oxygen sensor (DrDAQ Oxygen Sensor, Pico Technology, UK) in the room and in the chamber outflow. The chambers were opened for 20–30 min daily for regular animal care.

For in vitro models of PAH, isolated pulmonary arteries (PA) from male Wistar rats were isolated from the left lung. Vessels were then placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (4.5 g/L), non-essential amino acid solution (1×), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml), and maintained for 48 h in a normoxic incubator (21% $O_2$ and 5% $CO_2$) or a hypoxic incubator (3% $O_2$ and 5% $CO_2$ in the presence of 10 μM Su5416) before assessing vascular reactivity.

### In silico analysis for miR target prediction

Target endothelial gene prediction analysis was completed using the MicroRNA.org target prediction resource, utilizing miRanda sites and miRSVR scoring. The target sites predicted using miRanda are scored for the likelihood of mRNA downregulation using a regression model trained on the sequence and contextual features of the predicted miRNA: mRNA duplex.

### Cell culture

PASMCs were isolated from explants of PA from 5 different control rats. Briefly, cells were isolated from endothelium-denuded vessels digested in a $Ca^{2+}$-free solution containing (in mg/ml) collagenase 1.125, elastase 0.1, and albumin 1 for 4 min at 4 °C followed by 1 min at 37 °C. Following digestion, tissues were washed in the $Ca^{2+}$-free HEPES solution containing (in mM) NaCl 130, KCl 5, HEPES 10, $MgCl_2$ 1.2, and glucose 10 (pH adjusted to 7.3 with NaOH) and disaggregated using a wide bore, smooth-tipped pipette, to make a cell suspension. Cell suspension was plated into 35-mm Petri dishes and incubated in a humidified atmosphere of 5% $CO_2$ in air at 37 °C in DMEM containing 20% heat-inactivated FBS, pyruvate (1.1 mg/ml), 1% non-essential amino acids, streptomycin (100 mg/ml), penicillin (100 unit/ml), and amphotericin B (250 ng/ml) for 1 week. Cells were subcultured in 75-$cm^2$ sterile flasks in DMEM supplemented with 10% FBS and used within passages 2–3.

### Transfection of miR into PASMCs

PASMCs were transfected with miR-1 (hsa-miR-1-3p mirVanaTM miRNA mimic, MC10617) or a scramble miR (miRNA mimic negative control, 4464058) from Applied Biosystems using Lipofectamine™ RNAiMAX (Life Technologies) following manufacturer's instructions. miRNAs with a final concentration of 10 nM and Lipofectamine™ RNAiMAX were mixed with Opti-MEM (Life Technology) supplemented with 2% FBS and 1% antibiotic/antimycotic solution. The mixture of miRNA/LipofectamineTM RNAiMAX was kept at room temperature for 5 min to form the transfection complexes and then added to the cells. After incubation for 24 h at 37 °C in a humidified incubator (21% $O_2$/5% $CO_2$), transfection complexes were replaced with complete growth media (DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution) and kept for 24 h.

### Transfection of PA

PA from 5 different rats were isolated from the left lung and cut into rings. For the study of endothelial function, to introduce miR-1 and scramble miR (final concentration 100 nM) into isolated PA, reverse permeabilization was used as previously reported [23]. Briefly, PA were exposed to three successive solutions (4 °C) containing (in mM) (i) miRNA duplexes, 10 EGTA, 120 KCl, 5 $Na_2ATP$, 2 $MgCl_2$, 20 HEPES (pH 6.8; 30 min); (ii) miRNA duplexes, 120 KCl, 5 $Na_2ATP$, 2 $MgCl_2$, and 20 HEPES (pH 6.8; 180 min); and (iii) miRNA duplexes, 120 KCl, 5 $Na_2ATP$, 10 $MgCl_2$, and 20 HEPES (pH 6.8; 30 min). Subsequently, PA were bathed in a fourth solution containing (in mM) 120 NaCl, 5 KCl, 5 $Na_2ATP$, 10 $MgCl_2$, 5.6 glucose, and 10 HEPES (pH 7.1, 4 °C), in which [$Ca^{2+}$] was gradually increased from 0.001 to 0.01, 0.1, and 1 mM every 15 min. Vessels were then placed in DMEM culture medium supplemented with glucose (1 g/L), non-essential amino acid solution (1×), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml), and maintained for 48 h in a normoxic incubator (21% $O_2$ and 5% $CO_2$) before assessing vascular reactivity. Previously, the transfection efficiency was assessed of this protocol using a fluorescent microRNA (Dharmacon miRIDIAN mimic transfection control-Dy547, CP-004500-01-05). We found that endothelial function was preserved, and transfection was achieved successfully for endothelial cells.

