Lipid nanoparticle delivery of a microRNA-145 inhibitor improves experimental pulmonary hypertension

Jared M. McLendon a,d,*,1, Sachindra R. Joshi a,d,1, Jeff Sparks e, Majed Matar e, Jason G. Fewell e, Kohtaro Abe d, Masahiko Oka c,d, Ivan F. McMurtry b,c,d, William T. Gerthoffer a,d

a Department of Biochemistry and Molecular Biology, University of South Alabama College of Medicine, Mobile, AL 36688, USA
b Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL 36688, USA
c Center for Lung Biology, University of South Alabama College of Medicine, Mobile, AL 36688, USA
d Department of Internal Medicine, University of South Alabama College of Medicine, Mobile, AL 36688, USA

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1. Introduction

Patients with pulmonary arterial hypertension (PAH) suffer from abnormally high pulmonary arterial blood pressure (pulmonary hypertension, PH) that leads to right ventricular dysfunction [1]. Normally thin-walled, highly compliant pulmonary arteries undergo wall thickening, become less compliant and more contractile [2–4]. This vascular remodeling in PAH is a result of multigenic mechanisms affecting multiple cell types including, smooth muscle hypertrophy, enhanced endothelial cell proliferation, decreased endothelial cell apoptosis, perivascular inflammation and altered progenitor cell differentiation. The pulmonary arteriopathy increases vascular resistance and pressure, which increases the workload on the right ventricle leading to dysfunction and relentless progression to right ventricular (RV) failure. Novel therapies for PAH should repair arteriopathy, reduce PH, and prevent RV failure.

One new class of anti-remodeling agents being tested is small oligonucleotides that exploit the RNA interference (RNAi) pathways [5]. Identifying suitable RNAi targets in PAH has relied on genetic
and biochemical studies of critical pathways [6] and expression surveys of miRNAs in animal models of PH [5]. MiRNAs are attractive targets because they are important epigenetic regulators of protein abundance, which defines both normal and abnormal cellular phenotypes and organ function. Thus, reprogramming miRNA regulators of vascular wall cell phenotype is appealing because epigenetic regulation by miRNAs is readily reversible, and maladaptive changes in miRNA expression can be modified with oligonucleotide mimics or inhibitors.

Several miRNAs that contribute to pulmonary vascular remodelling have been described in studies of clinical and experimental PAH. A survey of miRNA expressions in total lung extracts from rat and mouse models of chronic hypoxia-induced PH found downregulation of miR-21 in both [7]. Later, this group found upregulation of miR-145 in experimental and human PAH, and that miR-145 is necessary for muscularization of pulmonary arteries in mice exposed to chronic hypoxia [8]. Another study showed that miR-204 was downregulated in both experimental and human PAH. A miR-204 mimic delivered intratracheally reduced disease severity [9]. A later study of the miR-17 – 92 cluster showed that intravenous delivery of a miR-17 antagonist was an effective treatment of chronic hypoxia PH in mice and monocrotaline-induced PH in rats [10]. Additional studies of miR-20a [11], miR-21 [12,13], miR-328 [14], miR-424 and miR-503 [15] provide solid proof of principle that RNAi agents regulating miRNA expression can attenuate experimental PH by modifying expression of cell signaling pathways, contractile proteins, and regulators of the cell cycle. However, many of these RNAi therapies, including anti-miR-145, have not been tested for effective reversal of PAH or repair of the occlusive remodeling that occurs in rats with Sugen5416/hypoxia-induced PH. In addition, these prior studies have not addressed key challenges including delivery, dose optimization, and toxicity, which are necessary for translation of miRNA manipulation into effective RNAi based therapies.

