Apolipoprotein A-I Mimetic Peptide 4F Rescues Pulmonary Hypertension by Inducing MicroRNA-193-3p

Salil Sharma, PhD; Soban Umar, MD, PhD; Francois Potus, MSc; Andrea Iorga, BSc; Gabriel Wong, BA; David Meriwether, MS; Sandra Breuils-Bonnet, MSc; Denise Mai, BSc; Kaveh Navab, MD; David Ross, MD; Mohamad Navab, PhD; Steve Provencher, MD; Alan M. Fogelman, MD; Sébastien Bonnet, PhD; Srinivasa T. Reddy, PhD; Mansoureh Eghbali, PhD

Background—Pulmonary arterial hypertension is a chronic lung disease associated with severe pulmonary vascular changes. A pathogenic role of oxidized lipids such as hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids is well established in vascular disease. Apolipoprotein A-I mimetic peptides, including 4F, have been reported to reduce levels of these oxidized lipids and improve vascular disease. However, the role of oxidized lipids in the progression of pulmonary arterial hypertension and the therapeutic action of 4F in pulmonary arterial hypertension are not well established.

Methods and Results—We studied 2 different rodent models of pulmonary hypertension (PH): a monocrotaline rat model and a hypoxia mouse model. Plasma levels of hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids were significantly elevated in PH. 4F treatment reduced these levels and rescued preexisting PH in both models. MicroRNA analysis revealed that microRNA-193-3p (miR193) was significantly downregulated in the lung tissue and serum from both patients with pulmonary arterial hypertension and rodents with PH. In vivo miR193 overexpression in the lungs rescued preexisting PH and resulted in downregulation of lipoxygenases and insulin-like growth factor-1 receptor. 4F restored PH-induced miR193 expression via transcription factor retinoid X receptor α.

Conclusions—These studies establish the importance of microRNAs as downstream effectors of an apolipoprotein A-I mimetic peptide in the rescue of PH and suggest that treatment with apolipoprotein A-I mimetic peptides or miR193 may have therapeutic value. (Circulation. 2014;130:776-785.)

Key Words: apolipoprotein A-I mimetic peptide 4F ■ lipoproteins ■ microRNAs ■ miR-193, human ■ pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a multifaceted disease caused by increase in pulmonary arterial pressure leading to right ventricular (RV) hypertrophy, RV failure, and death. PAH is a clinical condition associated with severe pulmonary vascular disorder due to excessive proliferation of pulmonary artery smooth muscle and endothelial cells, migration of pulmonary vascular smooth muscle cells, remodeling of small pulmonary arteries with diminished apoptosis, enhanced inflammation, and fibrosis in the lung tissue. Vascular disorders result from complex interactions between oxidized lipoproteins, monocytes/macrophages, and injured endothelium and smooth muscle cells. The role of oxidized phospholipids in atherosclerosis and other inflammatory diseases is well studied. Biological metabolites of arachidonic acid and linoleic acid, including hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs), play an important role in the pathogenesis of atherosclerosis by mimicking the inflammatory properties of oxidized phospholipids. An apolipoprotein A-I (apoA-I) mimetic peptide, 4F, is known to bind to oxidized lipids including HETEs and HODEs very effectively and decrease their levels and therefore mitigate an anti-inflammatory response. Although pulmonary hypertension (PH) is associated with inflammation, the role of HETEs and HODEs in PH is not fully understood.

Clinical Perspective on p 785

MicroRNAs (miRNAs) are small noncoding, single-stranded RNAs 19 to 25 nucleotides in length. They regulate several physiological and disease pathways, including apoptosis, cell migration, vascular development, cell proliferation, and cancer via regulating a network of target genes. MiRNAs can downregulate gene expression by binding to the 3’
untranslated region of target mRNAs. This coupling results in translational inhibition or transcript degradation.\textsuperscript{10,11} Recently, progress in delineating the important role of microRNAs in cardiovascular disease progression, including PH, has been made.\textsuperscript{12–14} Several miRs including miR-21, miR-204, and miR-328 have been reported to regulate pathogenic signaling in the development and progression of PH.\textsuperscript{15–17}

This study was designed to provide insights into the pathophysiology associated with increased lipoygenase products in the progression of PH and the development of novel therapeutic approaches to limit their accelerated effect on the diseased state. We found that PH is associated with increased plasma levels of oxidized lipids in rodents, and 4F therapy restores their levels and rescues preexisting PH. We also used miRNA microarray analysis to identify novel miRNAs as downstream effectors of 4F. We found that microRNA-193-3p (miR193) is downregulated in the lung tissue and plasma in both patients with PAH and rodents with PH. In vivo overexpression of miR193 in the lungs rescued preexisting PH. 4F restored PH-induced miR193 expression, most likely via transcription factor retinoid X receptor \(\alpha\) (RXR-\(\alpha\)) regulating lipoygenases and cell proliferation in PH.

**Methods**

The online-only Data Supplement provides more details on all methods.

**Animals**

Adult young male Sprague-Dawley rats (weight, 200–250 g; aged 2–3 months) and young male C57Bl/6 mice (aged 2–3 months) from Charles Rivers Laboratories were used. All experimental protocols received institutional review and committee approval.

**Human Tissue Samples**

Lung tissues and buffy coat were obtained from PAH patients and control subjects who had given signed consent previously in agreement with the ethical protocol (CER 20773; 20735), as described.\textsuperscript{18,19} Non-PAH lung tissues (controls) were obtained during lung resection for tumors from the noncancerous segments. Clinical characteristics of PAH patients and control subjects are given in Table I in the online-only Data Supplement.

**In Vitro Overexpression and Knockdown Studies**

Human pulmonary artery smooth muscle cells (HPASMCs; passages 3–5) were cultured in media 231 and transfected with scrambled control or the stage of the disease, as we published recently.\textsuperscript{20,21}

**Cardiac and Pulmonary Hemodynamics**

B-mode, M-mode, and pulmonary pulsed-wave Doppler echocardiography were performed with the use of a Visual Sonics Vevo 2100 device equipped with a 30-MHz linear transducer to accurately monitor the stage of the disease, as we published recently.\textsuperscript{20,21}

**Gross Histological Evaluation**

The RV wall, the left ventricular wall, and the interventricular septum were dissected, and the ratio of the RV to left ventricle plus interventricular septum weight \([\text{RV/(LV+IVS)}]\) was calculated as an index of RV hypertrophy.

**Real-Time Polymerase Chain Reaction**

Total RNA from lungs was isolated with the use of Trizol and reverse transcribed with gene-specific primers with the use of the Omniscript reverse transcription kit (Qiagen). A microarray screen of miRNA expression in the total lung tissue of rats was performed with the use of non-Affymetrix single-channel arrays (MirBASE 17.0 MicroRNA Array, Ocean Ridge Biosciences). The primer sequences are given in Table II in the online-only Data Supplement.

**Immunohistochemistry and Imaging**

Whole hearts and lungs were fixed in paraformaldehyde, immersed in 20% sucrose, mounted with the use of OCT compound, and transversely sectioned with a cryostat. Tissue sections were stained for immunofluorescence, immunoperoxidase, standard hematoxylin-eosin, and Masson trichrome staining. The images were acquired with the use of a light microscope or a laser-scanning confocal microscope (Nikon).