### Recording of arterial reactivity

For contractile tension recording, PA rings (1.7–2 mm long, ~ 0.8 mm internal diameter) were mounted in a wire myograph, as previously reported [20]. Vessels were stretched to give an equivalent transmural pressure of 30 mmHg. Preparations were first stimulated by raising the $K^+$ concentration of the buffer (to 80 mM) in exchange for $Na^+$. Vessels were washed three times and allowed to recover before a new stimulation. Then, endothelial function was assessed by cumulative addition of acetylcholine (ACh, 1 nM−10 μM) in PA rings as described [18].

### Lung histology

The left lung was inflated in situ with 4% formol saline through the left bronchus and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and examined by light microscopy, and elastin was visualized by its green auto-fluorescence [22].

### Detection of $O_2^-$ production

$O_2^-$ release in rat PASMCs was quantified by dihydroethidium (DHE) fluorescence. Briefly, confluent PASMCs in 96-well plates were transfected with scramble or miR-1 (10 nM), as described above. Forty hours post-transfection, cells were incubated as described above with

HEPES buffered solution supplemented with 1.5 mM $CaCl_2$ at 37 °C for 30 min in the absence or presence of SOD mimetic, MnTMPyP (100 μM). Then, DHE (30 μM) or the nuclear stain 4,6-diamidin-2-phenylindol dichlorohydrate (DAPI, 0.3 μM) was added. The fluorescent intensity was followed for 1 h at an excitation and emission wavelengths of 530 nm and 620 nm, respectively, for DHE and of 355 nm and 460 nm, respectively, for DAPI, using a plate spectrofluorimeter (Fluoroskan Ascent, Thermo Scientific). DHE fluorescence was normalized by the fluorescence of DAPI.

### RNA extraction and quantitative RT-PCR

Total RNA and microRNA were extracted from PASMCs using miRNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. RNA concentration and quality were checked using NanoDropTM 1000 Spectrophotometers (Thermo Scientific, Massachusetts, USA). For mRNA determination, 1 μg of RNA was reverse transcribed into cDNA using iScriptTM cDNA Synthesis Kit (Biorad, California, USA) following the manufacturer's instructions. For miRNA amplification, complementary DNA analysis was synthesized from total RNA using specific stem-loop reverse transcription primers (Taqman MicroRNA Reverse Transcription Kit, Applied Biosystems, Table 1). Gene expression was determined by quantitative real-time PCR (qRT-PCR) with a Taqman system (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) in the Genomic Unit of the Complutense University (Madrid) using specific primers (Table 1). The delta-delta Ct method was used to quantify relative changes. mRNA expression was normalized by the expression of β-actin with a Taqman probe number #69 (Roche, Cat: 04688686001), and U6 for miRNA analysis.

### Western blot analysis

Homogenates were run on a sodium dodecyl sulfate-polyacrylamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, incubated with primary rabbit polyclonal antibody against SOD-1 (1:10000, #SOD-101, Stressgen, San Diego, CA) overnight and then with the secondary peroxidase conjugated antibodies. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Blots were imaged using an Odissey Fc System (Li-COR, Biosciences) and were quantified by densitometry using Quantity One software. Samples were re-probed for the expression of smooth muscle α-actin (1:10000, A2547, Sigma-Aldrich, Spain).

### Drugs

All drugs were from Sigma-Aldrich (Spain) except Su5416 (Tocris, UK).

### Statistical analysis

Data are expressed as means ± s.e.m. Statistical comparisons were performed using two-tailed unpaired *t* tests or two-way ANOVA followed by Bonferroni test as appropriate. $P < 0.05$ was considered statistically significant.

## Results

### Endothelial dysfunction in in vitro and in vivo models of pulmonary hypertension

Figure 1a shows hematoxylin and eosin–stained lung sections from control or Hyp/Su5416 animals. The increase of medial wall thickness can be observed by hematoxylin and eosin staining (Fig. 1a, above), and elastin was visualized by its

**Table 1** Details of primers employed for the PCR

| Gen | | Taqman assay |
|---|---|---|
| *Gap junction protein, alpha 1 (Gja1)* | | Rn01433957_m1 |
| *Superoxide dismutase 1 (Sod1)* | | Rn00566938_m1 |
| *Caveolin 2 (Cav2)* | | Rn00590969_m1 |
| *Krüppel-like factor 4 (Klf4)* | | Rn00821506_g1 |
| Gen | Primer forward 5′–3′ | Primer reverse 5′–3′ |
| *Actin, beta (Actb)* | 5′-GCCCTAGACTTCGAGCAAGA-3′ | 5′-TCAGGCAGCTCATAGCTCTTC-3′ |
| miRNA | Taqman assay | Sequence |
| *U6* | 001973 | GTGCTCGCTTCGGCAGCACATATACTAAAATTGGA ACGATACAGAGAAGATTAGCATGGCCCCTGCGCAA GGATGACACGCAAATTCGTGAAGCGTTCCATATTTT |
| *miR-1* | 002222 | UGGAAUGUAAAGAAGUAUGUAU |

