miR-223 reverses experimental pulmonary arterial hypertension

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PULMONARY ARTERIAL HYPERTENSION (PAH) is a serious condition characterized by obstruction of the precapillary pulmonary arterioles (PA) due to excessive vasoconstriction, inflammation, and imbalance between cells’ proliferation and apoptosis within the arterial wall. This remodeling and obstruction of the PAs leads to increases in pulmonary vascular resistance, right ventricular (RV) failure, and death of patients. Despite recent therapeutic advances, most patients exhibit persistently poor exercise capacity and quality of life, with a 3-yr survival rate of 65% (12).

Growing evidence underlines the importance of DNA damage in PAH etiology (9, 19, 23). Indeed, the sustained inflammation, RAGE expression (21), and metabolic stress (30) observed in PAH result in DNA damage-dependent activation of poly(ADP-ribose)polymerase 1 (PARP-1) in PA smooth muscle cells (PASMCs) of PAH patients (23). We recently showed that PARP-1 overexpression was responsible for enhanced PAH-PASMC proliferation and suppressed apoptosis (23). Nevertheless, the mechanisms that regulate this overexpression in PAH remain elusive.

In cancer, PARP-1 expression is regulated by microRNA-223 (miR-223), a microRNA involved in DNA damage response (34). According to TargetScan 6.2, PARP-1 is in fact a predicted target of miR-223. Moreover, downregulation of miR-223 has been shown to mediate mechanical stretch-stimulated proliferation of vascular smooth muscle cells (33). Interestingly, Wang et al. (38) reported that miR-223 knockout mice spontaneously develop inflammatory lung pathology with increased inflammatory cell infiltration (14) as seen in PAH (4, 24). Moreover, miR-223 downregulation in macrophages activates the signal transducer and activator of transcription 3 (STAT3) (6), another critical transcription factor implicated in inflammation and PAH physiopathology (27, 28). Hence, miR-223 downregulation is associated with proliferative and inflammatory disorders (11), both crucial pathomechanisms of PAH development (20). We thus hypothesized that decreased miR-223 expression in PAH leads to PARP-1 upregulation promoting the proliferation/apoptosis imbalance of PAH-PASMC and thus triggers PAH development.

Using a multidisciplinary and translational approach, we demonstrated in human PAH lungs, distal PAs, and PASMC that miR-223 is downregulated. This downregulation accounts for PARP-1 upregulation, promoting PAH-PASMC proliferation and resistance to apoptosis. Finally, in vivo, ectopic upregulation of miR-223 using nebulized miR-223 mimics improves monocrotaline-induced (MCT) PAH in rats.1

1 This article is the topic of an Editorial Focus by Kimberly A. Smith, Jason X.-J. Yuan, and Paul T. Schumacker (32a).
Table 1. Clinical characteristics of PAH patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 13)</th>
<th>PAH (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [female (%)]</td>
<td>8 (61.5%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Age, yr</td>
<td>46 ± 17</td>
<td>51 ± 18</td>
</tr>
<tr>
<td>Subclass of PAH [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPAH</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>HPAH</td>
<td>2 (20%)</td>
<td></td>
</tr>
<tr>
<td>APAH</td>
<td>5 (50%)</td>
<td></td>
</tr>
<tr>
<td>Functional class [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3 (30%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>7 (70%)</td>
<td></td>
</tr>
<tr>
<td>mPAP, mmHg</td>
<td>47 ± 5</td>
<td></td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>11 ± 6</td>
<td></td>
</tr>
<tr>
<td>PVR, dyn·s/cm²</td>
<td>777 ± 225</td>
<td></td>
</tr>
<tr>
<td>CO, l/min/m²</td>
<td>4.2 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>SvO₂, %</td>
<td>2.4 ± 0.5</td>
<td>55 ± 10</td>
</tr>
<tr>
<td>6MWD, m</td>
<td>248 ± 87</td>
<td></td>
</tr>
<tr>
<td>Medication [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin receptor antagonist</td>
<td>5 (50%)</td>
<td></td>
</tr>
<tr>
<td>PDE5 inhibitor</td>
<td>6 (60%)</td>
<td></td>
</tr>
<tr>
<td>Epoprostenol</td>
<td>1 (10%)</td>
<td></td>
</tr>
<tr>
<td>Medication not known</td>
<td>2 (20%)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. PAH, pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; HPAH, heritable pulmonary arterial hypertension; APAH, associated pulmonary arterial hypertension; mPAP, mean pulmonary arterial pressure; RAP, right atrial pressure; PVR, pulmonary vascular resistance; CO, cardiac output; CI, cardiac index; SvO₂, venous oxygen saturation; 6MWD, 6-min walk distance; PDE5, phosphodiesterase-5.

