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

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Abstract

Background—Pulmonary Arterial Hypertension (PAH) is a chronic lung disease associated with severe pulmonary vascular changes. A pathogenic role of oxidized lipids such as hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs) is well established in vascular disease. Apolipoprotein A-I (apoA-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 PAH and the therapeutic action of 4F in PAH is not well established.

Methods and Results—We studied two different rodent models of Pulmonary Hypertension (PH); a monocrotaline (MCT) rat model and a hypoxia mouse model. Plasma levels of HETEs and HODEs were significantly elevated in PH. 4F treatment reduced these levels and rescued pre-existing PH in both models. MicroRNA analysis revealed that miR193-3p (miR193) was significantly downregulated in the lung tissue and in serum from both PAH patients and in PH rodents. *In-vivo* miR193 overexpression in the lungs rescued pre-existing PH and resulted in

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downregulation of lipoxygenases and insulin-like growth factor-1 receptor. 4F restored PH-induced miR193 expression via transcription factor retinoid X receptor alpha (RXRα).

Conclusions—These studies establish the importance of microRNAs as downstream effectors of an apoA-I mimetic peptide in the rescue of PH and suggest that treatment with apoA-I mimetic peptides, or miR193 may have therapeutic value.

Keywords

Oxidized lipids; ApoA-I mimetic peptide; 4F peptide; miR193-3p; Pulmonary Arterial Hypertension

Introduction

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[1]. 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[2], remodelling of small pulmonary arteries with diminished apoptosis, enhanced inflammation and fibrosis in the lung tissue[3]. 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[4,5]. 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[6]. 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[7] and therefore mitigate an anti-inflammatory response[8,9]. Although pulmonary hypertension (PH) is associated with inflammation, the role of HETEs and HODEs in PH is not fully understood.

MicroRNAs (miRNAs) are small non-coding, single-stranded RNAs 19–25 nucleotides long 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. MicroRNAs can downregulate gene expression by binding to the 3’ untranslated region of target mRNAs. This coupling results in translational inhibition or transcript degradation[10,11]. Recently, progress in delineating the important role of microRNAs in cardiovascular disease progression, including PH has been made[12–14]. Several miRs including miR-21, miR-204 and miR-328 have been reported to regulate pathogenic signalling in the development and progression of PH[15–17].

This study was designed to provide insights into the pathophysiology associated with increased lipoxygenase 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 pre-existing PH. We also used microRNA-microarray

analysis to identify novel miRNAs as downstream effectors of 4F. We found that miR193 is downregulated in the lung tissue and plasma in both PAH patients as well as in PH rodents. In vivo overexpression of miR193 in the lungs rescued pre-existing PH. 4F restored PH-induced miR193 expression, most likely via transcription factor RXRα regulating lipoxygenases and cell proliferation in PH.

Materials and methods

The online Supplemental Material provides more details on all methods.

Animals

Adult young male Sprague-Dawley rats (200–250g, 2–3 months old), and young male mice C57Bl/6 (age 2–3 months old) 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 previously given signed consent in agreement with the ethical protocol (CER 20773; 20735) as described[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 supplemental Table 1.

In-vitro overexpression and knockdown studies

Human Pulmonary Artery Smooth Muscle Cells (HPASMCs,passage 3–5) were cultured in Media 231 and transfected with scrambled controls, mimic-193 and inhibitor-193 oligonucleotides at a final concentration of 50nM.

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[20,21].

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.

Real time PCR

Total RNA from lungs was isolated using Trizol and reverse transcribed with gene-specific primers using the Omniscript RT kit (Qiagen). A microarray screen of microRNA expression in the total lung tissue of rats was performed using non-Affymetrix single channel arrays (MirBASE 17.0 MicroRNA Array Ocean Ridge Biosciences). The primer sequences are given in Supplemental Table 2.

Immunohistochemistry and imaging

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

Cell culture and Proliferation Assays

HPASMC 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 (Supplemental Table 1). PASMC phenotype was confirmed by using α-smooth muscle actin staining. Cells were cultured in Medium 231 in the presence or absence of 4F (D-4F, 1μg/ml) or miR193 mimic or inhibitor. HETE and HODE incubation (100ng/ml) in the presence and absence of 4F was achieved in lipoprotein deficient serum. Cell proliferation was measured by standard MTT Cell Proliferation Assay (ATCC) or Ki-67 antibody (Millipore AB9260).

Chromatin immunoprecipitation

ChIP and DNA extraction were carried out using the ChIP-IT high sensitivity kit from Active Motif. The samples were analyzed by qRT-PCR and the results were presented as the mean ± SE normalized to input.

Plasma Sample Preparation and **LC/MS/MS Analysis** for Determination of Oxidized Lipids and Leukotrine Analysis

Plasma samples for measurements of oxidized lipids as well as internal standards mixture were loaded onto a preconditioned 1cc Oasis HLB solid-phase extraction (SPE) cartridge on a vacuum manifold (Waters), and processed for LC/MS/MS analysis. For quantification of HETEs/HODEs, plasma samples were added to 300 μl of 2:1 v/v chloroform/methanol containing 0.01% BHT and similarly processed.

Plasma samples for leukotrines were acidified and BHT was added to a final concentration of 20 μM. The samples were loaded onto solid-phase extraction cartridges (Waters, 186001880) and processed for 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.

Statistical Analysis—Means were compared across groups using one way analysis of variance (ANOVA) for three or more groups or t-tests for two groups for data that followed the normal distribution and met variance homogeneity. Significance was assessed using the Tukey criterion for pairwise mean comparisons under the ANOVA model. Normality was assessed using the Kolmogorov-Smirnov test. When homogeneity of variance was not met under the ANOVA model, a robust ANOVA was carried out[22]. Survival curves were estimated using the Kaplan-Meier. When the data did not follow the normal distribution, p values were computed using the non parametric Kruskal- Wallis (Mann-Whitney) methods.

Two tailed p values < 0.05 were considered significant, after adjustment. Values are expressed as mean ± SE. Computations were carried out using SPSS SigmaStat for Windows, version 3.0 (SPSS Inc, Chicago Ill) and JMP version 11.0 (SAS Inc, Cary NC).