RNAi-based therapies are often limited by ineffective delivery to the site of action, off-target effects, and the potential for renal, hepatic, and immune system toxicity. These limitations can be addressed by employing oligonucleotide delivery systems for targeting specific organs in vivo. Delivery systems that have advanced the most towards clinical validation include cationic lipid nanoparticles/LNPs and GalNAc-siRNA conjugates that have been shown to preferentially deliver oligonucleotides to the liver (see review [16]). However, a recently described liposomal system (Star:Star-mPEG) has been shown to direct the biological activity of systemically administered small interfering RNA (siRNA) to the lung. Specifically, intravenous delivery of siRNA formulated with these liposomal nanoparticles resulted in RNAi-mediated knockdown of several transcripts and proteins including caveolin-1, CD-31, and Tie-2 [17,18]. Although the precise mechanism of lung specific activity of the Star:Star-mPEG system has not been elucidated, it has been suggested that it is not the result of increased lung uptake of siRNAs from the blood, but rather a decreased clearance from lungs relative to other organs. Regardless, knockdown of proteins specifically in the lung and widely expressed by endothelial cells demonstrates effective delivery of siRNA to pulmonary vascular cells. These data suggest a strong potential for developing lung directed RNAi based therapies, but the application of Star:Star-mPEG mediated delivery of RNAi therapies for pulmonary vascular diseases such as PAH remains unknown.

A miR-145 antagonist was identified as an ideal candidate to test the hypothesis that Star:Star-mPEG mediated delivery of RNAi-based drug will be effective therapy against pulmonary vascular disease. The miR-145 – miR-145 cluster is a critically important regulator of multiple processes that control vascular wall structure and function [19]. Expression of miR-145 promotes vascular smooth muscle cell differentiation and a contractile smooth muscle cell phenotype by regulating expression of KLF4/5, myocardin and SRF-dependent, smooth muscle-restricted contractile proteins [19–21]. MiR-145 promotes vascular development [22–24], coronary collateral formation [25], stem cell and smooth muscle progenitor cell differentiation [21,26], and antagonizes vascular damage responses. In PAH, hypertensive pulmonary arteries undergo medial hypertrophy, are hypercontractile, and have increased expression of miR-145 and dysregulated expression of miR-145 target genes [10,30]. These studies collectively show that miR-145 is a key node in signaling pathways that regulate vascular wall structure and function in normal development and in cardiovascular disease. This reversal study used Star:Star-mPEG to deliver a highly selective antisense oligonucleotide against miR-145 in the Sugen5416/hypoxia rat model of severe PAH to test for delivery, toxicity and efficacy against occlusive arteriopathy. Some results have been previously reported in abstract form [27–29].

2. Materials and methods

2.1. Oligonucleotides

The locked nucleic acid (LNA)/DNA oligonucleotides used were modified with a phosphorothioate backbone. The nonsilencing control sequence was 5′-ACGTCTATACCGCCA-3′ (Exiqon, Inc., batch #609214). The anti-miR-145 sequence was 5′-CTCGGAAAACTGGA-3′ (Exiqon, Inc., batch #607391, Woburn, MA).

2.2. Liposome preparation and formulation

Star and Star-mPEG were synthesized and characterized as described previously [16,17]. To generate liposomes Star and Star-mPEG lipids were mixed in chloroform at (10:1) molar ratio. Following overnight evaporation the lipid film was rehydrated with water for injection and sonicated in a water bath sonicator for 30 min. Liposomes were further sonicated using a probe sonicator for 10–30 min producing liposomes with particle sizes of 40–60 nm. The liposomes and antimir-145 were diluted in 5% dextrose and mixed together in equal volumes to produce a formulation that was at 0.2 mg/mL antimir-145 concentration at a 20:1 N:P ratio (Nitrogen:Phosphate). The formulation was further concentrated to 0.8 mg/mL using Amicon ultra centrifugal filter units. This formulation procedure typically produced lipid nanoparticles of 80–100 nm in diameter with a zeta-potential of 30–50 mV.

2.3. Rat model of pulmonary arterial hypertension

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. Experimental severe PAH was induced in adult male Sprague–Dawley rats by the two-hit model of Sugen-5416 (20 mg/kg, SC) and chronic hypoxia (10% O2) as described previously [30,31]. Eight weeks after induction of PAH, rats were randomized into three treatment groups – 1) vehicle control (5% dextrose) (PAH), 2) miR-145 inhibitor (AntimiR-145), or 3) nonsilencing control oligonucleotide (Nonsilencing). The rats were treated at 8, 10, and 12 weeks by IV injection and subjected to serial echocardiography and blood collection for toxicity testing. At week 13, hemodynamic measurements were recorded by simultaneous catheterization of the right ventricle via the jugular vein [31] and of the left ventricle via the carotid artery [32] (Fig. 1). Detailed hemodynamic methods are described in supplement.