**Cell Culture and Proliferation Assays**

HPASMCs were either purchased (cryopreserved, Invitrogen or Cell Application, San Diego, CA) or isolated from <1000-μm-diameter small pulmonary arteries from PAH or control subjects (Table I in the online-only Data Supplement). Pulmonary artery smooth muscle cell phenotype was confirmed with the use of α-smooth muscle actin staining. Cells were cultured in medium 231 in the presence or absence of 4F (D–4F; 1 μg/mL) or miR193 mimic or inhibitor. HETE and HODE incubation (100 ng/mL) in the presence and absence of 4F was achieved in lipoprotein-deficient serum. Cell proliferation was measured by a standard MTT cell proliferation assay (ATCC) or Ki-67 antibody (Millipore AB9260).

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation and DNA extraction were performed with the use of the ChiP-IT high-sensitivity kit from Active Motif. The samples were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), and the results were presented as mean±SE normalized to input.

**Plasma Sample Preparation and Liquid Chromatography–Tandem Mass Spectrometry Analysis for Determination of Oxidized Lipids and Leukotriene Analysis**

Plasma samples for measurements of oxidized lipids as well as internal standards mixture were loaded onto a preconditioned 1 cm3 Oasis HLB solid-phase extraction cartridge on a vacuum manifold (Waters) and processed for liquid chromatography–tandem mass spectrometry analysis. For quantification of HETEs/HODEs, plasma samples were added to 300 μL of 2:1 vol/vol chloroform/methanol containing 0.01% butylated hydroxytoluene and processed similarly.

Plasma samples for leukotrienes were acidified, and butylated hydroxytoluene was added to a final concentration of 20 μmol/L. The samples were loaded onto solid-phase extraction cartridges (Waters, 186001880) and processed for liquid chromatography–tandem mass spectrometry analysis.

Liquid chromatography–tandem mass spectrometry was performed with the use of a mass spectrometer (4000 QTRAP; Applied Biosystems, Foster City, CA) equipped with an electrospray ionization source.

**Statistical Analysis**

Means were compared across groups with the use of 1-way ANOVA for ≥3 groups or \(t\) tests for 2 groups for data that followed the normal distribution and met variance homogeneity. Significance was assessed with the Tukey criterion for pairwise mean comparisons under the ANOVA model. Normality was assessed with the Kolmogorov-Smirnov test. When homogeneity of variance was not met under the ANOVA model, a robust ANOVA was performed.22 Survival curves were estimated by the Kaplan-Meier method. When the data did not follow the normal distribution, \(P\) values were computed with nonparametric Kruskal-Wallis (Mann-Whitney) methods. Two-tailed \(P\)
values <0.05 were considered significant after adjustment. Values are expressed as means±SE. Computations were performed with the use of SPSS SigmaStat for Windows, version 3.0 (SPSS Inc, Chicago, IL) and JMP version 11.0 (SAS Inc, Cary, NC).

Results

4F Rescues Monocrotaline-Induced PH in Rats and Hypoxia-Induced PH in Mice by Improving Cardiac and Pulmonary Structure and Function

To examine whether 4F therapy can rescue preexisting PH, we started 4F therapy (50 mg/kg per day) at day 21 (Figure 1A) because we have shown previously that severe PH is already established by day 21 in the monocrotaline model (60 mg/kg) in rats.20,21,23 4F therapy rescued PH because RV systolic pressure (46.2±2.6 mmHg in 4F versus 68.0±7.9 mmHg in PH; P<0.01) and RV hypertrophy index (0.4±0.2 in 4F versus 0.68±0.01 in PH; P<0.001) were significantly lower than those in untreated monocrotaline rats (Figure 1B and 1C). In non-PH rats, 4F therapy had no effect on RV systolic pressure (27.1±1 in 4F-control versus 26.0±1.6 in control; P=NS), RV hypertrophy index (0.26±0.01 in 4F-control versus 0.31±0.03 in control; P=NS), or kidney or liver function (Figure 1B and 1C and Table III in the online-only Data Supplement). The improvement in lung and RV structure and function mediated by 4F led to 80% survival until day 30, whereas in untreated PH rats, the mortality started at day 24 and sharply increased to 45% by day 28 and to 75% by day 30 (Figure 1D). Moreover, pulmonary arteriolar medial hypertrophy was reversed in 4F-treated animals (Figure 1E).

4F therapy was also effective in rescuing hypoxic mice because the RV systolic pressure (25.1±2.7 mmHg in 4F versus 34.5±3.5 mmHg in PH; P<0.01) and RV hypertrophy index (0.4±0.02 in 4F versus 0.42±0.03 in PH; P<0.05) were both significantly decreased when the hypoxic mice received a daily 4F injection from day 14 to 21 compared with untreated hypoxic mice (Figure 1F through 1H).

4F Rescue of PH Is Associated With Decreased Plasma Levels of Oxidized Lipids

PH was associated with significantly elevated plasma levels of HETEs and HODEs, which were reversed by 4F therapy in PH rats (Figure 2 and Figure I and Table IV in the online-only Data Supplement). Among the leukotrienes, plasma LBT4 level was significantly elevated only in PH rats, and 4F treatment was not able to reverse LBT4 level (Figure II in the online-only Data Supplement). In summary, severe PH is associated with an increase in plasma levels of HETEs and HODEs, which were restored by 4F treatment.

MiR193 Is Downregulated in Lung and Plasma in Experimental PH and in PAH Patients, and 4F Therapy Restores Lung miR193 Levels in Rodent Models

To determine the effect of PH and 4F on microRNA expression in the lungs of rats, miRNA microarray (non-Affymetrix) was performed in the control, PH, and 4F rescue groups at day 30. The expression of 366 miRNAs was successfully detected. Multiple miRNAs were differentially regulated between the control and PH groups. Among these, previously reported miR2117 (3.8-fold upregulation), miR193 (2.7-fold downregulation), and miR451 (2.6-fold downregulation) were dysregulated in PH (Figure 3A). 4F treatment was not able to restore miR21 (Figure 3B), miR322, or miR451 but was efficient in restoring
PH-induced miR193 downregulation in both monocrotaline and hypoxia models (Figure 3C and 3D). These data suggest that 4F selectively regulates the expression of miR193 in both models of PH. MiR193 was also downregulated in the lungs of PAH patients compared with control subjects (Figure 3E). Interestingly, miR193 was also detectable in circulating blood and was significantly lower in PH rats and idiopathic PAH patients compared with controls (Figure 3F and 3G).

4F Treatment and miR193 Overexpression Inhibit Proliferation of HPASMCs
PH is characterized by an increase in the proliferation of pulmonary artery smooth muscle cells. Treatment of HPASMCs with oxidized lipids stimulated proliferation that was prevented by 4F (Figure 4A and Figure IIIA in the online-only Data Supplement).

Next we examined whether overexpression of miR193 could inhibit proliferation in HPASMCs. Transfection with miR193 mimic led to an ≈5-fold increase in the expression level of miR193 (Figure IV in the online-only Data Supplement). Interestingly, both 4F treatment and overexpression of miR193 in HPASMCs inhibited cell proliferation, whereas miR193 knockdown stimulated proliferation (Figure 4B and Figure IIB in the online-only Data Supplement). Overexpression of miR193 in HPASMCs isolated from idiopathic PAH patients also resulted in inhibition of proliferation, whereas knockdown of miR193 in HPASMCs isolated from control subjects stimulated proliferation (Figure 4C).