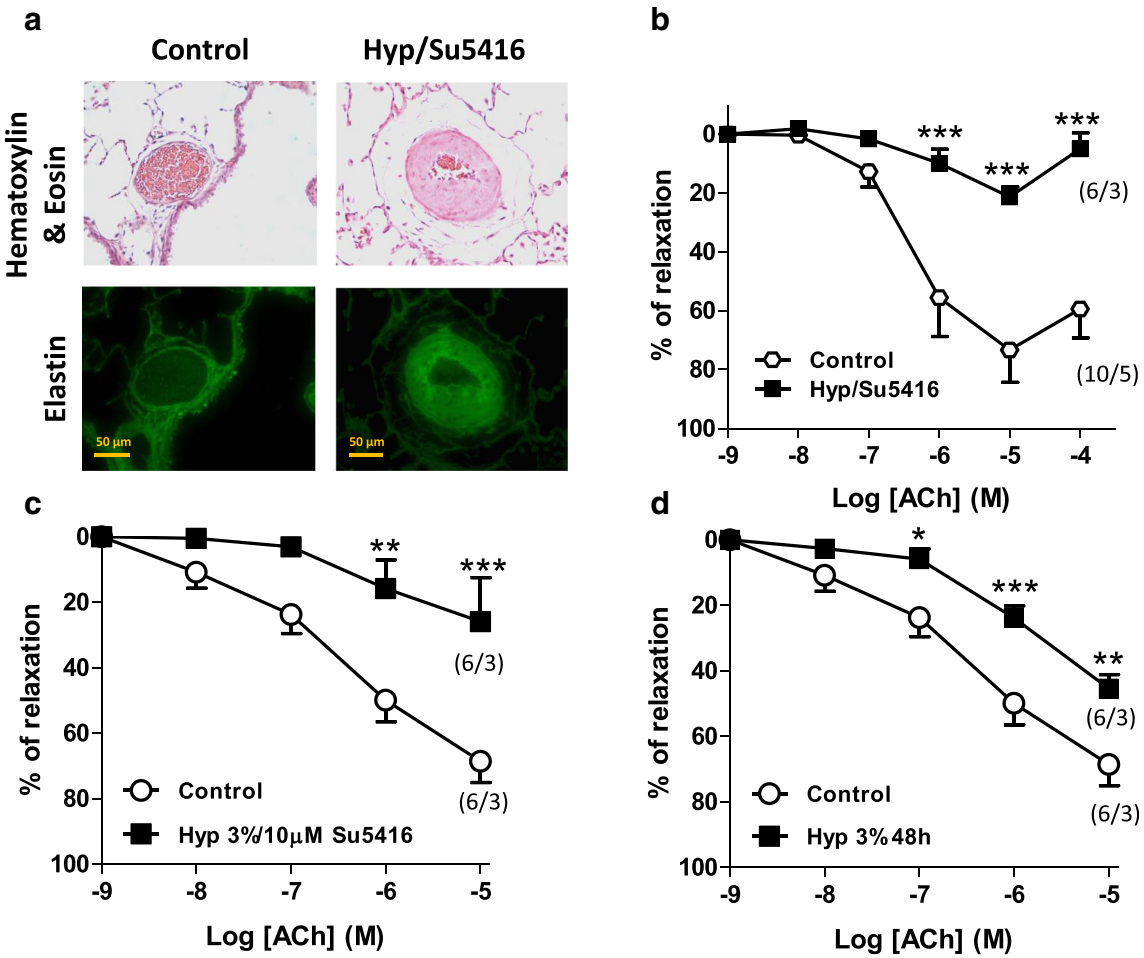


**Fig. 1** Vascular remodeling and endothelial dysfunction in vivo and in vitro models of pulmonary hypertension associated to upregulated miR-1. **a** Representative images (× 40) of vascular remodelling of pulmonary arteries in Hyp/Su5416 animals compared with control. Concentration-response curves to cumulative addition of ACh (1 nM–100 µM) as an indicator of endothelial function in rat PAs constricted by phenylephrine (1 µM) from **b** in vivo model of Hyp/Su5416 and in rat PAs constricted by 5-HT (10 µM) from **c** in vitro model Hyp 3% for 48 h/10 µM Su5416 and **d** in vitro model of Hyp 3% for 48 h. Results are means ± S.E.M. The number of pulmonary arteries/animals in each group is indicated in parenthesis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control