Note that some patients took more than one type of medication.

**EXPERIMENTAL PROCEDURES**

All experiments were performed in accordance with the ethical standards of Laval University and were approved by the IUCPQ Biosafety and Ethics Committee (CER 20773).

**Human tissue samples.** Tissues were obtained from patients who had previously given signed informed consent. Small PAs (<1,000 µm diameter) were freshly dissected from explanted PAH lungs (n = 8), and non-PAH lung tissues (controls) were obtained during lung resection for tumors from the noncancerous segments (n = 8) as listed in Table 1.

**Cell culture and treatments.** PAH-PASMCs were isolated from PAs with a diameter smaller than 1,000 µm from 5 PAH patients (2 idiopathic PAH, 1 PAH associated with scleroderma, and 2 heritable PAH) all included in Table 1. Diagnosis was confirmed according to current guidelines and all patients exhibited a mean pulmonary arterial pressure (mPAP) ≥25 mmHg and a pulmonary artery wedge pressure ≥15 mmHg. Control PASMCs (n = 5 cell lines) were purchased from Cell Application (San Diego, CA). All PASMCs were used from third to sixth passage. PASMCs were grown in high-glucose DMEM supplemented with 10% FBS (Sigma) and 1% antibiotic/antimicrobial (Life Technologies). The implication of miR-223 was assessed using miRDIAN miR-223 mimic (200 nM for 48 h) (Thermo Fisher Scientific). For each experiment, we used a proper control (mimics or hairpin inhibitor negative control from Thermo Fisher Scientific). To modulate hypoxia-inducible factor 1α (HIF-1α), PASMCs were transfected either with siRNAs (from AMBION) at a final concentration of 20 nM with calcium chloride, or with an adenovirus encoding for CA5 (AdCA5), a constitutively active mutant form of HIF-1α (gift from Dr. E. D. Michelakis) [10 plaque-forming units (PFU)/cell], hereinafter named AdHIF1. The AdHIF1 carries a mutated form of HIF-1α (CA5), which is resistant to proline hydroxylation and protosomal degradation under normoxic conditions, thus allowing its stabilization [15]. A siRNA negative control (scrambled siRNA - siSCRN) and an adenovirus encoding LacZ were respectively used as negative controls for these experiments.

**Proliferation and apoptosis measurements.** To study the effect of miR-223 on PASMC proliferation and apoptosis in vitro, cultured human PAH-PASMCs were exposed to 10% FBS (proliferation) or 0.1% FBS (starvation, which is known to promote apoptosis) as previously described (1). Proliferation was measured by manual counting of proliferative cells after immunofluorescence staining using Ki67 antibody (Millipore, 1:400). Apoptotic cells were quantified by immunofluorescence using ApoAlert Annexin V-FITC Apoptosis kit (Clontech) and Apoptag apoptosis detection kit (TUNEL, Millipore). Results are expressed in percent (%) calculated as follows: (number of positive cells divided by the number of DAPI-positive cells) × 100. The percentage of positive PASMCs for TUNEL or Ki67 was determined by dividing the number of cells showing nuclear staining over the total number of cells (assessed with DAPI). All experiments were at least performed in triplicate in three different cell lines.

**Quantitative RT-PCR and immunoblotting.** Total RNAs (miRNAs and mRNAs) and protein were extracted from PAH or control PASMCs and lung samples, as previously described (23). To measure miR-223 expression, stem-loop qRT-PCR for mature miRNAs was performed on a real-time PCR system (Applied Biosystems). miRNAs were reverse transcribed and amplified using probes and reagents from Applied Biosystems for genomic analyses. 18S and U6 were used as housekeeping genes for miRNA and mRNA studies, respectively (Applied Biosystems). We quantified protein expression of PARP-1 by immunoblot using a rabbit anti-PARP-1 (Cell Signaling, 1:1,000). The signal was detected using the ChemiDoc system (Bio-Rad) and normalized to protein content using amidoblack stain. All experiments were at least performed in triplicate.