Gain of miR193 Rescues PH in Both Monocrotaline and Hypoxia Models
To test whether gain of miR193 level can reverse symptoms of PH, synthetic miR193 RNA molecules (miR193 mimic) were selectively delivered to the lung by intratracheal administration of 10 mg/kg body wt at days 17, 21, and 26 in the monocrotaline model or at days 14 and 18 in the hypoxia model (Figure 5A and 5B). To verify that miR193 was indeed overexpressed in the lungs, we measured miR193 pulmonary tissue expression, we measured miR193 pulmonary expression in the lungs of control (CTRL), PH, and 4F rescue rats in the monocrotaline model of PH. Relative miR21 expression in the lungs is confirmed with the use of quantitative reverse transcriptase polymerase chain reaction analysis in the monocrotaline (MCT) model. Relative miR193 expression in the lungs is confirmed with the use of quantitative reverse transcriptase polymerase chain reaction analysis in the monocrotaline (MCT) model and hypoxia model (D). E, Relative miR193 expression in the lungs of PAH patients (n=5) and in control subjects (n=7). F, MiR193 expression in the plasma of control (n=8) and PH (n=6) rats in MCT model. G, MiR193 expression in the buffy coat of control (n=10) and idiopathic PAH (IPAH) patients (n=9). For B through G, *P<0.05, **P<0.01 vs CTRL; ###P<0.001 vs PH (n=6–8 animals per group).
pressure (38.2±7.2 mm Hg in miR193-overexpression rats versus 73.3±7.6 in PH in monocrotaline rats [P<0.01]; 25.5±0.9 mm Hg in miR193-overexpression mice versus 33.2±2.5 in PH in hypoxic mice [P<0.05] Figure 5E and 5F) and RV hypertrophy index (0.38±0.03 in miR193-overexpression rats versus 0.68±0.01 in PH in monocrotaline rats [P<0.001]; 0.32±0.02 in miR193-overexpression mice versus 0.40±0.02 in PH in hypoxic mice [P<0.01]) were significantly reduced.
(Figure 5G and 5H). There was no significant change in left ventricular weight or left ventricular weight/body weight ratio among control, PH, miR193-overexpression, and 4F rescue groups (Figure VI in the online-only Data Supplement). MiR193 gain also prevented the increased lung arteriolar muscularization and adverse RV remodeling occurring in PH rats (Figure 5I and Table V in the online-only Data Supplement).

**MiR193 Targets Lipoxygenases and Insulin-Like Growth Factor-1 Receptor In Vivo**

To determine the mechanism by which miR193 improves function in PH models, we explored the targets of miR193 in vivo. We found that miR193 gain reversed PH-induced increased expression of multiple lipoxygenases including ALOX5, ALOX12, and ALOX15 in both monocrotaline and hypoxia models of PH, thus implicating a role for miR193 in inhibiting the production of oxidized lipids (Figure 6A and 6B). 4F was also as efficient as miR193 overexpression in reversing PH-induced upregulation of these lipoxygenases (Figure 6A and 6B).

To explore miR193 targets directly involved in cell proliferation, we identified insulin-like growth factor-1 (IGF1) receptor (IGF1R) as a predicted target of miR193 in silico (Target Scan; http://www.targetscan.org/). IGF1R signaling is vital for proliferation, survival, and migration of many cell types, including smooth muscle cells. IGF1R signaling has also been implicated in PH.25,26 Indeed, miR193 gain was able to suppress the levels of IGF1R transcripts in both monocrotaline rats and hypoxia mice (Figure 6A and 6B). Administration of 4F reversed PH-induced upregulation of IGF1R in the monocrotaline but not in the hypoxia model (Figure 6A and 6B).

**4F Induces Expression of miR193 in HPASMCs by Suppressing Peroxisome Proliferator-Activated Receptor/RXR-α Signaling Pathway**

The apoA-I mimetic peptide 4F is known to bind and decrease levels of oxidized lipids.9 To assess whether induction of miR193 by 4F is mediated via different HETEs and HODEs, miR193 expression was assessed in HPASMCs treated with 5-, 12-, 15-HETEs and 9-, 13-HODEs (100 ng/mL) alone or together with 4F. Exposure of the cells to 5-, 12-, 15-HETEs and 9-, 13-HODEs resulted in decreased expression of miR193 compared with the control cells (Figure 7A). In the presence of 4F, the various HETEs and HODEs were unable to downregulate miR193 (Figure 7A). To determine the molecular mechanism by which HETEs and HODEs downregulate miR193 expression, we examined the expression of

![Figure 6](image-url)  
**Figure 6.** MicroRNA-193 (miR193) and 4F target lipoxygenases in vivo. Quantitative reverse transcription polymerase chain reaction assessment of transcript levels of lipoxygenases ALOX5, ALOX12, and ALOX15 and insulin growth factor-1 receptor (IGF1R) in the lungs of control (CTRL), pulmonary hypertension (PH), miR193-overexpression (OE), and 4F groups in monocrotaline (MCT) rat model of PH (A) and in hypoxia mouse model of PH (B). *P<0.05, **P<0.01, ***P<0.001 vs CTRL; #P<0.05, ##P<0.01, ###P<0.001 vs PH (n=5–8 animals per group).

![Figure 7](image-url)  
**Figure 7.** 4F induces microRNA-193 (miR193) by inhibiting the expression of retinoid X receptor α (RXR-α). A. MicroRNA-193 (miR193) expression was assessed in human pulmonary artery smooth muscle cells (HPASMCs) treated with 5-, 12-, 15-hydroxyeicosatetraenoic acid (HETE) or 9-, 13-hydroxyoctadecadienoic acid (HODE) for 48 hours in the presence or absence of 4F. For A and B, *P<0.05, **P<0.01, ***P<0.001 vs CTRL; #P<0.05, ##P<0.01, ###P<0.001 vs HETE/HODE. B. RXR-α transcripts were assessed in HPASMCs treated with 5-, 12-, 15-HETE or 9-, 13-HODE for 48 hours in the absence or presence of 4F. For A and B, *P<0.05, **P<0.01, ***P<0.001 vs CTRL; #P<0.05, ##P<0.01, ###P<0.001 vs HETE/HODE. C. RXR-α enrichment at the miR193 promoter is determined by quantitative reverse transcription polymerase chain reaction in HPASMCs treated with 15-HETE in the presence or absence of 4F by chromatin immunoprecipitation. *P<0.05, **P<0.01, ***P<0.001 vs RNA polymerase II; ###P<0.001 vs Immunoglobulin G (n=3 independent experiments).
RXR-α and myc-associated factor X (MAX), the top 2 transcription factors that previously have been validated to bind to the promoter region of miR193 and thereby suppress miR193 expression in cancer cells. We found that various HETEs and HODEs induce the expression of the transcription factor RXR-α in HPASMCs and that this induction is suppressed by 4F (Figure 7B).

However, expression of the transcription factor MAX was not induced by HETEs and HODEs (Figure VII in the online-only Data Supplement). To validate that RXR-α could directly bind to the promoter of miR193 in HPASMCs, we performed chromatin immunoprecipitation experiments. We found that treatment of HPASMCs with 15-HETE results in enrichment of RXR-α on the promoter of miR193, which is inhibited in the presence of 4F (Figure 7C).