2.4. Morphometry

Detailed morphometry methods are described supplemental methods. Briefly, pulmonary arteries were stratified into three groups based on the size of the outer diameter (OD), 1) < 50 μm, 2) 50–100 μm, and 3) 100–200 μm. Outer diameter and wall thickness (media plus intima) were measured as described previously [33]. Vascular occlusion density (vessels/mm² tissue cross sectional area) was assessed in VVG-stained sections as described previously [34,35]. The number of animals/group was: Normoxia – 5, PAH – 7, AntimiR-145 – 9, Nonsilencing Control – 8. The number of vessels measured in each animal ranged from 118–240 with median = 149 vessels. The total number of
vessels/treatment group was: Normoxia — 831, PAH — 1102, AntimiR-145 — 909. The total cross-sectional area (mm²) of lung sections measured was: Normoxia — 357, PAH — 513, AntimiR-145 — 468, Nonsilencing Control — 421. The measurements and scoring of occlusive lesions were performed by an investigator blinded to treatments.

2.5. In situ hybridization, RNA isolation, RT-QPCR, and antimiR-145 quantification

Tissue processing, histology, and in situ hybridization assays were performed using standard procedures. Details are described in supplemental methods. RNA isolation, reverse transcription, and quantitative PCR were performed as described in supplemental methods. A sandwich hybridization assay was used to quantify antimiR-145 in tissue samples as described in the supplemental methods.

3. Results and discussion

Oligonucleotide based therapy has become an attractive therapeutic approach for PH. This study focuses on inhibition of miR-145 expression for three reasons. First, dynamic expression of miR-145 has a dominant role in regulating smooth muscle phenotype in vivo and in vitro. Second, expression of miR-145 is increased in clinical and experimental PAH. Third, chronic hypoxia induced PH in mice is prevented by genetic knockout of miR-145 or administration of a miR-145 inhibitor. Here we designed experiments to test for the therapeutic efficacy of miR-145 inhibition in a rat model of established, advanced PAH that develops severe pulmonary hypertension, due in part to widespread occlusive neointimal lesions, and more closely mimics the human disease pathology.

The Sugen5416/hypoxia rat model of PAH combines features of classic PH models (induced by hypoxia or monocrotaline) by eliciting vascular injury via endothelial damage followed by hypoxic vasoconstriction for 3 weeks. The severe pulmonary hypertension that is induced is sustained for at least 10 weeks after return to normoxia. Two important features of the Sugen5416/hypoxia rat model are the slow but continuous progression of arteriopathy and the progressive right heart remodeling and dysfunction that is similar to severe PAH in humans [31,34,36]. The progressive nature of PAH in the Sugen5416/hypoxia rat model is associated with increased perivascular inflammation and increasing density of obstructive vascular lesions similar to complex neointimal and plexiform lesions in humans. Consistent with previous reports in other experimental mouse and rat models [8,9], we used several approaches (microarray, qPCR, and ISH) to demonstrate increased expression of miR-145 in hypertensive pulmonary arteries from Sugen5416/hypoxia rats (data not shown). For these reasons we used the Sugen5416/hypoxia model to test the efficacy and toxicity of the miR-145 antagonist. Although no animal model recapitulates all features of human disease, we suggest, with the above limitations in mind, that the results are a valid initial preclinical efficacy and toxicity trial of a novel class of anti-remodeling therapy for PAH.