**Immunofluorescence.** Human PASMCs and rat lung sections (5 µm thick) were used for immunofluorescence staining. PASMCs were fixed with 1% formaldehyde and permeabilized with 0.2% Triton X-100. Rat lungs were fixed with 4% formaldehyde. In lung samples, only small and distal PAs (smaller than 100 µm) were investigated. Phospho-histone H2A.X (γ-H2AX) (Cell Signaling, 1:250), Ki67 (Millipore, 1:400), and smooth muscle actin (Sigma, 1:250) were used as secondary antibodies, and Alexa Fluor 488 and 594 were used as primary antibodies, and Alexa Fluor 488 and 594 were used as secondary antibodies. γ-H2AX is a DNA damage marker and Ki67 marks proliferative cells. Histology measurements were performed as previously described (7, 27). Briefly, PA wall thickness was measured using 2 measurements/artery in 10 arteries/animal in at least 5 animals per group.

**Animal model and hemodynamic measurements.** Male Sprague-Dawley rats (250–300 g) received a single subcutaneous injection of monocrotaline at 60 mg/kg (Sigma Aldrich) at day 0 to induce pulmonary hypertension (MCT-PAH). Clinical trial standards were used: rats were randomly distributed between groups, and all methods and analyses were performed blinded to the condition. There was a minimum of 10 rats per group. PAH progression was monitored every week by noninvasive two-dimensional Doppler echocardiography. In this curative study, rats were randomly selected to receive either intratracheal nebulization of mimic-223 (Life Technologies, 1 nmol in 50 µl of sterile water) or its negative control as previously described (7) on day 15, when PAH signs have already appeared on echocardiography. A second dose was administered on day 21. Before death (day 28), all rats underwent a comprehensive pressure-volume evaluation by closed chest right heart catheterization (Sciscens Catheters). Briefly, rats were anesthetized with 3–4% isoflurane induction and maintained with 2% during procedures. A high-fidelity catheter [1.9F PV-6.0 mm Elec Spacing (no. FTH-1912B-6018), Transonic Science Instruments] allowing measurement of instantaneous RV pressure and volume was advanced through the jugular vein, and measurements...
miR-223 expression is decreased in PAH

A: miR-223 expression is decreased in PAH

B: HIF-1α triggers a decrease in miR-223 expression

C: miR-223 regulates PARP-1 expression

Fig. 1. Hypoxia-inducible factor 1α (HIF-1α) triggers miR-223 downregulation in pulmonary arterial hypertension (PAH), which leads to poly(ADP-ribose)polymerase 1 (PARP-1) overexpression. A: miR-223 expression is decreased (qRT-PCR) in human lungs (n = 8 per group), distal pulmonary arteries (PAs) (n = 5 control and n = 6 PAH), as well as in isolated human pulmonary artery smooth muscle cells (PASMCs) (n = 5 per group). B: HIF-1α overexpression using AdHIF1 [10 plaque-forming units (PFU)/cell, 48 h] triggers a decrease in miR-223 expression measured by qRT-PCR in control cells (n = 4), and HIF-1α inhibition (siHIF-1α, 20 nM, 48 h) in PAH-PASMC increases miR-223 expression (n = 5). C: miR-223 targets PARP-1 expression as artificial decrease of miR-223 (AntagomiR-223, 200 nM, 48 h) in control (Ctrl) cells (n = 3) leads to increased PARP-1 expression. Conversely, restoring miR-223 expression in PAH-PASMC using a miR-223 mimic (mimic-223, 200 nM, 48 h) decreases PARP-1 expression at the protein and mRNA levels. *P < 0.05, **P < 0.01, and ****P < 0.0001.
A miR-223 regulates DNA-damage sensitivity

Control | PAH | PAH+mimic-neg | PAH+mimic-223

γ-H2AX in red and DAPI in blue (20x)

% γ-H2AX positive cells

Control | PAH | PAH+mimic-neg | PAH+mimic-223

** B miR-223 regulates the proliferation/apoptosis imbalance in PAH-PASMCs

Proliferation

Control | Control+AntagomiR-Ctrl | Control+AntagomiR-223

PAH | PAH+mimic-Ctrl | PAH+mimic-223

Ki67 in red and DAPI in blue (10x)

% Ki67 positive cells

Control | Control+AntagomiR-Ctrl | Control+AntagomiR-223 | PAH | PAH+mimic-Ctrl | PAH+mimic-223