**Exogenous HETE Therapy Is Sufficient to Induce PH**

Our in vitro data show that HETEs stimulate proliferation of HPASMCs and also downregulate the expression of miR193 in these cells. To confirm our in vitro data, we examined the role of exogenous HETE therapy on RV systolic pressure and miR193 expression in the lung tissue by feeding mice a 15-HETE-containing diet for 3 weeks. We found that RV systolic pressure was significantly higher in mice on the 15-HETE diet compared with controls (Figure 8A). The lung weight was also significantly increased in mice on the 15-HETE diet (0.27±0.02 g in 15-HETE diet group versus 0.19±0.01 g in controls; P<0.05; Figure 8A). The lung weight was also significantly increased in mice on the 15-HETE diet (0.27±0.02 g in 15-HETE diet group versus 0.19±0.01 g in controls; P<0.05; Figure 8B). Finally, we found that the 15-HETE diet significantly downregulated the expression of miR193 in the lungs of mice compared with controls (Figure 8C).

**Discussion**

In the present study, we provide evidence that the oxidized metabolites of arachidonic and linoleic acids, including HETEs and HODEs, were elevated in a rodent model of PH. More importantly, we found that 4F therapy, starting after the establishment of severe PH, is effective in rescuing advanced PH in both monocrotaline-treated and hypoxia-induced animal models (Figure 1). We also report that increasing oxidized fatty acid levels in vivo is sufficient to induce PH in mice (Figure 8). Our findings implicate for the first time the involvement of oxidized metabolites in the etiology of PH. Additionally, we have identified miR193 as a novel downstream effector of 4F. Downregulation of miR193 was observed in the lung tissue and plasma of PAH patients and in rodents. 4F therapy successfully restored miR193 to levels comparable to those in healthy control animals (Figure 3). Overexpression of miR193 in the lungs of monocrotaline-treated rats and chronically hypoxic mice rescued preexisting PH (Figure 5), thus confirming the important role of this miRNA in PH. We also found that miR193 gain of function in cultured HPASMCs from idiopathic PAH patients attenuates proliferation, whereas miR193 knockdown stimulates proliferation in cells isolated from control subjects (Figure 4C). The lipoxygenase pathway is one of the key pathways regulated by miR193 because the PH-induced transcriptional upregulation of lipoxygenases, including ALOX5, ALOX12, and ALOX515, was fully reversed by either 4F therapy or gain of miR193 in the lungs of hypoxic mice (Figure 6). We also provide evidence that miR193 expression is inhibited in pulmonary smooth muscle cells by oxidized metabolites, most likely via induction of the transcription factor RXR-α, a known inhibitor of miR193. However, ablation of RXR-α induction by 4F restored the miR193 expression level (Figures 7 and 8).

Oxidized phospholipids are a major class of oxidized lipids that have proinflammatory properties. Oxidized phospholipids are also associated with DNA damage, which is implicated in PAH etiology, and are found in the vasculature of animal models of atherosclerosis and in many other inflammatory diseases. Oxidized phospholipids trigger vascular and nonvascular cells to initiate an inflammatory reaction...
that involves activation of cytosolic phospholipase A, and the 12-lipoxygenase pathway.\textsuperscript{30} Oxidized metabolites of arachidonic and linoleic acids can be generated by specific oxidation by enzymes, including lipoxygenases.\textsuperscript{31,32} These oxidized metabolites, such as 12-HETE, act in a manner similar to that of oxidized phospholipids in the induction of inflammation.\textsuperscript{33,34} Studies have shown that normal high-density lipoprotein can prevent the inflammatory reaction characteristic of atherosclerosis and that this is associated with decreased levels of oxidized lipids both in vitro and in vivo.\textsuperscript{4,28,35,36} ApoA-I mimetic peptides, such as 4F, are 18-amino acid peptides that do not have sequence homology with apoA-I but mimic the class A amphipathic helices contained in apoA-I. The 4F peptide is effective in mitigating inflammation by binding oxidized lipids with high affinity and removing these from tissues.\textsuperscript{3,37-39} Administration of the 4F peptide has shown beneficial effects in many pathological conditions in animal models including type I diabetes mellitus, type II diabetes mellitus, and obesity-, hyperlipidemia-, and sickle cell disease–induced vascular dysfunction.\textsuperscript{40-44} Our study has shown for the first time that levels of oxidized lipids 5-,12-, 15-HETE and 9-, 13-HODE are significantly increased in the plasma of PH rats. Conversely, increasing oxidized lipids in vivo by feeding mice with 15-HETE for 3 weeks led to PH. These data strongly implicate the involvement of oxidized metabolites in the etiology of PH. 4F therapy of PH rats was associated with restoration of the levels of HETEs and HODEs. Because leukotrienes are also produced as metabolites of arachidonic acid, we examined the effect of 4F treatment on the levels of leukotrienes. Among the 3 leukotrienes tested, the plasma LTB4 level was the only one that was significantly higher in PH, but 4F was not able to restore the LBT4 level (Figure II in the online-only Data Supplement), suggesting that regulation of leukotrienes by 4F does not play a role in PH.

The role of a few miRNAs has been investigated recently in the development of PH.\textsuperscript{45} MiR21 induction was found to repress RhoB and Rho-kinase activity and caused decreased angiogenesis and vasodilation, thereby aggravating symptoms of PH.\textsuperscript{13} In another elegant study, downregulation of miR204 was shown to correlate with the severity of PH by the activation of Src kinase and nuclear factor of activated T cells, causing PAH/PA smooth muscle cell proliferation and resistance to apoptosis.\textsuperscript{15} The role of miR328 has been well studied in PH resulting from chronic hypoxia. MiR328 was shown to play a key role in pulmonary arterial constriction and remodeling by regulating L-type calcium channel-α1C expression in hypoxic PH.\textsuperscript{14} However, the role of miR193 in PH has not been studied previously. We focused on miR193 because of its significant downregulation in PH and its remarkable normalization/induction after 4F treatment. The fact that miR193 displayed a similar aberrant expression in 2 different rodent models of PH, both of which were rescued by 4F treatment, suggests that the aberrant expression of miR193 may not be limited to a specific insult but may be caused by many common and converging pathways during the progression of PH. Interestingly, miR193 was also downregulated in human samples of PAH. Another major finding of this study is that miR193 was identified as a downstream effector of 4F, playing a key role in the remodeling and rescue of PH via its direct effect on lipoxygenases and oxidized lipid production. Recent studies have implicated the role of miR193 in cancer-related events, including an inhibitory effect on cell proliferation, cell growth, and cell cycle progression and a proapoptotic effect in various cancer cell lines by regulation of serine/arginine-rich splicing factor 2, E2F transcription factor 1, and an antiapoptotic Bcl-2 family member Mcl-1.\textsuperscript{46} In our studies, we report that miR193 regulates various lipoxygenases including ALOX-5, -12, and -15 in 2 rodent models of PH (Figure 6). Although we found that PH regulates a number of miRNAs (miR193, miR21, miR322), the regulation of oxidized lipids by 4F is mediated mainly through miR193 (Figure 3).

Several circulatory miRs have been reported to serve as plasma biomarkers.\textsuperscript{47,48} Our data show that miR193 is expressed in the buffy coats of human healthy subjects and in rats, and its level is downregulated in the blood of both idiopathic PAH patients and PH rats (Figure 3). Future studies with larger sample size are needed to examine whether miR193 levels could serve as a biomarker for PAH.