green auto-fluorescence (Fig. 1a, bottom). A marked vascular remodelling with an increase of medial wall thickness was present in PA from the in vivo Hyp/Su5416 animal model compared with control PA. We analyzed the endothelium-dependent relaxant response to ACh in isolated PA pre-contracted with serotonin (5-HT, 10 µM). The relaxation induced by ACh was attenuated in PAs from Hyp/Su5416 indicating that PA from Hyp/Su5416 rats overexpressing miR-1 exhibit endothelial dysfunction (Fig. 1b, $P < 0.001$). In addition, the relaxant responses to ACh were attenuated in PA incubated for 48 h at 3% $O_2$ in the presence (Fig. 1c) or absence (Fig. 1d) of Su5416 ($P < 0.001$) compared with PA incubated under normoxic conditions (5% $CO_2$ in air at 37 °C).

## miR-1 induces endothelial dysfunction in rat pulmonary arteries

The endothelium-dependent relaxation induced by ACh was also studied in PA transfected with miR-1 compared with scramble miR. PA transfected with miR-1 showed a decreased relaxant response to ACh (Fig. 2a, $P < 0.01$), indicating that miR-1 induces endothelial dysfunction. Forty-eight hours post-transfection with the miR-1 mimetic, the miR-1 levels were markedly increased compared with scramble miR transfected PASMC (Fig. 2b, $P < 0.001$). We also evaluated the production of ROS in PASMCs by DHE fluorescence. The DHE fluorescent signal was strongly reduced in the presence of the SOD mimetic MnTMPyP, suggesting that it was essentially due to $O_2^-$ production. miR-1 increased DHE

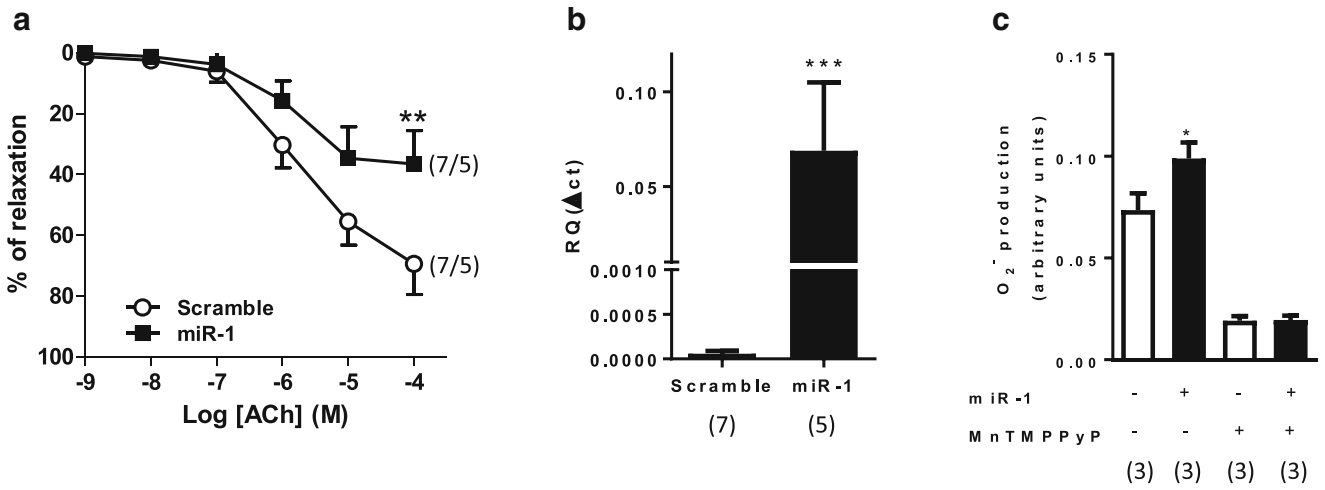


**Fig. 2** miR-1 induces endothelial dysfunction in rat pulmonary arteries. Effects of miR-1 on **a** endothelium-dependent relaxation to ACh (1 nM–100 μM) in rat PA rings precontracted with 5-HT (10 μM) transfected with miR-1 or scramble miR (negative control) for 48 h. **b** miR-1 expression analyzed by RT-PCR in PASMC transfected with scramble miR or miR-1. **c** $O_2^-$ production in transfected rat PASMCs. $O_2^-$ release was quantified by DHE (30 μM) fluorescence. Average values of DHE fluorescence were normalized to the DAPI (30 μM) fluorescence in rat PASMCs incubated in the presence or absence of the SOD mimetic MnTMPyP (100 μM). Results are means ± S.E.M. The parentheses indicate the number of arteries or PASMC cell cultures, and the number of animals from which these arteries were obtained, respectively. *$P < 0.05$, **$P < 0.01$

fluorescence compared with scramble miR (Fig. 2c, $P < 0.05$), and this increase was abolished in the presence of MnTMPyP.