Apoptosis

Control | Control+AntagomiR-Ctrl | Control+AntagomiR-223

PAH | PAH+mimic-Ctrl | PAH+mimic-223

AnnexinV in green and DAPI in blue (10x)

% AnnexinV positive cells

Fig. 2. miR-223 regulates DNA damage sensitivity and controls cell proliferation and apoptosis. A: using γ-H2AX immunofluorescence staining, we measured DNA damage after ectopic increase of miR-223 (miR-223 mimic, 200 nM, 48 h) in PAH-PASMC (n = 5) and showed that there is more DNA damage after miR-223 restoration, suggesting that cells may stop proliferating and undergo apoptosis. Scale bar, 10 μm. B: using a bidirectional approach, we assessed proliferation (Ki67) and apoptosis (AnnexinV) of PAH-PASMC after miR-223 modulation. By mimicking the disease in control cells (AntagomiR-223 treatment, 200 nM, 48 h), we showed that decreased miR-223 expression triggers proliferation and resistance to apoptosis. On the other hand, reversing this PAH phenotype is possible using a miR-223 mimic (200 nM, 48 h). Scale bars, 20 μm. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
were recorded continuously using LabScribe2 software (iWorks System), as this technique is the gold standard in the diagnosis and evaluation of PAH in humans. We measured mPAP, RV systolic pressure, and RV cardiac output (CO). Total pulmonary resistance was calculated by dividing mPAP by the CO. Right ventricle hypertrophy was assessed post mortem as the weight ratio of the right ventricle free wall to the left ventricle plus septum. Histology measurements were performed as previously described (23). Briefly, PA wall thickness was measured in 10 arteries/animal in at least 10 animals per group using hematoxylin and eosin (H&E) staining on lung sections. PA wall thickness was calculated by the percentage of the PA wall area on total artery area.

Statistics. Data are presented in fold changes or as means ± SE. For comparison of two means, we used an unpaired t-test. For comparison between more than two means, we used an one-way ANOVA followed by a Holm-Sidak multiple comparisons test. In cultured cell-based experiments, n indicates number of PAH patients or cell lines. In the in vivo studies, n indicates number of animals. Significance was set as P < 0.05.

RESULTS

miR-223 expression is decreased in human PAH. To assess the implication of miR-223 in PAH, miR-223 expression was measured in lungs of 10 PAH and 8 control patients. A 2.7-fold decrease in miR-223 was observed in PAH lung tissue as compared with controls (Fig. 1A). Within the lungs, miR-223 expression was decreased in the PAH distal PA, as assessed by qRT-PCR in isolated arteries from six PAH patients and five controls (Fig. 1A). This finding was confirmed by measuring miR-223 levels in PAH-PASMC isolated from distal PA of five PAH patients and five control patients. A five-fold decrease in miR-223 levels was observed between PAH-PASMC and control cells (Fig. 1A).

Normoxic activation of HIF-1α represses miR-223 expression in human PAH. Despite lack of hypoxia, HIF-1α is activated in PAH-PASMC (2). HIF-1 is a critical transcription factor implicated in the downregulation of many miRNAs (5). Thus, using a gain and loss of function approach, we determined whether HIF-1α expression is accountable for decreased miR-223 expression. Interestingly, miR-223 expression was increased in PAH-PASMC after HIF-1α inhibition using silencing RNAs (siHIF-1α, 20 nM for 48 h) (qRT-PCR, n = 5) (Fig. 1B). To pinpoint the exact effect of HIF-1α on miR-223 expression, control PASMC were transfected with an AdHIF1α, avoiding any confounding effects of hypoxia, as previously described (15, 35). We demonstrated that ectopic overexpression of HIF-1α alone represses miR-223 expression in PASMC (n = 5) (Fig. 1B).

miR-223 downregulation promotes PARP-1 expression in human PAH-PASMC. We and others highlighted the role of DNA damage signaling and PARP-1 activation in PAH lung vascular cells (9, 19, 23), but the exact mechanism underlying this deregulation remains unknown. In silico analysis using TargetScan 6.2 revealed that PARP-1 is a predicted target of miR-223. Thus, to evaluate the implication of miR-223 in the regulation of PARP-1 expression in PAH, PARP-1 expression was measured in PAH-PASMC and control PASMC in the presence or absence of miR-223 mimic or antagonomiR, respectively. As expected, PAH-PASMC had a significant increase in PARP-1 mRNA and protein levels as compared with control cells (Fig. 1C). Moreover, when we restored miR-223 levels in PAH-PASMC using a miR-223 mimic (200 nM for 48 h), we decreased mRNA and protein levels of PARP-1 (Fig. 1C), suggesting that miR-223 downregulation accounts for PARP-1 overexpression in PAH-PASMC. Indeed, in control cells treated with miR-223 antagonomiR, PARP-1 expression was increased (Fig. 1C).