Results

4F rescues MCT-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 pre-existing PH, we started 4F therapy (50mg/kg/day) at day 21 (Fig. 1A), as we have previously shown that severe PH is already established by day 21 in MCT model (60 mg/kg) in rats[20,21,23]. 4F therapy rescued PH as RVSP (46.2±2.6mmHg in 4F *vs.* 68.0±7.9mmHg in PH, $p<0.01$) and RV hypertrophy index (0.4±0.02 in 4F *vs.* 0.68±0.01 in PH, $p< 0.001$) were significantly lower compared to untreated MCT rats (Fig. 1B, C). In non-PH rats, 4F therapy had no effect on RVSP (27.1±1 in 4F-CTRL vs. 26.0±1.6 in CTRL, *p*=n.s.), RV hypertrophy index (0.26±0.01 in 4F-CTRL vs. 0.31±0.03 in CTRL, *p*=n.s.), kidney or liver function (Fig. 1B, C, Supplemental Table 3). 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, sharply increased to 45% by day 28 and to 75% by day 30 (Fig. 1D). Moreover, pulmonary arteriolar medial hypertrophy was reversed in 4F treated animals (Fig. 1E).

4F therapy was also very effective in rescuing hypoxic mice as the RVSP (25.1±2.7mmHg in 4F *vs.* 34.5±3.5mmHg in PH, $p<0.05$) and RV hypertrophy index (0.35±0.01 in 4F *vs.* 0.42± 0.03 in PH, $p<0.05$) were both significantly decreased when the hypoxic mice received daily 4F injection from day 14 to 21 compared to untreated hypoxic mice (Fig. 1F–H)

4F rescue of PH is associated with decreased plasma levels of Oxidized Lipids

PH was associated with significantly elevated plasma HETEs and HODEs which was reversed by 4F therapy in PH rats (Fig. 2A–E, Supplemental Fig. 1 and Table 4). Among the leukotrines, plasma LBT4 level was only significantly elevated in PH rats and 4F treatment was not able to reverse LBT4 level (Supplemental Fig. 2). In summary, severe PH is associated with an increase in plasma levels of HETEs and HODEs which is restored by 4F treatment.

MiR193 is downregulated in the lung and plasma in experimental PH as well as 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, microRNA microarray (non affymetrix) was performed in CTRL, PH and 4F rescue group at day 30. The expression of 366 microRNAs was successfully detected. Multiple microRNAs were differentially regulated between control and PH group. Among these, previously reported miR-21[17] (3.8 folds up), miR193 (2.7 folds down) miR322 (2.6 folds down) and miR451 (2.6s fold down) were dysregulated in PH (Fig. 3A). 4F treatment was not able to restore miR21 (Fig. 3B), miR322 or miR451, but was very efficient in restoring PH-induced miR193 downregulation both in MCT and hypoxia model (Fig. 3C, D). 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 to control subjects (Fig. 3E). Interestingly, miR193 was also detectable in circulating blood and was significantly lower in PH rats and idiopathic PAH patients compared to controls (Fig. 3F, G).

4F treatment and miR193 overexpression inhibits proliferation of HPASMCs

PH is characterized by an increase in the proliferation of pulmonary artery smooth muscle cells[24]. Treatment of HPASMCs with oxidized lipids stimulated proliferation which was prevented by 4F (Fig. 4A, Supplemental Fig. 3A).

Next we examined whether overexpression of miR193 could inhibit proliferation in HPASMCs. Transfection with miR193-mimic lead to ~5 fold increase in expression level of miR193 (Supplemental Fig. 4). Interestingly, both 4F treatment and overexpression of miR193 in HPASMCs inhibited cell proliferation, whereas miR193 knockdown stimulated proliferation (Fig. 4B, Supplemental Fig. 3B). Overexpression of miR193 in HPASMCs isolated from idiopathic PAH patient also resulted in inhibition of proliferation, whereas knockdown of miR193 in HPASMCs isolated from CTRL subjects stimulated proliferation (Fig. 4C).

Gain of miR193 rescues PH in both MCT and hypoxia model

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 weight at days 17, 21 and 26 in the MCT model or at days 14 and 18 in the hypoxia model (Fig. 5A, B). To verify that miR193 was indeed overexpressed in the lung tissue, we measured miR193 pulmonary levels by qRT-PCR. Since miR193 is downregulated in PH, our results revealed that miR193 delivery led to ~7 fold and ~2.5 fold increase in its expression in MCT and in the hypoxia model respectively, when compared to the PH group (Fig. 5C, D). The overexpression of miR193 in the lung tissue was primarily localized to the pulmonary arteries (Supplemental Fig. 5). Gain of miR193 in the lungs effectively rescued pre-existing PH as the RV systolic pressures (38.2±7.2mmHg in miR193-OE *vs.* 73.3±7.6 in PH in MCT-rats, $p<0.01$; and 25.5±0.9mmHg in miR193-OE *vs.* 33.2±2.5 in PH in hypoxic mice, $p<0.05$ Fig. 5E, F), and RV hypertrophy index (0.38±0.03 in miR193-OE *vs.* 0.68±0.01 in PH in MCT-rats, $p<0.001$; and 0.32±0.02 in miR193-OE *vs.* 0.40±0.02 in PH, $p<0.01$ in hypoxic mice) were significantly reduced (Fig. 5G, H). There was no significant change in the LV weight or LV weight/body weight ratio among CTRL, PH, miR193-OE and 4F rescue groups (Supplemental Fig. 6). MiR193 gain also prevented the increased lung arteriolar muscularization and adverse right ventricular remodelling occurring in PH rats (Fig. 5I, Supplemental Table 5).

MiR193 targets lipoxygenases and insulin-like growth factor-1 receptor (IGF1R) *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 MCT and hypoxia models of PH, thus implicating a role for miR193 in inhibiting the

production of oxidized lipids (Fig. 6A, B). 4F was also as efficient as miR193-OE in reversing PH-induced upregulation of these lipoxygenases (Fig. 6A, B).

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 signalling is vital for proliferation, survival, and migration of many cell types, including smooth muscle cells. IGF1R signalling has also been implicated in PH[25,26]. Indeed, miR193 gain was able to suppress the levels of IGF1R transcripts in both MCT rats and hypoxia mice (Fig.6A, B). Administration of 4F reversed PH-induced upregulation of IGF1R in the MCT but not in the hypoxia model (Fig. 6A, B).

4F induces the expression of miR193 in HPASMCs by suppressing PPAR-RXRα signalling pathway

The apolipoproteinA-I mimetic peptide 4F is known to bind and decrease levels of oxidized lipids[6]. In order 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 (100ng/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 to the control cells (Fig. 7A). In the presence of 4F, the various HETEs and HODEs were unable to downregulate miR193 (Fig. 7A). To determine the molecular mechanism by which HETEs and HODEs downregulate miR193 expression, we examined the expression of RXRα and myc-associated factor X (MAX), the top two transcription factors that have previously been validated to bind to the promoter region of miR193 and thereby suppress miR193 expression in cancer cells[27]. We found that various HETEs and HODEs induce the expression of the transcription factor RXRα in HPASMCs and this induction is suppressed by 4F (Fig. 7B).