3.1. Lipid nanoparticle-mediated delivery of antimiR-145 accumulates active inhibitor in lung tissue

AntimiR-145 targeting the seed sequence of hsa-miR145-5p was formulated with Star:Star-mPEG lipid nanoparticles delivered IV at a dose of 2 mg antimiR-145/kg body weight. To achieve a therapeutic effect we administered 3 doses at 2-week intervals. We assumed that knockdown of target proteins would have a 2–4 day time to 50% inhibition and sustained inhibition for at least 10 days as previously reported using Star:Star-mPEG delivery of siRNA to mouse lung [17,18]. We also assumed the tissue half-life of antimiR-145 would be similar to tiny locked nucleic acids (LNA) in vivo, which is 4–6 days [37]. Therefore, dosing every 14 days should allow adequate time to alter expression of proteins but still achieve sufficient trough levels of antimiR-145 between doses to sustain antagonism of native miR-145. AntimiR-145 treated rats received doses at weeks 8, 10, and 12. Control groups received three injections of dextrose (PAH group) or three injections of a nonsilencing control oligonucleotide (Nonsilencing). Normal control
animals were exposed only to normoxia (21% O₂) for the entire 13-week protocol (Normal). At 13 weeks, heart and lungs were harvested en bloc and tissue processed for quantitative analysis.

Uptake of antimiR-145 oligonucleotide after the subchronic dosing protocol was verified in lung and heart tissues by using a hybridization assay. AntimiR-145 levels were significantly higher in lung tissue than in the right or left ventricle (Fig. 2A). To determine whether antimiR-145 treatment reduced tissue levels of native miR-145-5p, lung and heart levels were assayed by quantitative RT-PCR (Taqman miRNA assay). Retention of antimiR-145 in lungs significantly reduced lung levels of endogenous miR-145 at 13 weeks (Fig. 2B). Interestingly, miR-145 levels in heart tissue were also significantly lower in antimiR-145 treated animals (data not shown). These results show intravenous administration of Star:Star-mPEG lipid nanoparticles effectively delivered antimiR-145 to the lung, and to a lesser degree the heart, which reduced steady-state levels of endogenous miR-145.

AntimiR-145 distribution in lung tissue was determined using in situ hybridization (ISH) with probes specific for antimiR-145 sequence. Fig. 2C-a,b shows antimiR-145 in multiple cell types in the lung parenchyma and blood vessel walls demonstrating effective antimiR-145 delivery to rat lung. In contrast, animals that received three injections of dextrose showed no staining for antimiR-145 (Fig. 2C-c, d). Considering that ISH analysis was conducted one week after the last injection, and 6 weeks from the start of therapy, the significant accumulation of antimiR-145 in lung tissue shown here supports previous reports of increased siRNA retention in mouse lungs following Star:Star-mPEG mediated delivery. Altogether, these results show Star:Star-mPEG is a useful system to deliver oligonucleotide to the lung in rats.

Next we measured the distribution of antimiR-145 in extra pulmonary tissues using in situ hybridization with probes specific for the antimiR-145 sequence. Fig. 3 shows no staining for antimiR-145 in liver, spleen, and kidney from animals treated with dextrose (Fig. 3G, H and I, respectively). Surprisingly, rats treated with antimiR-145 complexed with Star:Star-mPEG show abundant accumulation of antimiR-145 in liver, spleen, and kidney (Fig. 3D, E, F, respectively). Serial sections stained with a scramble probe were negative (Fig. 3A–C) indicating appropriate staining conditions. The significant accumulation of antisense oligonucleotide in these non-pulmonary tissues was surprising given the previous reports in mice that indicated lung specific biological activity of systemically delivered siRNAs. There are several differences that might account for this discrepancy. First, in the previous studies, biodistribution and efficacy were assessed acutely after a single injection. Here the animals received three injections over a 5-week period, allowing more time for lung clearance and systemic organ accumulation. Although the dose of oligonucleotide delivered was identical (2 mg oligonucleotide per kg body weight) the total mass of oligonucleotide and concentration were substantially different (mouse, 40 μg oligonucleotide at a concentration of 0.2 mg/mL, and rat, 800 μg oligonucleotide at a concentration of 0.8 mg/mL, times 3 injections totaling 2400 μg oligonucleotide). Because this is the first study using this more complex dosing schedule, it is unclear how the change in oligonucleotide concentration, the mass of oligonucleotide administered, and the increased time might have affected the clearance rate of oligonucleotide from the lung and accumulation in kidney and liver. Second, in previous studies, the drug formulation was modified double stranded RNA complexed with Star:Star-mPEG. Here the animals received a single stranded antisense oligonucleotide with mixed LNA and DNA bases and a phosphorothioate backbone specifically designed to resist degradation in vivo. The chemical and physical properties of this modified oligonucleotide might reduce the clearance from liver, kidney, or spleen and explain the sustained distribution in these organs not observed in previous mouse studies. Third, in previous studies, the relative mass of siRNA retained as compared to unit tissue was increased in lungs over other tissues. An in situ hybridization approach in histological samples will not reflect this normalization difference and a quantitative approach is necessary.