Moreover, PARP-1 is a well-known player in the DNA repair machinery and its sustained expression and activation contributes to cell survival and proliferation. PAH-PASMCs treated with miR-223 mimic, which decreased PARP-1 expression (Fig. 1C), exhibit a significant increase in DNA damage compared with both untreated and mimic control-treated PAH-PASMC (Fig. 2A). This confirms again the impact of miR-223 on PARP-1 and on the DNA repair machinery. This arrest in DNA repair (by inhibiting PARP-1) in PAH-PASMC will enhance DNA damage, thus the cell will react to overload of DNA damage by stopping proliferation and triggering apoptosis.

miR-223 promotes proliferation and resistance to apoptosis in PAH-PASMCs. To study the effect of miR-223 on PASMC proliferation and apoptosis in vitro, cultured human PAH-PASMCs were exposed to 10% FBS (a condition known to promote proliferation) or 0.1% FBS (a “starvation” condition that promotes apoptosis). As expected, PAH-PASMC exhibited increased proliferation, as well as less apoptosis as com-

Fig. 3. In vivo, ectopic delivery of miR-223 improves survival in rats with monocrotaline-induced PAH. A: a miR-223 mimic was delivered to the lungs of monocrotaline-induced PAH (MCT-PAH) and showed an increase in miR-223 expression (n = 10 per group). Analyses (qRT-PCR) were performed at the end of the protocol (after the 2 wk of mimic administration). B: this increase in miR-223 expression led to decreased mortality in nebulized rats. *P < 0.05 and **P < 0.01.
pared with control cells (Fig. 2B). Using a gain and loss of function approach, control cells were treated with miR-223 antagomiR (200 nM for 48 h) to assess whether decreased miR-223 levels alone can trigger the PAH-related deregulation in proliferation and apoptosis. On the other hand, a therapeutic approach was used on PAH-PASMCs using a miR-223 mimic (200 nM, 48 h) to measure the impact of restoring miR-223 levels in PAH-PASMC phenotype. Our results showed that control cells \((n = 5)\) treated with a miR-223 antagomiR doubled their proliferation rate as compared with cells treated with a random antagomiR (AntagomiR-Ctrl) (Fig. 2B). They also exhibited resistance to starvation-induced apoptosis (Fig. 2B). On the other hand, PAH-PASMCs \((n = 5)\) treated with miR-223 mimic showed less proliferation level and higher apoptosis rates (Fig. 2B). Following miR-223 mimic treatment, the proliferation and apoptosis rates of PAH cells were comparable to the ones found in non-PAH cells, demonstrating a potential therapeutic impact of miR-223 level restoration in diseased cells.

**Ectopic delivery of miR-223 mimic reverses experimental PAH.** To test whether the reestablishment of miR-223 level can improve symptoms of PAH in a rat model of the disease, synthetic miR-223 RNA molecules were selectively delivered to the lungs of MCT-PAH rats. Administration of miR-223 mimic by intratracheal nebulization was performed on \(\text{day 14}\) (when PAH is established) and \(\text{day 21}\) post-MCT injection. To verify the efficiency of our delivery method, we confirmed that mimic-223 administration by nebulization increases miR-223 expression in the lungs (Fig. 3A). We observed that the local delivery of synthetic miR-223 in MCT-PAH rats improves overall survival compared with MCT-PAH rats nebulized with the random miRNA (mimic-Ctrl) (Fig. 3B). Ectopic delivery of miR-223 mimic was also associated with a reduction in mPAP as well as with RV hypertrophy of MCT-PAH rats.