## In silico *analysis* for the prediction of miR-1 endothelial targets

In order to assess the potential pathophysiological impact of the upregulation of miR-1 levels, we performed an in silico analysis for the prediction of miR-1 targets using the microRNA.org database. Among those mRNAs whose downregulation could be involved in endothelial dysfunction, we selected the *Sod1*, *Gja1*, *Cav2*, and *Klf4* mRNAs (Table 2) which encode for superoxide dismutase 1, Gap junction protein α1 or connexin 43 (Cx43), caveolin-2, and Krüppel-like factor 4. The table contains the gene symbol, gene description, complementary sequence, and mirSVR score for each gene. A more negative mirSVR score indicates a more favorable union site.

**Table 2** Selected mRNA targets of miR-1 in *Homo sapiens* that may be involved endothelial dysfunction (*SOD1, GJA1, CAV2,* and *KLF4*). Data predicted from www.microRNA.org

| Gene symbol | Gene description | Complementary sequence | mirSVR score |
|---|---|---|---|
| *SOD1* | Superoxide dismutase 1, soluble | 3′ uauguaugaagaaaUGUAAGGu 5′ hsa-miR-1<br>\|\|\|\|\|\|\|<br>5′ ----------ACAUUCCc 3′ SOD1 | −0.81 |
| *GJA1* | Gap junction protein α1 (connexin 43) | 3′ uauguauGAAGA-AAUGUAAGGu 5′ hsa-miR-1<br>\|\| \|\| \|\|\|\|\|\|<br>5′ uaaguccCUGCUAAAACAUUCCa 3′ GJA1<br>3′ uauGUA-UGAAGAAAU-GUAAGGu 5′ hsa-miR-1<br>\|\|\| \|\|\|\|:\|\| \| \|\|\|\|\|\|<br>5′ uacCAUCACUUUUUCAUCAUUCCu 3′ GJA1<br>3′ uaUGUAUGAAGAAA-UGUAAGGu 5′ hsa-miR-1<br>\|\| \|:\|\| \|\|\| \|\|\|\|\|\|\|<br>5′ uuACUAAUUUGUUUGACAUUCCa 3′ GJA1 | |
| *CAV2* | Caveolin 2 | 3′ uauguaUGAAG--AAAUGUAAGGu 5′ hsa-miR-1<br>\|\| \|\| \|\| \|\|\|\|\|\|\|<br>5′ aucagaACCUCAAUUGACAUUCCu 3′ CAV2 | −0.54 |
| *KLF4* | Krüppel-like factor 4 (gut) | 3′ uaugUAUGAAGAAAUGUAAGGu 5′ hsa-miR-1<br>\|\| \|\|\|\| \| \|\|\|\|\|\|<br>5′ cuggAU-CUUC-UAUCAUUCCa 3′ KLF4 | −0.17 |

### miR-1 directly targets superoxide dismutase 1 (SOD1)

After the evaluation of ROS production, we analyzed if miR-1 altered the expression of *Sod1*, an enzyme responsible for the metabolism of superoxide ions. Our data showed that there were no changes in the expression of *Sod1* mRNA in PASMCs transfected with miR-1 compared with scramble miR, analyzed by qRT-PCR (Fig. 3a). However, we found a decrease in protein SOD1 levels in PASMCs transfected with miR-1 compared with scramble miR as analyzed by western blot and normalized by α-actin expression (Fig. 3b, $P < 0.05$).

### Changes in miR-1-targeted endothelial genes

As additional potential mechanisms for impaired endothelial function, we analyzed the mRNA expression of several target genes of miR-1 (Fig. 4). Impairment of endothelial-dependent relaxation in PA transfected with miR-1 was associated with a reduction in the expression of the genes encoding for connexin43 (Fig. 4a, $P < 0.001$), caveolin-2 (Fig. 4b, $P < 0.01$), and Krüppel-like factor 4 (Fig. 4c, $P < 0.05$) in rat PASMCs transfected with miR-1 compared with scramble miR, analyzed by qRT-PCR and normalized by β-actin expression.