3.2. No toxicity observed following antimiR-145 treatment

One of the most important impediments to translating novel RNAi-based therapies from animal models of disease to clinical use in humans is off-target effects on liver, kidney, and immune system functions.

![Fig. 3. Biodistribution of antimiR-145 in extrapulmonary organs.](image)
Uncomplexed locked-nucleic acid oligonucleotides can potentially cause liver damage [38], and lipid nanoparticles < 100 nm, similar to those used in this study, are avidly taken up by the liver [39]. Small oligonucleotides including miRNAs are detected by pathogen-associated molecular pattern receptors including toll-like receptors [40], which suggests potential for immunotoxicity. To assess the potential for subchronic toxicity of the antimiR-145/Star:Star-mPEG formulation, comprehensive metabolic panels were performed on blood samples collected at weeks 10 and 12 of the protocol. Complete blood counts with differential counts, serum electrolytes, metabolites, and proteins were measured in rats with PAH treated with vehicle, antimiR-145, or nonsilencing control oligonucleotide. The nonsilencing control group was also included to distinguish between sequence-specific effects of the antimiR-145 and effects of the lipid nanoparticles. Body weight, and spleen weight were not affected by antimiR-145 treatment (Fig. 4A) or nonsilencing control (Fig. 4A). Similarly, white blood cell count, serum electrolytes and metabolites, and marker proteins (Fig. 4B) were unaffected by antimiR-145 treatment. The nonsilencing control is not shown for the sake of clarity, but no values were different from mean values of PAH or antimiR-145 treated groups. Complete analyses of blood cells and analytes are shown in Supplementary Table 1. In spite of significant retention of oligonucleotides in these organs, there was no evidence of subchronic renal, hepatic, cardiac, metabolic, or hematologic toxicity of antimiR-145 or the Star:Star-mPEG lipid nanoparticle carrier after 5 weeks of therapy. The results confirm lack of obvious toxicity reported in previous studies of Star:Star-mPEG lipid nanoparticles in mice [17, 18]. Low toxicity combined with significant vascular anti-remodeling effects and significant improvement of RV structure and function suggest further translational development of antimiR-145 agents in treatment of PAH is warranted.

3.3. AntimiR-145 therapy repairs pulmonary arteriopathy

Developing an effective anti-remodeling therapy of PAH arteriopathy requires that established changes in arterial structure in late-stage PAH be reversible, or that further pathological remodeling be halted and normal vascular repair processes be allowed to proceed. A miR-145 antagonist was designed because miR-145 is required for vascular smooth muscle and progenitor cell differentiation, and its expression is increased in experimental and clinical PAH. Although inhibition of miR-145 prevents muscularization of pulmonary arteries in response to chronic hypoxia, it is unknown whether inhibition of miR-145 will repair established arteriopathy. Also the effect of antimiR-145 on occlusive neointimal remodeling, as seen in clinical PAH, is unknown. We hypothesized blocking miR-145 function would inhibit a variety of pathways important in regulating vascular remodeling. The Star:Star-mPEG/antimiR-145 complex was injected into the tail vein of rats with severe PAH beginning at week 8, because at 8 weeks RV systolic pressure is maximally elevated and pulmonary hypertension persists through 13 weeks [31,36]. Additionally, arterial wall thickening and RV dysfunction are fully developed at 8 weeks based on previously reported morphological, hemodynamic and echocardiographic findings [34,36]. Effects of antimiR-145 on vascular wall structure and the density of occlusive lesions were assessed in lung tissue at weeks 8 and 13. Lung sections