However, expression of the transcription factor MAX was not induced by HETEs and HODEs (Supplemental Fig. 7). To validate that RXRα 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 RXRα on the promoter of miR193 which is inhibited in the presence of 4F (Fig. 7C).

Exogenous HETE therapy is sufficient to induce PH

Our *in-vitro* data show that HETEs stimulates proliferation of HPASMC 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 RVSP and miR193 expression in the lung tissue by feeding mice with 15-HETE containing diet for 3 weeks. We found that RVSP was significantly higher in mice on the 15-HETE diet compared to mice on regular chow (34.3±2.8mmHg in 15-HETE diet *vs.* 22.1±2.4mmHg in CTRL, $p<0.05$), Fig. 8A). The lung weight was also significantly increased in mice on the 15-HETE diet (0.27±0.02gr in 15-HETE diet *vs.* 0.19±0.01gr in CTRL, $p<0.05$, Fig. 8B). Lastly, we found that 15-HETE diet significantly downregulated the expression of miR193 in the lungs of mice compared to controls (Fig. 8C)

Discussion

Here, we provide evidence that the oxidized metabolites of arachidonic and linoleic acids, including HETEs and HODEs, were elevated in a rodent model of PH (Fig. 2). Treatment with the HDL mimetic peptide 4F was able to effectively reduce the levels of these oxidized metabolites in this rodent model of PH (Fig. 2). More importantly, we found that 4F therapy, starting after the establishment of severe PH, is very effective in rescuing advanced PH in both MCT and hypoxia-induced animal models (Fig. 1). We also report that increasing oxidized fatty acids levels *in-vivo* is sufficient to induce PH in mice (Fig. 8). Our findings implicate for the first time the involvement of oxidized metabolites in the etiology of PH. Additionally, we have identified microRNA 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 healthy control animals (Fig. 3). Overexpression of miR193 in the lungs of MCT-treated rats and chronically hypoxic mice rescued pre-existing PH (Fig. 5), thus confirming the important role of this microRNA in PH. We also found that miR193 gain of function in cultured human pulmonary smooth muscle cells from IPAH patients attenuates proliferation, whereas miR193 knockdown stimulates proliferation in cells isolated from control subjects (Fig. 4C). The lipoxygenase pathway is one of the key pathways regulated by miR193, as 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 (Fig. 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 RXRα, a known inhibitor of miR193[27]. However, ablation of RXRα induction by 4F restored miR193 expression level (Figs. 7, 8).

Oxidized phospholipids (Ox-PL) are a major class of oxidized lipids that have pro-inflammatory properties. Ox-PLs are also associated with DNA damage which is implicated in PAH etiology[19] and are found in the vasculature of animal models of atherosclerosis and in many other inflammatory diseases[28,29]. OX-PLs trigger vascular and nonvascular cells to initiate an inflammatory reaction that involves activation of cytosolic phospholipase A_2 and the 12-lipoxygenase pathway[30]. Oxidized metabolites of arachidonic and linoleic acids can be generated by specific oxidation by enzymes including lipoxygenases[31,32]. These oxidized metabolites, such as 12-HETE act similarly to Ox-PL in the induction of inflammation[33,34]. Studies have shown that normal HDL can prevent the inflammatory reaction characteristic of atherosclerosis and this is associated with decreased levels of oxidized lipids both *in-vitro* and *in-vivo*[4,28,35,36]. ApoA-I mimetic peptides, such as 4F are 18-amino-acid (AA) peptides that do not have sequence homology with apoA-I, but mimic the class A amphipathic helixes contained in apoA-I. The 4F peptide is very effective in mitigating inflammation by binding oxidized lipids with very high affinity and removing these from tissues[6,37–39]. Administration of the 4F peptide has shown beneficial effects in many pathological conditions in animal models including type I diabetes, type II diabetes and obesity, hyperlipidemia and sickle cell induced vascular dysfunction[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 lead 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. Since leukotrienes are also produced as metabolites of arachidonic acid, we examined the effect of 4F treatment on the levels of leukotrienes. Among the three leukotrienes tested, the plasma LTB4 level was the only one that was significantly higher in PH but 4F was not able to restore LBT4 level (Supplemental Fig. 2), suggesting that regulation of leukotrienes by 4F does not play a role in PH.

The role of a few microRNAs has recently been investigated in the development of PH[45]. MiR21 induction was found to repress RhoB and Rho-kinase activity and caused decreased angiogenesis and vasodilation, thereby aggravating symptoms of PH[17]. 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 (NFAT) causing PAH-PASMC proliferation and resistance to apoptosis[15]. The role of miR328 has been well studied in PH resulting from chronic hypoxia. MiR328 was shown to play a key role pulmonary arterial constriction and remodelling by regulating L-type calcium channel-α1C expression in hypoxic PH[16]. However, the role of miR193 in PH has not previously been studied. We focused on miR193 because of its significant downregulation in PH and its remarkable normalization/induction following 4F treatment. The fact that miR193 displayed a similar aberrant expression in two 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 remodelling and rescue of PH via its direct effect on lipooxygenases and oxidized lipid production. Recent studies have implicated the role of miR193 in cancer-related events, including an inhibitory effect on cell proliferation, growth and cell cycle progression, and a pro-apototic effect in various cancer cell lines by regulation of serine/arginine-rich splicing factor 2 (SRSF2), E2F transcription factor 1 (E2F1) and an anti-apoptotic Bcl-2 family member Mcl-1[46]. In our studies, we report that miR193 regulates various lipoxygenases including ALOX-5,-12 and -15 in two rodent models of PH (Fig. 6). Although, we found that PH regulates a number of microRNAs (miR193; miR21; miR322), the regulation of oxidized lipids by 4F is mainly mediated through miR193 (Fig. 3).

Several circulatory miRs have been reported to serve as plasma biomarkers[47,48]. Our data shows that miR193 is expressed in the buffy coats of human healthy subjects and in rats and its level is downregulared in the blood of both idiopathic PAH patients and PH rats (Fig. 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 microRNAs by transcription factors[49,50]. Aberrations in this important step of microRNA regulation can contribute to the onset and progression of many diseases[51]. Peroxisome Proliferator-Activated Receptors (PPARs) are ligand-inducible transcription factors which can be activated by components of oxidized low-density

lipoproteins including 15-HETE[52]. PPARs heterodimerize with Retinoid X receptor alpha (RXRα) to regulate expression of many genes[53,54]. Interestingly, the transcription factor RXRα has previously been reported to inhibit the expression of miR193 by directly binding to its promoter in cancer cells[27]. We provide evidence that HETEs and HODEs induce the expression of RXRα in pulmonary artery smooth muscle cells. This induction results in an enrichment of RXRα on the miR193 promoter, thus causing its subsequent downregulation. However, 4F can decrease the overall content and binding of RXRα to miR193 promoter by sequestering HETEs and HODEs, ultimately leading to miR193 induction.