Many studies report the regulation of miRNAs by transcription factors.\textsuperscript{49,50} Aberrations in this important step of miRNA regulation can contribute to the onset and progression of many diseases.\textsuperscript{51} Peroxisome proliferator-activated receptors are ligand-inducible transcription factors that can be activated by components of oxidized low-density lipoproteins including 15-HETE.\textsuperscript{52} Peroxisome proliferator-activated receptors heterodimerize with RXR-α to regulate expression of many genes.\textsuperscript{53,54} Interestingly, the transcription factor RXR-α has previously been reported to inhibit the expression of miR193 by directly binding to its promoter in cancer cells.\textsuperscript{27} We provide evidence that HETEs and HODEs induce the expression of RXR-α in pulmonary artery smooth muscle cells. This induction results in an enrichment of RXR-α on the miR193 promoter, thus causing its subsequent downregulation. However, 4F can decrease the overall content and binding of RXR-α to miR193 promoter by sequestering HETEs and HODEs, ultimately leading to miR193 induction.

In a search for potential in silico targets of miR193 (http://www.targetscan.org/), we also identified IGF1R as a novel target. IGF1R signaling can regulate various biological pathways including cell growth, migration, and differentiation.\textsuperscript{26} Repression of IGF1R by miR328 and its involvement in apoptosis of HPASMCs in PH has been reported previously.\textsuperscript{16} The action of miR193 on IGF1R was similar to the response to 4F in the monocrotaline model of PH but not in the hypoxia mouse model (Figure 6). This example was the only one that we found in which the actions of miR193 and 4F were not congruent.

In conclusion, our study demonstrates that an important event associated with the pathophysiology of PH in rodent models is an increase in the production of oxidized lipid metabolites including HETEs and HODEs. We have shown that the high-density lipoprotein mimetic peptide 4F can successfully rescue advanced PH in 2 different models of PH by inducing the expression of miR193, most likely via the transcription factor RXR-α, which in turn inhibits various lipoxygenases. MiR193 inhibits HPASMC proliferation possibly by downregulating IGF1R (Figure 8). More importantly, gain of miR193 inhibits proliferation in pulmonary smooth muscle cells from patients with idiopathic PAH (Figure 4C). These
studies further establish the importance of miRNAs in PH and suggest that treatment with apoA-I mimetic peptides may have therapeutic value.

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Disclosures
Drs Fogelman, Navab, and Reddy are principals in Bruin Pharma, and Dr Fogelman is an officer in Bruin Pharma. Dr Provencher is a Founding Member of Quebec-Sante clinical scientist in Quebec, Canada, and Dr Bonnet holds Canada Research chairs. The other authors report no conflicts.

References
Pulmonary arterial hypertension is a chronic lung disease with progressive pulmonary vascular remodeling. A pathogenic role of oxidized lipids is well established in vascular diseases such as atherosclerosis. The high-density lipoprotein mimetic peptide known as 4F binds with high affinity to the oxidized lipids, reduces their levels, and thus improves vascular disease. Recently, small noncoding RNA molecules called microRNAs have emerged as potent regulatory molecules. Several microRNAs have been reported to regulate the development and progression of pulmonary hypertension (PH). In this study, we demonstrate that the plasma levels of oxidized lipids are increased in the experimental PH model. Furthermore, we show that 4F rescues advanced PH and reduces the levels of plasma oxidized lipids in PH. We also show that the microRNA-193-3p (miR193) is significantly downregulated in experimental PH and in the lung tissue and plasma in pulmonary arterial hypertension patients. A key finding is that 4F is able to restore expression of miR193 in the lungs to its levels in healthy controls via transcription factor retinoid X receptor α. Another important finding of this study is that overexpression of miR193 in the lung tissue of PH rodents is sufficient to rescue PH and inhibit proliferation of human pulmonary artery smooth muscle cells isolated from pulmonary arterial hypertension patients. MiR193 targets a number of lipoxigenases involved in the production of oxidized lipids. Our study has delineated the involvement of oxidized lipids in PH and highlights miR193 as the novel and nodal regulatory molecule by which mimetic peptide 4F entails its therapeutic effect in PH.
Apolipoprotein A-I Mimetic Peptide 4F Rescues Pulmonary Hypertension by Inducing MicroRNA-193-3p

Salil Sharma, Soban Umar, Francois Potus, Andrea Iorga, Gabriel Wong, David Meriwether, Sandra Breuils-Bonnet, Denise Mai, Kaveh Navab, David Ross, Mohamad Navab, Steeve Provencher, Alan M. Fogelman, Sébastien Bonnet, Srinivasa T. Reddy and Mansoureh Eghbali

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SUPPLEMENTAL MATERIAL

Materials and Methods

Animals and treatments

Adult male Sprague-Dawley rats (200-250g, 2-3 months old), and young male mice C57Bl/6 (age 2-3 months old) from the Charles Rivers Laboratories were used. In the monocrotaline (MCT) model, male rats were injected s.c. with MCT (60 mg/kg, Sigma) or PBS. MCT injected rats received daily D-4F therapy (50mg/kg/day, s.c., provided by Dr Fogelman’s group) from day 21 to 30 or left untreated to develop even more severe PH. Some PBS injected rats also received daily D-4F therapy (50mg/kg/day, s.c.) from day 21 to 30 to examine the effect of 4F on non-PH rats. This dose of 4F was selected based on the previous work from our collaborators showing D-4F concentrations from 4.5 to 45 mg/kg/day were very effective in reducing aortic artherosclerosis by~50% in apoE null mice and LDLR-KO mice1,2. In the chronic hypoxic model, mice were placed for 3 weeks in normobaric hypoxic chamber maintained with flow of hypoxic air (10% O2 and 90% N2). Chamber was opened once a week for cleaning and replenishment of food and water. Oxygen concentration was continuously monitored with blood gas analyzers. Soda lime was used to lower carbon dioxide concentration. Hypoxic mice from day 14-21 received daily D-4F therapy (50mg/kg/day, s.c.) or were left untreated. To increase oxidized fatty acid levels in-vivo, 15HETE-S was added to the mouse chow as described previously3. Male mice were fed with 15-HETE containing diet at a dose of 5µg/day (1mg HETE/gr of diet per day) as used previously3 or with regular chow for 3 weeks. To induce in-vivo overexpression of miR193, miR193 mimic oligonucleotide (mature sequence, 3’ aactggctacaagtccccagt-5’, Life technologies) was intratracheally administered to the animals (10 mg/kg body weight in 50 µl PBS on days 17, 21 and 26 for MCT model; and on days 14 and 18
for hypoxia model). For administration of miR193 to MCT rats or hypoxic mice, the animals were first anesthetized with isoflurane. After the tongues of the anesthetized animals were gently pulled forward with forceps, miR193 oligonucleotide was delivered through the oropharyngeal cavity into the trachea. The tongue was kept extended until all of the liquid was inhaled into the lungs. Overexpression of miR193 in the lungs was confirmed by qRT-PCR. Protocols received institutional review and committee approval. The investigation conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

**In vitro overexpression and knockdown studies**

HPASMCs (passage 3-5) were cultured in Media 231 supplemented with 1X Smooth Muscle Growth Factor (SMGS, Gibco Invitrogen) and 1% PS. Cells were starved overnight prior to transfection. Transfection was performed with scrambled controls, mimic-193 and inhibitor-193 oligonucleotides (Life technologies) at a final concentration of 50nM with lipofectamine (Invitrogen). Experiments were performed 48-hours post transfection.