Fig. 4. Lack of effect of antimiR-145 on leucocyte counts, electrolytes, metabolites and marker proteins assessed by comprehensive metabolic panels of rats with PAH. A. Lack of effect of antimiR-145 or nonsilencing control oligonucleotide on body weight and spleen weight. Blood was collected at week 12 after two doses of antimiR-145 (2 mg/kg) or nonsilencing control oligonucleotide (2 mg/kg) were administered at weeks 8 and 10. B. Assessment of leucocyte count with differential, serum electrolytes and metabolites and marker proteins. Data are presented as mean ± SEM and analyzed with 1 way ANOVA followed by Dunnett’s Multiple Comparison Test, N = 4–7.
cut at the hilum were stained with hematoxylin and eosin (H&E) or Verhoeff-van Gieson (VVG) stains. Diameter and wall thickness were measured in all arteries across the entire cross section using a previously described method [33]. Density of vessels per lung cross section of the total area surveyed was calculated. As previously reported, [31,34,36] we found significant pulmonary arteriopathy in Sugen5416/hypoxia rats. Wall thickness of vessels 20–200 μm diameter was increased, and there were increased numbers of occlusive lesions as illustrated in representative H&E stained vessels in Fig. 5A. Quantitative analysis of wall thickness of VVG stained vessels is shown by frequency distributions of the number of blood vessels/cross sectional area of lung tissue (Fig. 5B–C). The density of small diameter (50–100 μm) vessels decreased in PAH animals and was increased after antimiR-145 treatment with a shift to a more normal wall thickness (Fig. 5B). AntimiR-145 treatment had a more pronounced effect on wall thickness in larger vessels (100–200 μm) producing a significant shift of the distribution to more normal wall thicknesses. The effect was not complete repair, but the median wall thickness decreased, suggesting remodeling elicited by PAH was partially repaired in the antimiR-145 treated animals. The nonsilencing control group had no significant effect on wall thicknesses compared to untreated PAH animals and is not shown for the sake of clarity.

In addition to measuring vessel geometry, we used a simple scoring system to count the number of vessels that were not occluded (grade 0), occluded <50% (grade 1) or occluded >50% (grade 2). Any complex vascular lesions with obvious slit-like channels, which we describe as plexiform-like, were counted separately and normalized to total lung area surveyed. AntimiR-145 reduced the density of all three types of vascular lesions (Table 1). In 3 of 6 animals treated with antimiR-145 we found no plexiform-like lesions, whereas all 7 untreated PAH animals had multiple plexiform-like lesions. This suggests development of the complex occlusive lesions may have been prevented or repaired by the treatment.

3.4. AntimiR-145 therapy improves RV structure and function

To determine whether improved arterial wall structure correlated with a pulmonary-selective antihypertensive effect, right ventricular systolic pressure (RVSP) and systemic mean arterial pressure were measured by simultaneous catheterization of the right ventricle via the jugular vein and of the left ventricle via the carotid artery. Consistent