![A](image1.png) **miR-223 mimic administration improves hemodynamic measurements in MCT-PAH**

- **Mean PA pressure**
  - mPAP (mmHg) vs. day
  - Control
  - MCT-PAH
  - MCT-PAH + mimic-Ctrl
  - MCT-PAH + mimic-223

- **RV systolic pressure**
  - RSVP (mmHg) vs. day
  - Control
  - MCT-PAH
  - MCT-PAH + mimic-Ctrl
  - MCT-PAH + mimic-223

- **Cardiac output**
  - CO (mL/min) vs. day
  - Control
  - MCT-PAH
  - MCT-PAH + mimic-Ctrl
  - MCT-PAH + mimic-223

- **Total pulmonary resistance**
  - TPR (mmHg mL/min) vs. day
  - Control
  - MCT-PAH
  - MCT-PAH + mimic-Ctrl
  - MCT-PAH + mimic-223

![B](image2.png) **miR-223 decreases RV hypertrophy of MCT-PAH rats**

- **RV/LV+S ratio**
  - RV/LV+S ratio vs. day
  - Control
  - MCT-PAH
  - MCT-PAH + mimic-Ctrl
  - MCT-PAH + mimic-223
**A** miR-223 mimic administration decreases vascular remodeling in MCT-PAH

Fig. 5. Restoring miR-223 levels decreases lung vascular remodeling. A: we measured vascular remodeling by calculating the percentage of the PA wall area on total artery area in 10 arteries per animal in 10 to 13 animals per group. We also measured the amount (in %) of partially muscularized and fully muscularized arteries \( n = 10 \) to 13). miR-223 treatment decreased mean vascular remodeling and increased the amount of nonmuscularized arteries compared with rats treated with the random mimic (mimic-Ctrl). Scale bar, 40 μm. B: a decrease in proliferation (Ki67) and increase in apoptosis (TUNEL) are accountable for the decrease in vascular remodeling upon miR-223 mimic treatment. White arrows in the images are pointing out positive cells. Results are expressed in percent (%) calculated as follow: (number of positive cells within the artery divided by number of DAPI-positive cells) × 100, in 10 arteries per rats in 10 to 13 rats per group. Scale bars, 40 μm. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), and ****\( P < 0.0001 \).
systolic pressure when compared with MCT-PAH rats treated with nonspecific synthetic RNA molecules (Fig. 4A). These effects were associated with a significant increase in cardiac output and a reduction of total pulmonary resistance (Fig. 4A), confirming the therapeutic potential of restoring miR-223 levels in PAH. Beneficial effects were also found in the RV, as the hypertrophy was decreased upon mimic-223 treatment (n = at least 10 per group) (Fig. 4B). To determine whether the reduced vascular remodeling was accountable for the improvement in hemodynamic parameters allowed by miR-223 delivery, we measured PA medial wall thickness after an H&E stain. We observed that animals treated with the synthetic miR-223 displayed a significant reduction in medial thickness of small and distal PAs (Fig. 5A). Furthermore, rats that received synthetic miR-223 had decreased muscularization of their distal PAs and fewer were fully occluded. We also observed an increase in low-resistance nonmuscularized vessels as compared with MCT and random miRNA (mimic-Ctrl)-treated rats (Fig. 5A). The decrease in vascular remodeling in rats treated with synthetic miR-223 was due to a decrease in proliferation and increase in apoptosis levels within the vessel wall, as assessed by Ki67 and TUNEL, respectively (Fig. 5B).

DISCUSSION

We provide extensive evidences linking the abnormal expression of miR-223 to already known pathophysiological processes in PAH, including HIF-1α activation (2, 32), impaired DNA damage signaling (9, 19), and PARP-1 overexpression (23), as well as the proliferation/apoptosis imbalance of PAH-PASMC (37). Thus, our study not only demonstrates the importance of miRNAs especially miR-223 in PAH but also, on the basis of our in vivo data, suggests that it might represent a new therapeutic target for human PAH.

We demonstrated that the normoxic activation of HIF-1α induces miR-223 downregulation (Fig. 1B) and subsequent PARP-1/DNA damage signaling exacerbation (Figs. 1C and 2A), establishing a new link between these two crucial elements in PAH physiopathology. We demonstrated that restoring miR-223 in PAH-PASMCs decreased PARP-1 expression and thus DNA damage repair, promoting PAH-PASMC apoptosis and decreasing their proliferation (Fig. 2). In vivo, we showed that MCT-PAH rats treated with ectopic increase of miR-223 levels in their lungs exhibited decreased mortality (Fig. 3B), improvement in pulmonary hemodynamic parameters, as well as decreased RV hypertrophy and vascular remodeling (Figs. 4 and 5).