In a search for potential in silico targets of miR193 (targetscan.org), we also identified insulin growth factor 1 receptor (IGF1R) as a novel target. IGF1R signalling can regulate various biological pathways including cell growth, migration and differentiation[26]. Repression of IGF1R by miR-328 and its involvement in apoptosis of HPASMCs in PH has been previously reported[16]. The action of miR193 on IGF1R was similar to the response to 4F in the MCT model of PH, but not in the hypoxia mouse model (Fig. 6). This example was the only one that we found in which the action 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 HDL mimetic peptide 4F can successfully rescue advanced PH in two different models of PH by inducing the expression of miR193, most likely via the transcription factor RXRα, which in turn inhibits various lipoxygenases. MiR193 inhibits HPASMC proliferation possibly by downregulating IGF1R (Fig. 8). More importantly, gain of miR193 inhibits proliferation in pulmonary smooth muscle cells from idiopathic PAH patient (Fig. 4C). These studies further establish the importance of microRNAs in PH and suggest that treatment with apoA-I mimetic peptides may have therapeutic value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of Interest Disclosures: AMF, MN and STR are principals in Bruin Pharma and AMF is an officer in Bruin Pharma. SP is a Fonds de recherche du Québec-Santé (FRSQ) clinical scientist in Québec, Canada and SB holds Canada Research Chairs.

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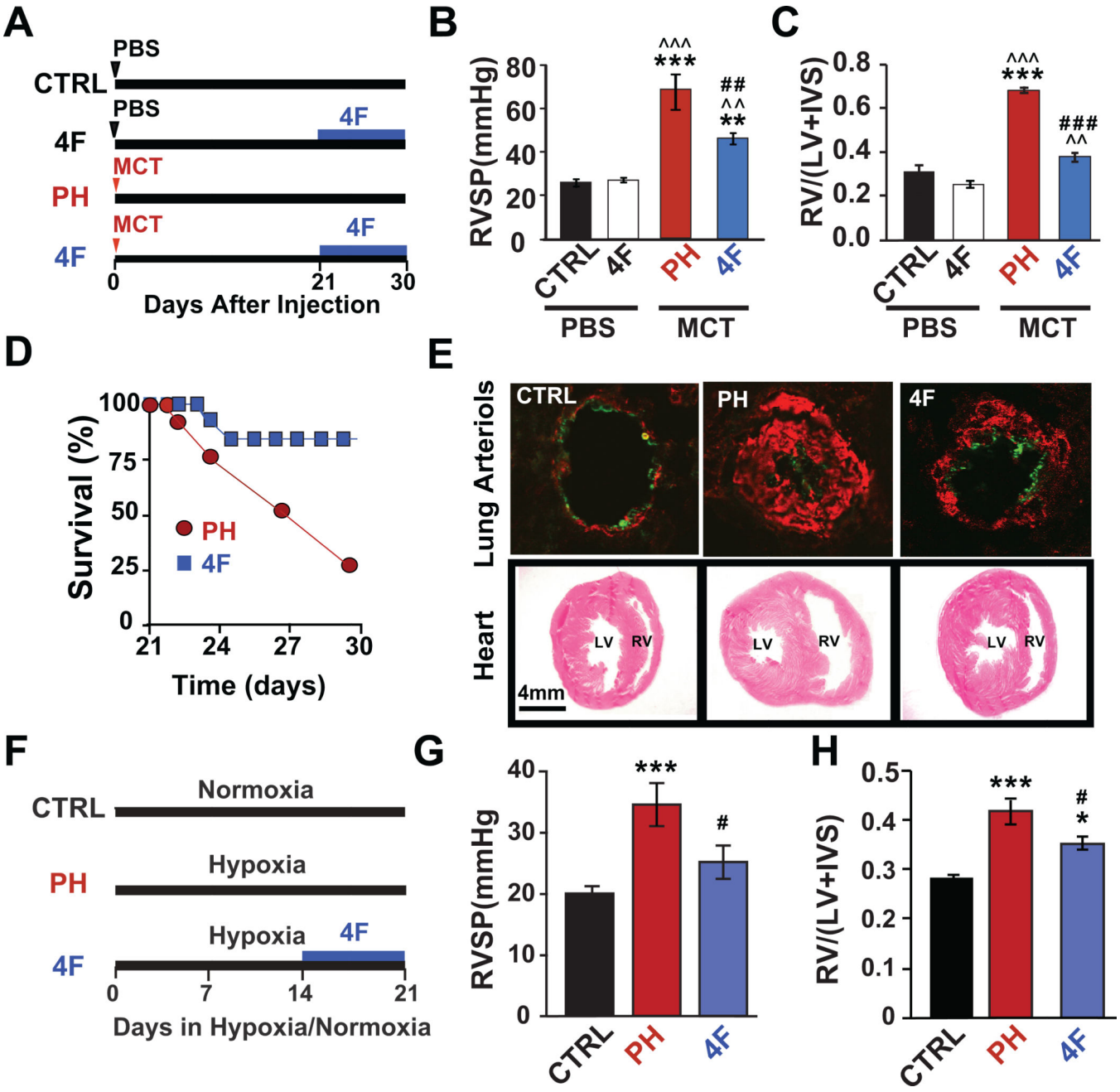

Figure 1.

4F rescues MCT-induced PH in rats and hypoxia-induced PH in mice by improving cardiac and pulmonary structure and function. **A**, Experimental protocol for MCT model: male rats were injected with monocrotaline (MCT) or phosphate-buffered saline (PBS) at day 0. The thick horizontal lines represent the length of each experimental group. The MCT injected animals were left untreated to develop pulmonary hypertension (PH group) or treated daily with 4F from day 21 to day 30. The PBS injected rats were either left untreated (CTRL) or received 4F from day 21 to day 30. All of the rats were sacrificed at day 30. B, RV systolic pressure (RVSP); and **C,** RV hypertrophy index (RV/(LV + IVS) where IVS is

interventricular septum and LV is the left ventricular wall. For panels B,C $^{**}p<0.01$, $^{***}p<0.001$ *vs.* CTRL; $^{\wedge\wedge}p<0.01$, $^{\wedge\wedge\wedge}p<0.001$ *vs.* 4F-CTRL; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* PH (N=5–8 rats per group); D, Survival plot. E, Lung arterioles and transverse whole heart sections. **F,** Experimental protocol for hypoxia model: male mice were subjected to hypoxia (10% O2) for 21 days and either left untreated (PH group) or treated with 4F from day 14 to day 21. Control mice were kept under normoxic conditions (21% O2). The horizontal lines represent the length of each experimental group. All mice were sacrificed at day 21. **G**, RV systolic pressure (RVSP) and **H,** RV hypertrophy index (RV/(LV + IVS). For panels G,H $^{*}p<0.05$, $^{***}p<0.001$ *vs.* CTRL; $^{\#}p< 0.05$ *vs.* PH (N = 5–8 mice per group).