![Fig. 5. AntimiR-145 therapy repairs pulmonary arteriopathy. A. Hematoxylin and Eosin stain of FFPE rat lung sections showing improvement of arteriopathy after antimiR-145 treatment at 13-week Sugen5416/hypoxia PAH rats in comparison to normal age-matched control rats and 13-week Sugen5416/hypoxia rats. B. Morphological measurements of pulmonary arteries in lung tissue from 13 week Sugen5416/hypoxia PAH rats showing increased arterial wall thickness in vessels sized 50–200 μm. Data are presented as frequency distributions analyzed by One-way nonparametric ANOVA followed by Dunn’s Test (*P < 0.05 compared to PAH). The nonsilencing control had no significant effect on wall thicknesses compared to untreated PAH animals and is not shown for the sake of clarity.](image-url)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Density of grade 1 and 2 occlusions and plexiform-like lesions per cross-sectional area of lung tissue. The number of lesions/mm² lung area for each animal was counted and averaged for each treatment group. Data are presented as mean ± SEM and analyzed by 1-way ANOVA followed by Dunn’s Multiple Comparison Test (*P &lt; 0.05 compared to PAH).</th>
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<tr>
<td>Total vessel per mm²</td>
<td>Grade 1 lesions</td>
</tr>
<tr>
<td>Normal (5)</td>
<td>2.52 ± 0.44</td>
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<tr>
<td>PAH (7)</td>
<td>2.33 ± 0.36</td>
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<tr>
<td>AntimiR-145 (6)</td>
<td>2.00 ± 0.19</td>
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<tr>
<td>Nonsilencing (8)</td>
<td>2.62 ± 0.17</td>
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![Diagram](diagram-url)
with previous findings [31,36], Sugen5416/hypoxia rats at 13 weeks showed a marked increase in RV systolic pressure (Fig. 6A). AntimiR-145 therapy reduced the RVSP (Fig. 6A) with no effect on systemic mean arterial pressure (Fig. 6B). This important finding suggests a pulmonary-selective antihypertensive effect of the Star:Star-mPEG-delivered antimiR-145 therapy. Chronic increases in afterload cause RV hypertrophy and a characteristic increase in the RV/LV + S ratio. AntimiR-145 treatment produced a modest decrease in the RV/LV + S ratio.

RV morphology and function in humans with PAH can be assessed by standard echocardiography methods that are also used in animal models of PAH [41,42]. Chronic RV volume and pressure overload cause characteristic structural changes in RV size and shape that impairs both RV and LV function and decreases cardiac output. Dysfunctional structural changes in the right and left ventricles were visualized at end diastole using a parasternal short axis view at the level of the papillary muscles and measuring RV inner diameter and the LV “eccentricity index” as previously described [43]. As shown in Fig. 6D, rats with severe PAH have a flattened septum, expanded RV inner diameter, and increased LV eccentricity characteristic of PH. Quantitative analysis of echocardiographic images shows antimiR-145 treatment decreased both RV inner diameter and LV eccentricity index (Table 2).

RV systolic function was also assessed by echocardiography to provide correlative data relevant to customary clinical measures of PAH severity in humans. The RV outflow tract (RVOT) was visualized using a modified parasternal long axis view and the flow velocity envelope obtained using pulsed-wave Doppler. The mid-systolic notch in the flow velocity envelope, characteristic of severe PAH, was eliminated by

Table 2

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<tr>
<th></th>
<th>RVID (mm)</th>
<th>LVEI (ratio)</th>
<th>RVOT-VTI (cm)</th>
<th>PAAT (ms)</th>
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<td>Normal (6)</td>
<td>3.63 ± 0.139*</td>
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<td>5.09 ± 0.153*</td>
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<td>PAH (9)</td>
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<td>2.07 ± 0.158</td>
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<td>AntimiR-145 (11)</td>
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<td>1.37 ± 0.094*</td>
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<td>Nonsilencing (8)</td>
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<td>1.80 ± 0.106</td>
<td>3.87 ± 0.235</td>
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Fig. 6. Effect of antimiR-145 on RV function. Hemodynamic measurement of: (A) right ventricular systolic pressure (RVSP) and (B) mean systemic arterial pressure (MSAP). (C) Assessment of RV hypertrophy by measurement of RV/LV + septum (S) weight ratio. Data are presented as mean ± SEM and analyzed with 1 way ANOVA followed by Dunnett’s Multiple Comparison Test (*P < 0.05 compared to PAH, N = 8-18). Representative echocardiographic images of (D) parasternal short axis views and (E) Pulsed wave Doppler traces from the right ventricular outflow tract.
antimiR-145 therapy (Fig. 6E). The velocity-time integral (RVOT-VTI) and pulmonary artery acceleration time (PAAT) were calculated from pulsed-wave Doppler signals. Rats with PAH showed a characteristic decrease in RVOT-VTI and RVOT-AT that was improved after antimiR-145 therapy (Table 2).