In the current study, we propose for the first time that HIF-1α implication in PAH is not exclusively directly related to apoptosis and proliferation as previously thought (2, 32). Indeed, we provide evidence that HIF-1α activation accounts for miR-223 downregulation, leading to PARP-1 overexpression in PAH-PASMC and thus to their proliferation and resistance to apoptosis. We recently demonstrated that PARP-1 promotes PAH-PASMC proliferation and resistance to apoptosis by decreasing miR-204, leading to a sustained activation of HIF-1α and nuclear factor of activated T cells (NFAT) (23). Therefore, these mechanisms were not explored again in the present study. Although this remains to be fully established, the implication of HIF-1α at two different levels of the proposed signaling cascade (upstream of miR-223 and downstream of PARP-1) suggests the existence of a feedforward mechanisms maintaining HIF-1α expression and subsequent decreased miR-223 level through PARP-1 (Fig. 6).

Although several other miRNAs are aberrantly expressed in PAH-PASMC, we focused our study on miR-223. The literature on miR-223 has focused on its role in cancer, hematopoiesis, and inflammatory diseases. Our study is thus the first exploring its role in vascular diseases associated with pulmonary vascular remodeling. Nonetheless, findings in other diseases support a role for miR-223 in vascular diseases like PAH.
For instance, it has been shown that miR-223 downregulation triggers the signal transducer and activator of transcription 3 (STAT3) activation (6), a critical transcription factor implicated in cell proliferation (28). In addition, in esophageal cancer cells, PARP-1 was identified as a direct target gene of miR-223 (34). Indeed, in esophageal adenocarcinoma cells, decreased cancer growth was observed in cells with enforced miR-223 expression and subsequent reduced PARP-1. These findings are in accordance with ours and with the already known pathophysiological processes of PAH including STAT3 and PARP-1 activation, reinforcing the importance of miR-223 in the etiology of PAH.

Other downstream targets of miR-223 not explored in the current study might also contribute to PAH development including F-box and WD repeat domain-containing 7 (FBW7), insulin-like growth factor 1 receptor (IGF-1R), and heat shock protein 90β (HSP90β), as they all play a role in cell proliferation and apoptosis in cancer (13, 18, 38), which share many similarities with PAH (31). Wang et al. (38) demonstrated that miR-223 is a repressor of FBW7, which regulates Kruppel-like factor 5 (KLF5) expression. Thus, we could also speculate that miR-223 downregulation can also lead to increased KLF5 expression observed in PAH (8). Moreover, downstream miR-223 targets IGF-1R and HSP90β seem to act on proliferation through the phosphatidylinositols 3-kinase, Akt, and mammalian target of rapamycin (PI3K/Akt/mTOR) pathway (13, 18) known to play a role in PAH pathophysiology (10, 26, 36).

Even though inflammation was not studied in the present work, we cannot exclude a role for miR-223 in the inflammatory processes implicated in PAH, notably through the activation of the PI3K/Akt/mTOR pathway. Indeed, miR-223 was first described as a key player in regulating inflammation in many inflammation-related disorders including DNA damage (11, 34, 39). Thus, decreased expression in miR-223 in PAH might also play a role in the inflammatory processes by regulating hematopoietic cell differentiation and cytokine production (11), such as IL-6 (6), which also plays a critical role in PAH via STAT3 (29). Thus, miR-223 could be seen as an integrative signaling hub merging several critical pathways implicated in PAH such as inflammation, hypoxia, as well as cellular stress and damage. This is consistent with the “second-hit” theory for the pathogenesis of PAH, in which the response to an environmental or endogenous trigger is enhanced in susceptible individuals (25). This may explain why prevalence of PAH is the most frequent in patients with chronic inflammation like scleroderma.

In conclusion, we provide evidence that miR-223 downregulation in PAH is accountable for PARP-1 overexpression and altered DNA damage response observed in PAH-PASMC. Restoring the expression of this miRNA in lungs of PAH rats reversed established PAH and provided beneficial effects on vascular remodeling, pulmonary resistance, RV hypertrophy, and survival. In addition, as PAH shares many similarities with systemic vascular diseases including HIF-1, STAT3, Akt activation (3, 16, 17, 22) and enhanced DNA damage, our findings are a step further in the understanding of vascular remodeling etiology in general and in finding new therapeutic targets for these deadly diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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