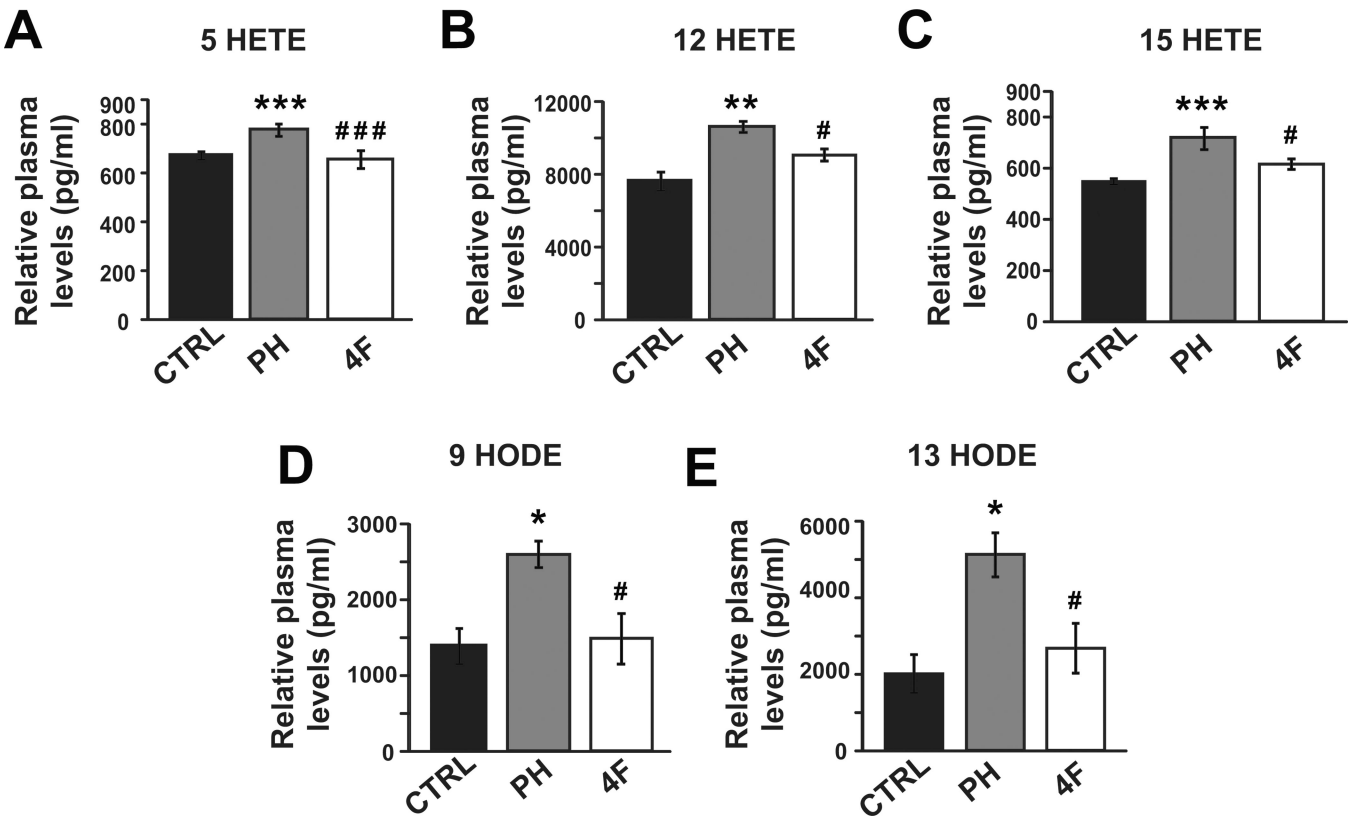

Figure 2.

4F therapy restores PH-induced increase in plasma levels of Oxidized Lipids. The relative levels of various oxidized lipids: **A,** 5-HETE; **B,** 12-HETE; **C,** 15-HETE; **D,** 9-HODE and **E,** 13-HODE were measured in the plasma of CTRL, PH and 4F groups in the MCT-induced model of PH by mass spectrometry. For panels A–E $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* CTRL; $^{\#}p< 0.05$, $^{\#\#\#}p< 0.001$ *vs.* PH (N= 6–8 animals per group).