## Discussion

Herein, we show that endothelial dysfunction is a common pathophysiological feature for several in vitro and in vivo models of pulmonary hypertension. miR-1, which is upregulated in the lungs from rats with PAH induced by Su5416 plus hypoxia, induced endothelial dysfunction and increased the production of ROS in rat pulmonary arteries. Several miR-1 targets, which could be involved in impaired endothelial function, i.e., *Sod*1, *Cav-2*, *Gja1*, and *Klf4* were downregulated.

miR-1 has been found upregulated in the plasma from 12 idiopathic PAH patients [31] and in lungs from the Hyp/Su5416 model, which best represents class 1 pulmonary hypertension (i.e., PAH) [10, 30]. Despite this fact, other studies affirm that the levels of miR-1 are downregulated in the buffy coat of a cohort of 31 subjects with pulmonary hypertension including all classes of pulmonary hypertension (COPD, interstitial lung disease, obstructive lung disease, scleroderma, etc.) [41] and in lungs from mice exposed to hypoxia (class 3 PH) as well as cultured human PASMC exposed to hypoxia in vitro [35]. The heterogeneity of miR-1 expression levels is greatly contributed by the difference in the cell tissues analyzed and the different conditions applied in each study. Overall, the data indicate that miR-1 does not appear to be a reliable biomarker of the disease. It may be due to the fact that a single miRNA can regulate hundreds of genes or proteins and conversely multiple miRNAs can regulate one protein [7]. These variable levels of miR-1 also may contribute to explain the heterogeneity in the different forms of the pathology.

Endothelial dysfunction is an early key player in the pathophysiology of multiple cardiovascular diseases, including pulmonary hypertension [12]. A reduced endothelial-derived nitric oxide is a widely used biomarker of endothelial dysfunction [14]. Nitric oxide bioavailability can be classically evaluated by the relaxation induced by ACh, which stimulates the release of NO from the endothelium. Both the in vivo model (present results and [3]) as well as in vitro model of Hyp/Su5416 showed NO-dependent endothelial dysfunction. We found that miR-1 reduced the ACh-induced relaxation in rat PA, reproducing the characteristics of PAH in both humans and animal models. The fact that incubation of isolated pulmonary arteries in vitro with Su5416 plus hypoxia [20] also increases miR-1 suggests that the effect of miR-1 is local. Regarding the cellular source of miR-1, our experiments do not address specifically the cell type involved. However, the effects of miR-1 impact on both endothelial and smooth

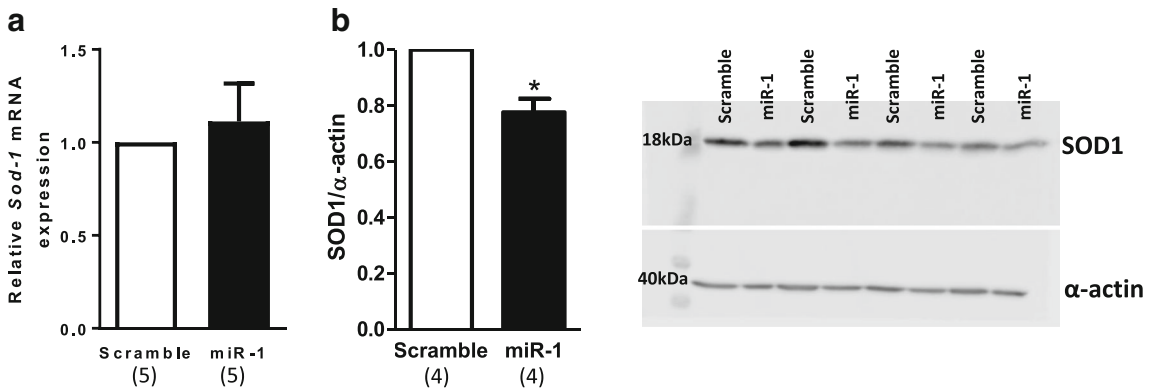


**Fig. 3** miR-1 targets superoxide dismutase 1 (SOD1). a mRNA expression by qRT-PCR *of Sod1* in rat PASMCs transfected with miR-1 or scramble miR (negative control). **b** SOD1 protein expression in rat PASMCs transfected with miR-1 or scramble miR analyzed by western blot and normalized by α-actin expression. Results are means ± S.E.M. The parentheses indicate the number of cell cultures used for analysis. *$P < 0.05$