**Isolation of pulmonary arteries**

Pulmonary arteries from rats were isolated by removing the heart and the lungs together after sacrifice. The lungs were perfused with cold PBS. The thymus, esophagus and fat were removed. The lungs were then pinned with the flat side up and the parenchyma was gently removed by trenching through the veins. The veins and bronchi were removed and pulmonary arteries were isolated. The pulmonary arteries were snap frozen and RNA was isolated using Trizol (Invitrogen). The entire process was carried out in cold PBS.

**Cardiac and pulmonary hemodynamics**
B-mode, M-mode and pulmonary pulsed-wave Doppler echocardiography were performed using a Visual Sonics Vevo 2100 equipped with a 30-MHz linear transducer to accurately monitor the stage of the disease as we published recently\(^4,5\).

**Gross histological evaluation**

The RV wall, the left ventricular wall and the interventricular septum were dissected and the ratio of the right ventricle to left ventricle plus septum weight \([RV/(LV+IVS)]\) was calculated as an index of RV hypertrophy.

**Cell culture and Proliferation Assays**

Human Pulmonary Artery Smooth Muscle Cells (HPASMC) were either purchased (Cryopreserved, Invitrogen or Cell Application (San Diego, CA)), or isolated from <1000-\(\mu\)m-diameter small pulmonary arteries from PAH or control subjects (Supplemental Table 1). PASMC phenotype was confirmed by using \(\alpha\) -smooth muscle actin staining. Cells were plated into a 96 well plate at a density of 1000 cells/well. Cells were serum starved overnight and proliferation induced by serum replenishment with Smooth Muscle Growth Supplement (5\%) in the presence or absence of 4F, (1\(\mu\)g/ml) or 48 hours post transfection with miR193 mimic(50nM) for overexpression and miR193 inhibitor for knock-down and respective controls. For incubation with 5-, 12- and 15- HETEs and 9-, 13-HODEs (100ng/ml) in the presence and absence of 4F (1\(\mu\)g/ml), lipoprotein deficient serum was used (2.5\%, Kalen Biomedical, LLC) for 2 hours. Cell proliferation was measured by the MTT Cell Proliferation Assay (ATCC)\(^4\). Proliferation rate without serum replenishment served as a negative control. All experiments were repeated with at least 8 replicates and at least 3 independent times.
Proliferation was also assessed by regular immunofluorescence staining using Ki-67 antibody. HPASMCs were fixed in acetone for 15 minutes at –20°C, washed with PBS, and incubated with 10% normal goat serum in PBS+0.1% Triton for 30 min to block the background. The cells were then incubated with anti-Ki-67 (Millipore AB9260, 1:100 dilution) primary antibody in PBS+0.1% Triton+ 1% normal goat serum (NGS) at 4°C overnight, washed with PBS+0.1% Triton three times, incubated with the goat anti-rabbit Alexa 568 (1:1000) secondary antibody as well as DAPI (1:2000) in PBS+0.1% Triton+ 1% NGS at room temperature for 1 hr. The cells were washed with PBS and were then mounted using Prolong gold (Molecular Probes). Imaging was achieved using a laser scanning confocal microscopes (Nikon). For the assessment Ki-67 positive cells, the ratio of Ki-67+DAPI positive cells over only DAPI-positive cells was measured using ImageJ.

Chromatin immunoprecipitation

ChIP was carried out using the ChIP-IT high sensitivity kit from Active Motif. Briefly, the chromatin fragments, derived from the sonication of untreated HPASM cells, 15-HETE treated alone or together with 4F (48 hours) were immunoprecipitated with 4ug of antibody against RNA pol II (sc-899X), IgG (sc2027X) and RXRα (sc553X, Santa Cruz Biotechnology Inc.; Santa Cruz, CA). DNA extraction was performed using the same kit. The samples were analyzed by qRT-PCR and the results were presented as the mean ± SE of three independent experiments normalized to input. The primer sequences are given in Supplementary Table 2.

Plasma Sample Preparation for Determination of Oxidized Lipids

A 100μL volume of plasma sample was transferred to a 2mL polypropylene tube, and spiked with 100μL of internal standards mixture (15(S)-HETE-d8, 13(S)-HODE-d4, 10ng/ml each) in methanol. Subsequently, the pH of the samples was adjusted to ~ pH 3.0 using acidified (HCl)
water. The samples were left for 15 min on ice for complete acidification and equilibration. For analysis of plasma total (free + esterified) HETEs/ HODEs, the samples were hydrolysed with 1 mol/L potassium hydroxide in water at 37°C for 30 minutes before the acidification. The resulting sample was loaded onto a preconditioned 1cc Oasis HLB solid-phase extraction (SPE) cartridge on a vacuum manifold (Waters). The SPE cartridge was equilibrated with 1 ml methanol followed by 1 ml water before the sample load. The sample was slowly loaded on the cartridge, and the cartridge was washed with 1 ml 5% methanol in water. HETEs/HODEs, 14,15-EET and arachidonic acid were subsequently eluted with 1 ml methanol. The eluate was then evaporated to dryness under a stream of argon. 100 μl of methanol was added to the dried extract, vortexed for 30 s, and the reconstituted extract was centrifuged at 13,200 rpm for 20 min at 4°C to remove any precipitate that could clog the LC/MS/MS instrument. The resulting supernatants were transferred to autosampler vials and processed for LC/MS/MS analysis. For quantification of HETEs/HODEs, 300 μl of chloroform/methanol (2:1, v/v) containing 0.01% BHT was added to 100 μl plasma. After the solution was thoroughly mixed and centrifuged, the lower chloroform phase was collected. Additional chloroform was added to the upper phase to remove residual lipids. The solution was mixed and centrifuged, and the lower phase was transferred to the previously isolated chloroform phase.

**Lipid extraction and sample preparation for leukotrienes analysis**

Plasma samples (75 μl) were brought up to 1 ml with ddH20 and 5 ng of LTB4-d4 (Cayman 320110) per sample was added as internal standard. The samples were acidified by the addition of 1 μl of 98% formic acid; BHT was added to a final concentration of 20 μM; the samples were placed on ice for 15 min and then centrifuged. Oasis HLB 3 cc (60 mg) solid-phase extraction cartridges (Waters, 186001880) on a vacuum manifold were equilibrated with 2 ml methanol
followed by 2 ml water. The samples were slowly loaded on the cartridges under vacuum. After
the samples had completely flowed through at 0.5 ml/min, the cartridges were washed with 2ml
5% methanol in water. Lipids were then eluted from the cartridges with 2 ml methanol. The
eluates were evaporated to dryness under argon at 37°C. Each dried lipid extract was then
resuspended in 100 µl of methanol using gentle vortexing. The samples were transferred to
autosampler vials (Fisher scientific, 03-396-74) for LC/MS/MS analysis.