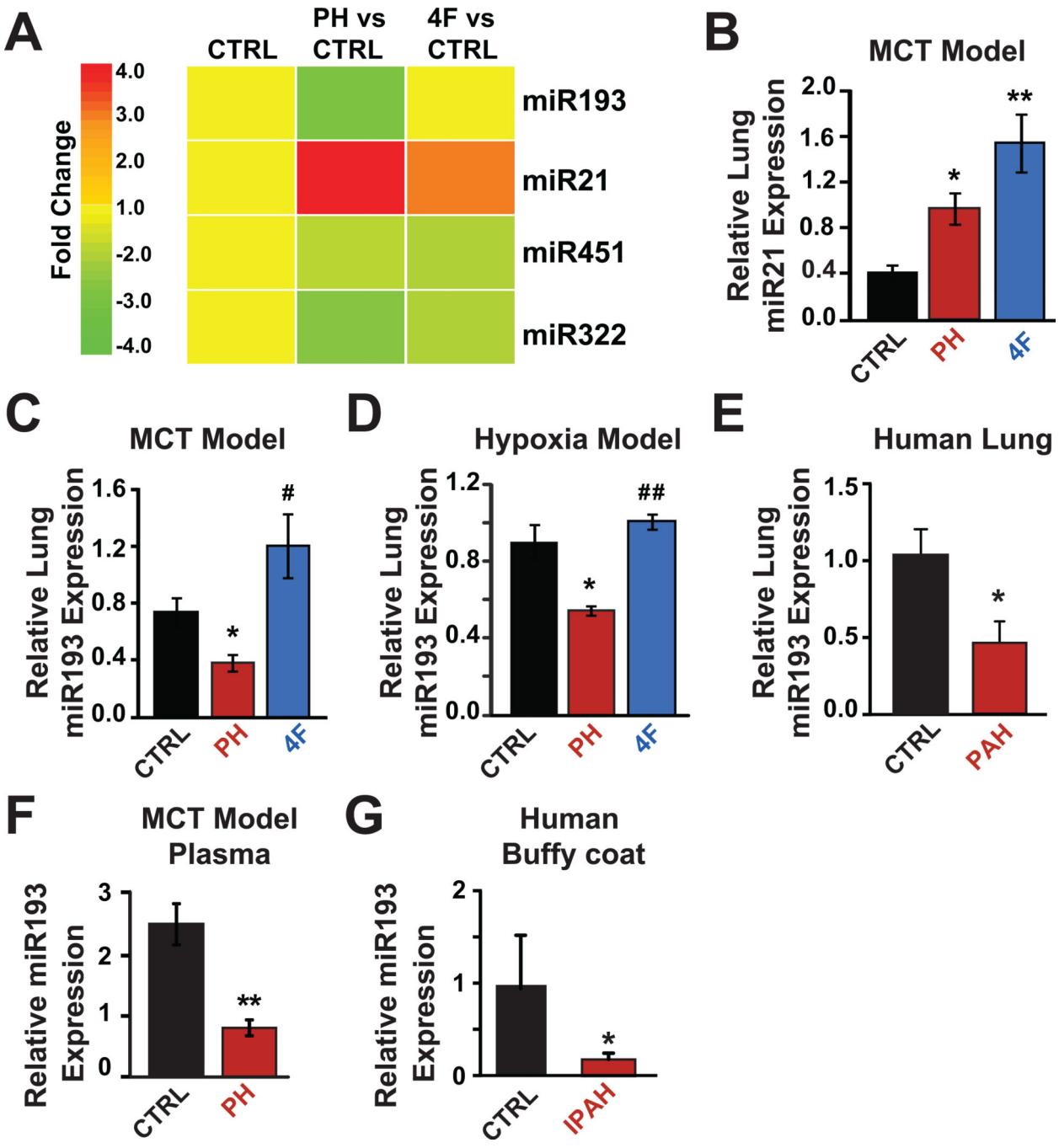

Figure 3.

MiR193 is downregulated in the lung and plasma in experimental PH as well as in PAH patients and 4F therapy restores lung miR193 levels. **A,** Heatmap generated from the microRNA microarray analysis in the lungs of CTRL, PH and 4F rescue rats in the MCT-model of PH. **B,** Relative miR21 expression in the lungs is confirmed using qRT-PCR analysis in the MCT model. Relative miR193 expression in the lungs is confirmed using qRT-PCR analysis in MCT model (**C**) and in 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 CTRL (n=8) and PH (n=6) rats in MCT model. **G,** MiR193 expression in the buffy coat of CTRL (n=10) and idiopathic PAH patients (n=9). For panels B–G $^{*}p< 0.05$, $^{**}p< 0.01$ *vs.* CTRL; $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ *vs.* PH (N= 5–8 animals per group).

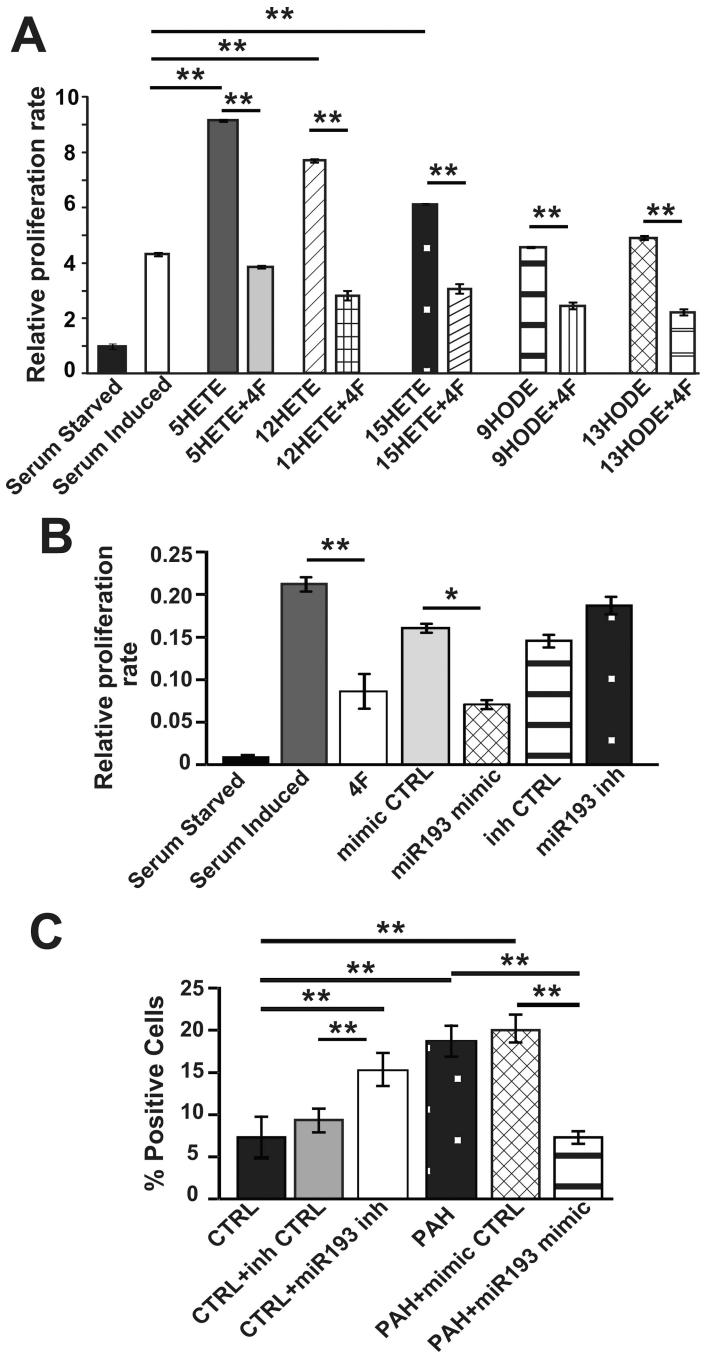

Figure 4.

4F inhibits HETEs and HODEs-induced proliferation of HPASMCs. Gain of miR193 inhibits proliferation of HPASMCs isolated from PAH patients. **A,** HPASMCs were serum starved overnight and proliferation was induced by serum replenishment with lipoprotein deficient serum (5%) in the presence of 5-, 12-, 15- HETE, 9- or 13-HODE (100ng/ml) alone or with 4F (1μg/ml) for 48 hours. Cell proliferation was measured by the MTT Cell Proliferation Assay. 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. **C**, HPASMCs isolated from idiopathic PAH or control subjects (Supplemental Table 1) and cultured in DMEM supplemented with FBS and 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. The relative proliferation was measured using Ki-67. For panels A–C $^{*}p<0.05$, $^{**}p<0.01$, N=3 independent experiments for each HPASMCs line.