Collectively, the results demonstrate subchronic treatment with antimiR-145 has beneficial cardiac effects in rats with PAH that result in increased cardiac function as well as partial repair of RV hypertrophy with no effect on systemic mean arterial pressure. One explanation for improved hemodynamic and echocardiographic findings in rats treated with antimiR-145 is that partial repair of the pulmonary arteriopathy reduces pulmonary arterial pressure (and/or increases vascular compliance), which decreases afterload on the RV. Normal repair mechanisms regulating cardiac structure may ensue resulting in reduced RV hypertrophy at the cellular and tissue levels. Although this hypothesis warrants further investigation, preliminary results show a reduction in RV fibrosis after antimiR-145 therapy (data not shown). An alternative hypothesis is that antimiR-145 therapy has direct effects in the heart. Although our results cannot exclude this possibility, they are inconsistent with work from Wang et al. which showed that direct inhibition of miR-145 in heart tissue causes a pro-fibrotic response [44]. We support the simplest hypothesis based on known principles of cardiovascular physiology that reduction in afterload allows for normal regulatory mechanisms to repair heart structure.

The positive therapeutic effect of antagonizing miR-145 in PAH correlates well with the known functions of native miR-145, but a limitation of the study is that cellular targets and molecular mechanisms of action of antimiR-145 treatment are not yet fully defined. Existing literature clearly describes the role of miR-145 in regulating smooth muscle cell phenotype. Additionally, pulmonary artery smooth muscle cells have dysregulated gene expression, altered signaling, cell structure and functions in PAH. Although our morphometric assessments demonstrate that antimiR-145 reduces arterial wall thickness, neither our work nor the existing literature fully defines cell targets and molecular effects of antimiR-145 in the pulmonary circulation. To address this issue, miR-145 target transcripts were assessed at 13 weeks after terminal hemodynamics studies were completed. Surprisingly, expression of verified miR-145 target transcripts and transcripts with theoretical miR-145 binding sites were not different between untreated PAH animals and those treated with antimiR-145. This outcome might be explained by the static number of arteries/mm² lung area after antimiR-145 treatment, but the distribution of wall thickness shifted towards thinner-walled vessels. Even though this shift in wall thickness apparently correlated with functional effects on lung perfusion and cardiac function, the change in smooth muscle cell mass may not be sufficient to alter mRNA levels in a whole lung sample. Another explanation for lack of effect on steady-state transcript levels may be due to kinetics of the drug effect. Taking tissue samples at week 13, which was 5 weeks after the initiation of therapy, might have missed early dynamic effects of the antimiR-145 treatment on miR-145 targets. A more detailed time-course of antimiR-145 treatment effects is required to test this idea.

A third possibility is that there are additional functionally important miR-145 targets not described yet that influence pulmonary vascular remodeling, either directly via regulating smooth muscle differentiation, or indirectly by modifying other cells that regulate vascular wall structure, such as endothelium, adventitial fibroblasts, perivascular leukocytes and vascular progenitor cells. A more detailed analysis of the transcriptome of vascular smooth muscle, endothelial, and other cell types in situ rather than in total lung will be required to discriminate between these various mechanisms.

4. Conclusion

We demonstrate here that Star:Star-mPEG delivery of a miR-145 inhibitor elicits therapeutic effects in experimental, severe PAH. Delivery of antimiR-145 to lung tissue reduced levels of endogenous miR-145, reduced wall thickness in arteries ranging in size from 50–200 μm and reduced the density of occlusive vascular lesions. The anti-remodeling effects of antimiR-145 were associated with repair of heart structure and improved cardiac function. These beneficial effects all occurred with no change in systemic mean arterial pressure, no change in mean body weight or spleen weight, and no evidence of renal, hepatic, or hematopoietic toxicity after 5 weeks of therapy. Although the dosing schedule has not been fully optimized, these results suggest a low frequency (biweekly), low dosing (2 mg/kg) approach is sufficient to achieve significant pulmonary vascular repair that improves cardiac function. Lung delivery, low toxicity, and robust efficacy make this formulation appealing for development as an RNAi therapy to reverse the pulmonary arteriopathy and RV failure of PAH to improve patient quality of life and survival.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.05.261.

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