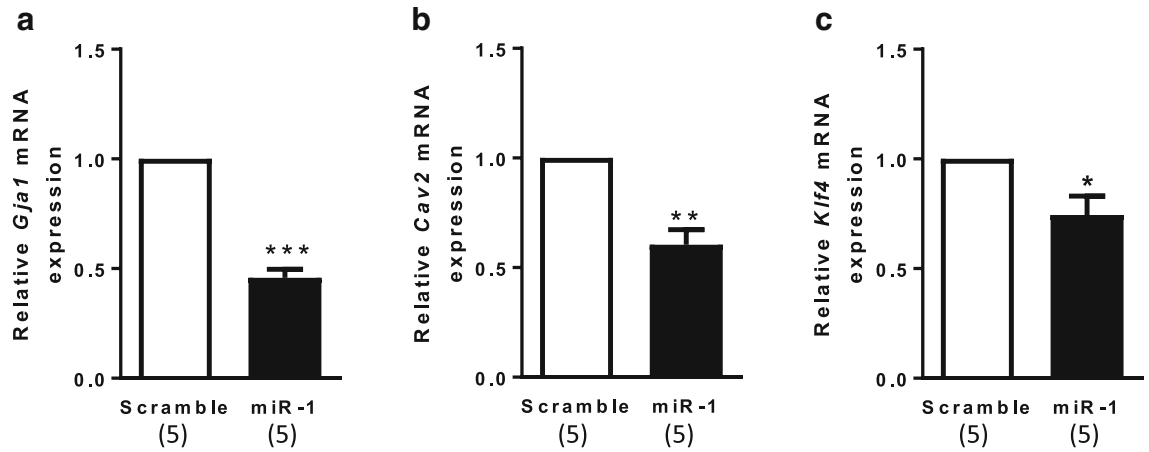


**Fig. 4** Changes in miR-1-targeted genes that encode connexin 43 (Gja1), caveolin-2 (Cav2), and Krüppel-like factor 4 (Klf4). mRNA expression by qRT-PCR of **a** *Gja1*, **b** *Cav2*, and **c** *Klf4* in rat PASMCs transfected with miR-1 or scramble miR (negative control). Results are means ± S.E.M. The parentheses indicate the number of cell cultures used for the analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control

muscle cells. Our functional studies clearly indicate that endothelial-dependent vasodilation is affected by miR-1.

NO inactivation by ROS, mainly superoxide, is a widely recognized mechanism for endothelial dysfunction [36]. We found increased production of superoxide by miR-1, suggesting that endothelial-derived NO is inactivated by superoxide. In fact, transgenic mouse overexpressing miR-1 show increased ROS levels which are likely to be due to a reduced superoxide metabolism by a miR-1 induced post-transcriptional repression of *Sod1* [40]. In line with this, SOD1-deficient mice exhibit PAH [27]. We found that *Sod1* mRNA was unchanged but SOD1 protein was reduced in PASMC transfected with miR-1. This suggests that the miR-1 interaction with 3′-UTR mRNA of *Sod1* results in reduced transduction but not mRNA