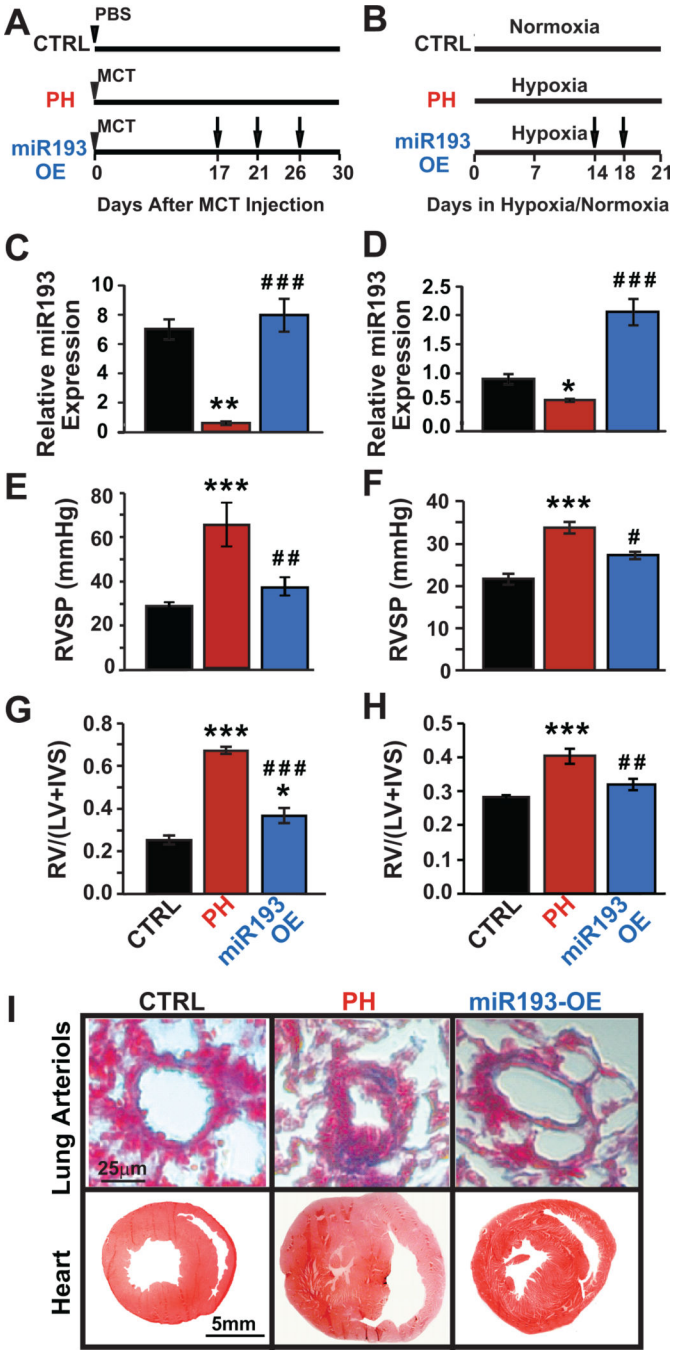

Figure 5.

Gain of miR193 rescues PH by improving cardiac and pulmonary structure. **A,** Experimental protocol for MCT model: male rats were injected with monocrotaline (MCT) or phosphate-buffered saline (PBS) at day 0 (arrowhead). The MCT injected animals were left untreated to develop severe PH (PH group), or received miR193 mimic (20nM) on days 17, 21 and 26 (miR193-OE group, arrows). All of the rats were sacrificed at day 30. **B,** Experimental protocol for the hypoxia model: mice were placed in hypoxia chamber (10% O2) for 21 days and were either left untreated to develop PH (PH group), or received miR193 mimic on days

14 and 18 (MiR193-OE group, arrows). Control mice were kept under normoxic conditions (21% O2). All of the mice were sacrificed at day 21. Expression of miR193 in the lungs was assessed by qRT-PCR in the MCT model (**C**) and in the hypoxia model (**D**). RV systolic pressure (RVSP) in the MCT model (**E**) and in the hypoxia model (**F**). The RV hypertrophy index (RV/(LV + IVS) where IVS is interventricular septum and LV is the left ventricular wall in the MCT model (**G**) and in the hypoxia model (**H**). **I**, Lung arterioles and heart cross sections in the MCT model. For panels C–H $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* CTRL; $^{\#}p<0.05$; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.*PH (n= 5–8 animals per group).