**LC/MS/MS Analysis**

LC/MS/MS was performed using a mass spectrometer (4000 QTRAP; Applied Biosystems,
Foster City, CA) equipped with electrospray ionization (ESI) source. The HPLC system utilized
an Agilent 1200 series LC pump equipped with a thermostatted autosampler (Agilent
Technologies, Santa Clara, CA). Chromatography was performed using a Luna C-18(2) column
(3µm particle, 150X3.0mm; Phenomenex, Torrance, CA) with a security guard cartridge (C-18;
Phenomenex) at 40°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase
B consisted of 0.1% formic acid in acetonitrile. The auto sampler was set at 4°C. The injection
volume was 10µl except for (±)14, 15- EET when 50µl was injected; the flow rate was controlled
at 0.4mL/min. The gradient program was as follows: 0-2min, 50% B; 2-3min, linear gradient
from 50% to 60% B; 3- 15min, linear gradient from 60-65% B; 15-17min, 65% B; 17-19min,
linear gradient from 65-100% B; 19-21min 100% B; 21-23min, linear gradient from 100% to
50% B; 23- 27min, 50% B. The data acquisitions and instrument control were accomplished
using Analyst 1.4.2 software (Applied Biosystems). Detection was accomplished by using the
multiple reaction monitoring (MRM) mode with negative ion detection; the parameter settings
used were: ion spray voltage=- 4500 V; curtain gas=25 (nitrogen); ion source gas 1=45; ion
source gas 2 =55; ion source gas 2 temperature= 450°C. Collision energy, declustering potential
and collision cell exit potential were optimized for each compound to obtain optimum sensitivity. The transitions monitored were mass-to-charge ratio (m/z): m/z 295.1-194.8 for 13-HODE; 295.0-171.0 for 9-HODE; 319.1-219.0 for 15- HETE; 319.1-115.0 for 5-HETE; 327.1-226.1 for 15(S)- HETE-d8; 299.0-197.9 for 13(S)-HODE-d4; 310.9 -166.5 for 13(S)-HPODE, 303.1-259.2 for arachidonic acid; 339.3-197.0 for LTB4-d4; 335.3-195.0 for LT5B4; 438.3-333.1 for LTE4; and 624.4-271.9 for LTC4. Standard curves for LTB4 (Cayman 10007240), LTC4 (Cayman 10007241), and LTE4 (Cayman 10007242) were generated from Lipid MAPS standards.

Supplemental Figure legends

Figure 1. Representative chromatograms depicting the picks for measurements of plasma oxidized lipids.

Figure 2. 4F treatment does not alter the levels of plasma leukotrienes in PH rats. The relative levels of various leukotrienes LTB4 (A), LTE4 (B) and LTC4 (C) were measured in the plasma of CTRL, PH and 4F rescued rats in the MCT model by mass spectrometry. **p<0.01 vs. CTRL (N= 5-8 animals per group).

Figure 3. 4F inhibits 15-HETE-induced proliferation of HPASMCs. Gain of miR193 inhibits proliferation of HPASMCs. A, HPASMCs were serum starved overnight and proliferation was induced by serum replenishment with lipoprotein deficient serum (5%) in the presence of 15- HETE (100ng/ml) alone or with 4F (1µg/ml) for 48 hours. Cell proliferation was measured using Ki-67 antibody. Proliferation rate without serum replenishment served as a negative control. B, HPASMCs were serum starved overnight and proliferation was induced by serum replenishment with lipoprotein deficient serum (5%) in the presence of 4F (1µg/ml) alone (4F group), or transfected with miR193 mimic oligonucleotides (50nM, miR193-OE group),
miR193 inhibitor oligonucleotides (50nM, miR193-KD group), or scrambled mimic and inhibitor control for 48 hours. Cell proliferation was measured using Ki-67 antibody. For both panels *p<0.05, **p<0.01, ***p < 0.001, N= 3 independent experiments.

**Figure 4.** Overexpression of miR193 in HPASMCs results in ~5 fold upregulation of miR193 transcript levels compared to scrambled mimic. miR193 expression is assessed in HPASMCs transfected with miR-193 mimic or scrambled mimic *p<0.05 vs. CTRL, N= 3 independent experiments.

**Figure 5.** Exogenous overexpression of miR193 in the lungs of PH rats is primarily in the pulmonary arteries. miR193 expression is assessed in the total lung, parenchyma and pulmonary arteries of MCT-induced PH rats treated with miR193 mimics. *p<0.05, N= 3 animals per group.

**Figure 6.** The LV weight and LV weight/body weight ratio is not changed among CTRL, PH, 4F and miR193 rescue groups. LV weight and LV weight/body weight ratio was assessed in CTRL, PH, 4F and miR193 rescue groups. (N= 5-6 animals per group, p=n.s.).

**Figure 7.** The expression of the transcription factor Max was not induced by HETEs and HODEs. Max transcript levels were assessed in HPASMCs treated with 5-, 12-, 15- HETES or 9-, 13- HODES for 48 hours in the absence or presence of 4F by qRT-PCR. ***p<0.001 vs. CTRL, #p< 0.05vs. HETE/HODE N= 3 independent experiments.

Reference List


Supplemental Figure 4

Relative miR193 Expression in HPASMC

CTRL-mimic  miR193-mimic

*
Supplemental Figure 5

miR193-OE in MCT rats

Relative miR193 Expression

- Total Lung
- Parenchyma
- Pulmonary Artery

* indicates statistical significance.
Supplemental Table 1: Clinical characteristics of PAH patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Buffy coat</th>
<th>Lung tissue</th>
<th>HPASMC Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=10)</td>
<td>IPAH (n=9)</td>
<td>Control (n=7)</td>
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<tr>
<td>Gender [female (%)]</td>
<td>70%</td>
<td>100%</td>
<td>14.30%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.7±2.2</td>
<td>60±9</td>
<td>62.7±7</td>
</tr>
<tr>
<td>Functional class [n (%)]</td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1 (11.11%)</td>
<td>6 (66.66%)</td>
</tr>
<tr>
<td>CO (L/min)</td>
<td>-</td>
<td>4.6±0.4</td>
<td>-</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>2.8±0.2</td>
<td>2.2±0.3</td>
<td>781±150</td>
</tr>
<tr>
<td>PVR (dyn×sec/cm⁵)</td>
<td>-</td>
<td>2.8</td>
<td>781±150</td>
</tr>
<tr>
<td>mPAP (mmHg)</td>
<td>48.8±6.5</td>
<td>764±78</td>
<td>47±0.4</td>
</tr>
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<td>PAPsys (mmHg)</td>
<td>81.3±10.1</td>
<td>75±2.4</td>
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<td>RAP (mmHg)</td>
<td>6.6±1.3</td>
<td>53±4.8</td>
<td>17</td>
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<td>SVO₂ (%)</td>
<td>64.3±3.7</td>
<td>224±26</td>
<td>-</td>
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<tr>
<td>6MWD (m)</td>
<td>330±44</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medication [n (%)]</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin receptor</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>antagonist</td>
<td>2 (22.22%)</td>
<td>3 (60%)</td>
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<tr>
<td>PDE5 inhibitor</td>
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<td>-</td>
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<tr>
<td>Epoprostanol</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
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</table>