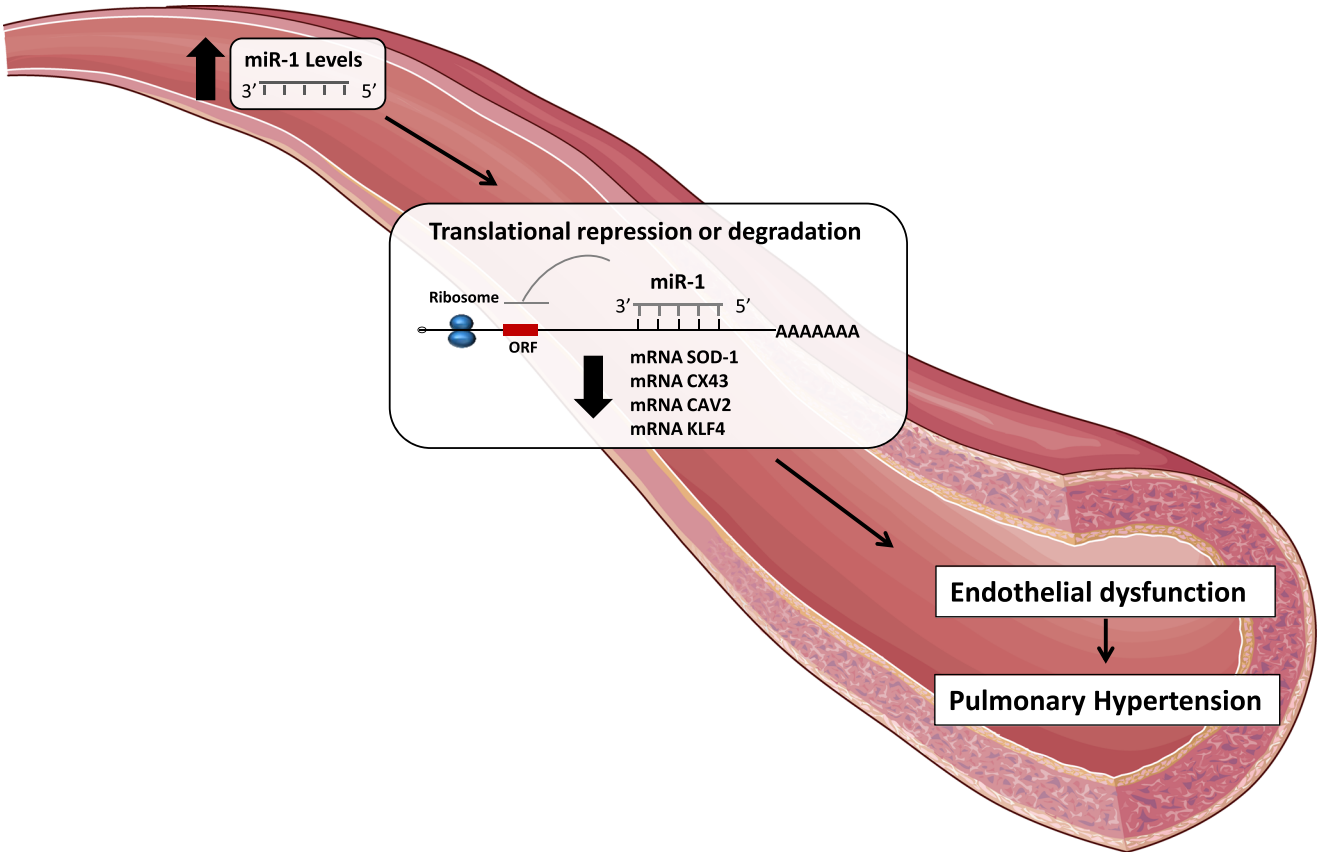


**Fig. 5** miR-1 upregulation in pulmonary arteries causes translational repression or degradation of SOD1, Cx43, CAV2, and KLF4 mRNA leading to endothelial dysfunction

degradation. The fact that the 3′-UTR sequence of SOD1 is a direct target of miR-1 has been already demonstrated by luciferase reporter assays in HEK293 cells [40].

Connexin43 is a gap junction protein necessary for the endothelial cell to cell communication which is another target of miR-1 [15]. It exhibits reduced expression in PAH patients resulting in altered gap junctional communication, barrier function, and angiogenesis [38]. We also confirmed that miR-1 reduced *Gja1*, the gene encoding Connexin43 expression at the mRNA level. Other miR-1 targets which may be interesting in the context of pulmonary vascular dysfunction include caveolin-2 and Klf4. We found that the expression of both genes was reduced by miR-1 in PA. Caveolin-2 is a membrane protein which co-expresses and heterooligomerizes with caveolin-1. Decreased caveolin-2 expression is found in the Hyp/Su5416 model of PAH [1]. Klf4 is a transcription factor expressed in the vascular endothelium, where it promotes anti-inflammatory and anticoagulant states, and increases endothelial nitric oxide synthase expression. Its expression was reduced in lungs from patients with PAH, and its loss exacerbated pulmonary hypertension in mice [34]. Therefore, we identified multiple miR-1 targets potentially involved in endothelial dysfunction. Another study in the context of erectile dysfunction supports the fact that the upregulation of miR-1 in the corpus cavernosum of rats produces endothelial dysfunction by downregulating the eNOS/NO/PKG and the PGE1/PKA pathways [25]. In contrast, other studies have shown that decreased miR-1 levels cause endothelial dysfunction by increased endothelin-1 (ET-1) mRNA in the case of diabetes [6] or by increased endothelial permeability in an atherosclerosis mice model [39].

In recent years, a large number of studies have proposed the potential use of miRNA mimics or antagonists (antagomirs) as therapeutic strategies in multiple conditions, including respiratory diseases [21]. Importantly, mimics and antagomirs can be formulated for inhaled administration, which may represent a more selective way to target the lung in respiratory diseases [4, 24, 26]. The identification of miR-1 as a potential factor contributing to key pathophysiological mechanisms of PAH such as endothelial dysfunction (present study) and ionic remodelling [20] suggests that antagonizing miR-1 (e.g., with antagomirs) may be a useful strategy for the treatment of PAH.

In conclusion, our data indicate that miR-1 induces endothelial dysfunction, increased production of ROS and downregulated *Sod1*, *Cav2*, and *Gja1* and *Klf4* expression. As depicted in Fig. 5, all these data suggest that miR-1 upregulation may play a pathophysiological role, at least in some forms of PAH.

**Author contributions** GMP, MC, BB, DMC, and SE performed and analyzed the experiments. GMP and MC drafted the manuscript. AC and FPV conceived the study and designed the experiments. FPV wrote the manuscript with significant conceptual contributions from GMP, MC, LM, MF, and AC.

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## Compliance with ethical standards

All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-251/15). All investigators understand the ethical principles.

**Conflict of interest** The authors declare that they have no conflict of interest.

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