Values are means ± SEM. IPAH, idiopathic pulmonary arterial hypertension; SSc-PAH, systemic scleroderma associated pulmonary arterial hypertension; CO, cardiac output; CI, cardiac index; PVR, pulmonary vascular resistance; mPAP, mean pulmonary arterial pressure; RAP, right atrial pressure; SvO₂, venous oxygen saturation; 6MWD indicates 6-minute walk distance; and PDE5, phosphodiesterase-5. Some patients take more than 1 type of medication.
**Supplemental Table 2.** Primers for quantitative expression analysis of RT-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ALOX5-F(rat)</td>
<td>CAGCAACACTATTTCTGAGAGAGTC</td>
</tr>
<tr>
<td>ALOX5-R(rat)</td>
<td>TCACTTTGAACGCACCCA</td>
</tr>
<tr>
<td>ALOX12-F(rat)</td>
<td>AGACAAACAGACCTACCGCTG</td>
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<tr>
<td>ALOX12-R(rat)</td>
<td>AGATCTGATCAAAGTCTTCCAGAC</td>
</tr>
<tr>
<td>ALOX15-F(rat)</td>
<td>CAGGAAACATAGGCCAGAGGA</td>
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<tr>
<td>ALOX15-R(rat)</td>
<td>GCAGTTGAAGTCATCTAGGCTT</td>
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<tr>
<td>IGF1R-F(rat)</td>
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<td>IGF1R-R(rat)</td>
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<td>ALOX5-F(mouse)</td>
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<tr>
<td>ALOX5-R(mouse)</td>
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<tr>
<td>ALOX12-F(mouse)</td>
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<tr>
<td>ALOX12-R(mouse)</td>
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<td>ALOX15-F(mouse)</td>
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<tr>
<td>ALOX15-R(mouse)</td>
<td>CTTTCCAGCGGTCACAG</td>
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<tr>
<td>IGF1R-F(mouse)</td>
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<tr>
<td>IGF1R-R(mouse)</td>
<td>ATCTTCGCGCTACCAGCA</td>
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<tr>
<td>miR193-promoter F (human)</td>
<td>GCTGAGGGCTGGTCT</td>
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<tr>
<td>miR193-promoter R (human)</td>
<td>GGGGGCCGAGAAGCTG</td>
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Supplemental Table 3. Metabolic panels of CTRL and 4F treated rats

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<th>CTRL</th>
<th>4F</th>
<th>P value</th>
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<td></td>
<td>n=6</td>
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<tr>
<td>ALB</td>
<td>3.12±0.05</td>
<td>3.07±0.06</td>
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<tr>
<td>ALP</td>
<td>227.00±20.31</td>
<td>226.33±20.29</td>
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</tr>
<tr>
<td>ALT</td>
<td>45.83±4.19</td>
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<td>AST</td>
<td>115.00±17.33</td>
<td>102.00±10.79</td>
<td>0.54</td>
</tr>
<tr>
<td>TBIL</td>
<td>0.10±0.00</td>
<td>0.10±0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>BUN</td>
<td>17.00±0.58</td>
<td>16.83±1.08</td>
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<tr>
<td>CO2</td>
<td>22.83±0.54</td>
<td>23.33±0.99</td>
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<tr>
<td>CA</td>
<td>10.10±0.18</td>
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<td>CREA</td>
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<td>GLU</td>
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<td>185.67±45.22</td>
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<td>NA</td>
<td>144.67±0.67</td>
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<tr>
<td>K</td>
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<td>CL</td>
<td>101.83±0.75</td>
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Values are means ± SEM. ALB-Albumin, ALP-Alkaline Phosphatase, ALT-Alanine Aminotransferase, AST-Aspartate Aminotransferase, TBIL-Total Bilirubin, BUN-Urea Nitrogen, CO2-Bicarbonate, CA-Calcium, Crea-Creatinine, GLU-Glucose, TP-Total Protein, Na-Sodium, K-Potassium, Cl-Chloride.
**Supplemental Table 4.** Plasma HETEs, HODEs and leukotrienes measurements in CTRL, PH and 4F rescued rats

<table>
<thead>
<tr>
<th></th>
<th>CTRL N=6</th>
<th>PH N=6</th>
<th>4F N=6</th>
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<tbody>
<tr>
<td><strong>5-HETE</strong></td>
<td>672.54±14.69</td>
<td>777.57±24.83***</td>
<td>656.50±35.10###</td>
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<tr>
<td><strong>12-HETE</strong></td>
<td>7623.03±533.33</td>
<td>10597.14±289.93</td>
<td>9052.04±347.23&quot;</td>
</tr>
<tr>
<td><strong>15-HETE</strong></td>
<td>549.54±11.06</td>
<td>719.62±43.90***</td>
<td>618.00±19.34#</td>
</tr>
<tr>
<td><strong>9-HODE</strong></td>
<td>1392.89±231.78</td>
<td>2597.47±175.20</td>
<td>1491.52±336.57&quot;</td>
</tr>
<tr>
<td><strong>13-HODE</strong></td>
<td>2017.81±504.71</td>
<td>5135.15±571.68&quot;</td>
<td>2678.67±654.75&quot;</td>
</tr>
<tr>
<td><strong>LTB4</strong></td>
<td>615.067±67.47</td>
<td>895.067±39.57**</td>
<td>635.867±265.02</td>
</tr>
<tr>
<td><strong>LTE4</strong></td>
<td>3873.2±791.23</td>
<td>3209.867±1050.34</td>
<td>2070.4±662.32</td>
</tr>
<tr>
<td><strong>LTC4</strong></td>
<td>1303.733±91.90</td>
<td>1183.2±297.89</td>
<td>1152.533±238.47</td>
</tr>
</tbody>
</table>

Values are means ± SEM. CTRL, control; PH, pulmonary hypertension; 4F, 4F rescue group; 5-HETE, 5-Hydroxyeicosatetraenoic acid; 12-HETE, 12-Hydroxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acids; 9-HODE, 9-hydroxyoctadecadienoic acids; 13-HODE, 13-hydroxyoctadecadienoic acids; LTB4, Leukotriene B4; LTE4, Leukotriene; LTC4, Leukotriene C4; *p<0.05, **p<0.01, ***p<0.001 vs. CTRL; #p<0.05, ###p<0.001 vs. PH.
Supplemental Table 5. Right ventricular hemodynamic parameters measured by M-mode echocardiography.

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>PH</th>
<th>miR193-OE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=4 rats</td>
<td>N=5 rats</td>
<td>N=5 rats</td>
</tr>
<tr>
<td>RV WT (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
<td>0.64±0.02</td>
<td>1.27±0.08***</td>
<td>0.94±0.05***#</td>
</tr>
<tr>
<td>Systolic</td>
<td>0.81±0.01</td>
<td>1.42±0.06***</td>
<td>1.13±0.06***#</td>
</tr>
<tr>
<td>RV diameter (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
<td>1.72±0.05</td>
<td>2.66±0.12*</td>
<td>1.49±0.12</td>
</tr>
<tr>
<td>Systolic</td>
<td>1.11±0.03</td>
<td>2.61±0.18*</td>
<td>0.99±0.13###</td>
</tr>
<tr>
<td>RVEF (%)</td>
<td>68.22±0.83</td>
<td>29.67±0.88***</td>
<td>72±3.9###***#</td>
</tr>
<tr>
<td>RVFS (%)</td>
<td>35.30±0.66</td>
<td>13.24±0.45***</td>
<td>38.5±3.2***#</td>
</tr>
</tbody>
</table>

Values are means ± SEM. CTRL, control; PH, pulmonary hypertension; miR193-OE, miR193 overexpression; RV WT, right ventricular wall thickness; RVEF, right ventricular ejection fraction; RVFS, right ventricular fractional shortening. *p<0.05, **p<0.01, ***p<0.001 vs. CTRL; #p<0.05, ##p<0.01, ###p<0.001 vs. PH.