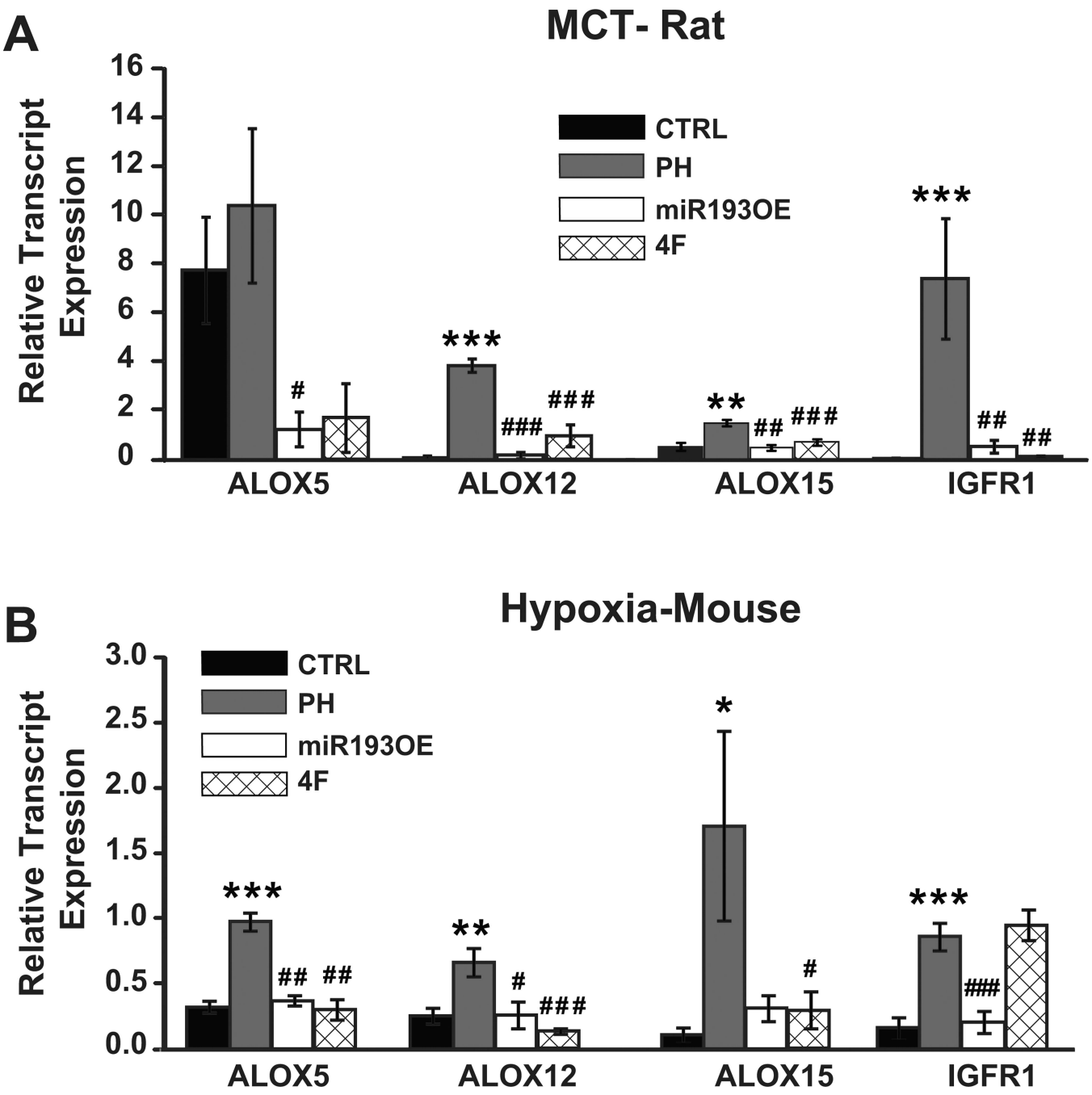

Figure 6.

MiR193 and 4F targets lipoxygenases *in-vivo*. qRT-PCR assessment of transcript levels of lipoxygenases ALOX5, ALOX12 and ALOX15 and insulin growth factor 1 receptor (IGF1R) in the lungs of CTRL, PH, miR193-OE and 4F groups in 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).

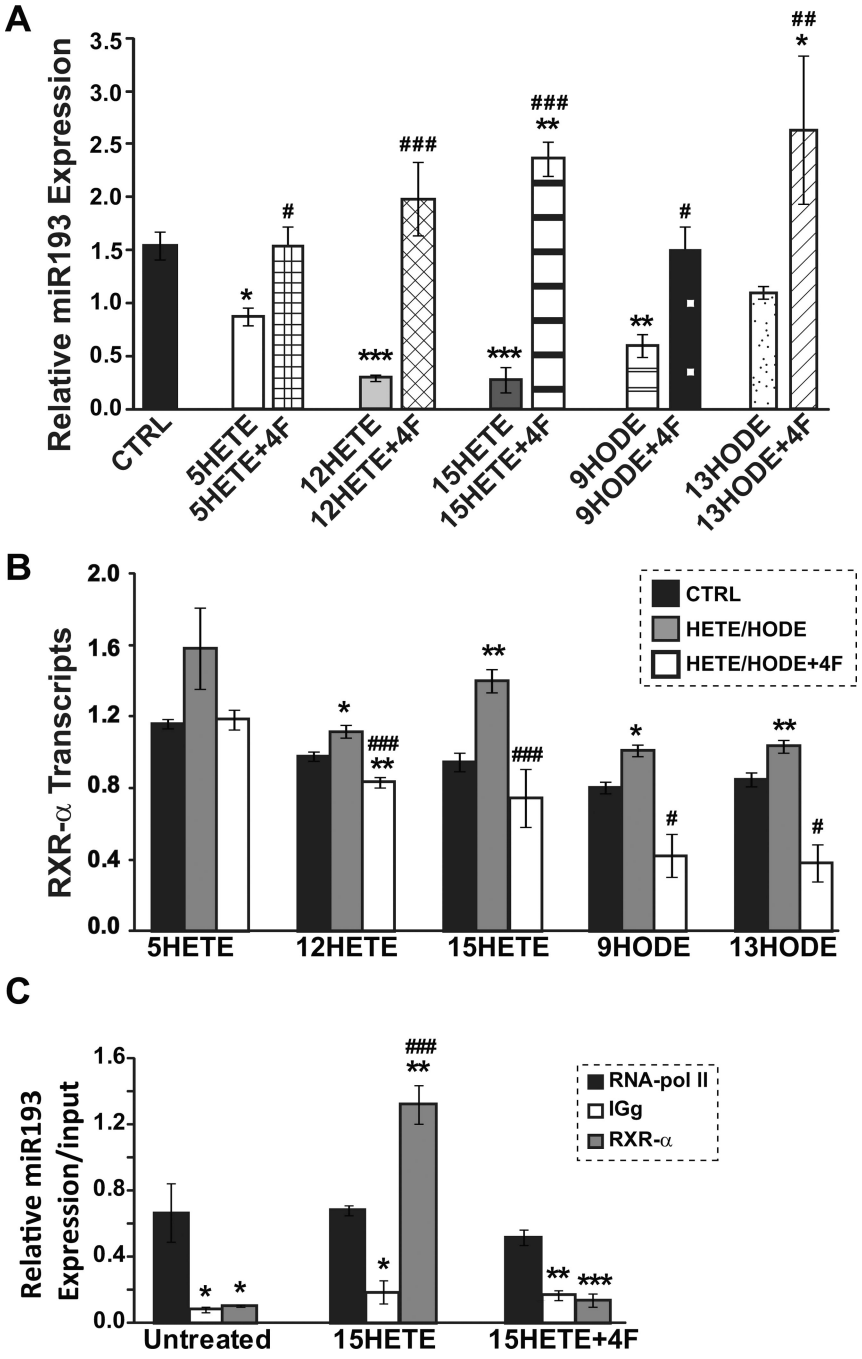

Figure 7.

4F induces miR193 by inhibiting the expression of RXRα. **A**, MiR193 expression was assessed in HPASMCs treated with 5-, 12-, 15- HETE or 9-, 13- HODE for 48 hours in the absence or presence of 4F. **B**, RXRα 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 panels A, 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**, RXRα enrichment at the miR193 promoter is determined by qRT-PCR 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-pol II; $^{\#\#\#}p<0.001$ *vs.* IGg N= 3 independent experiments.

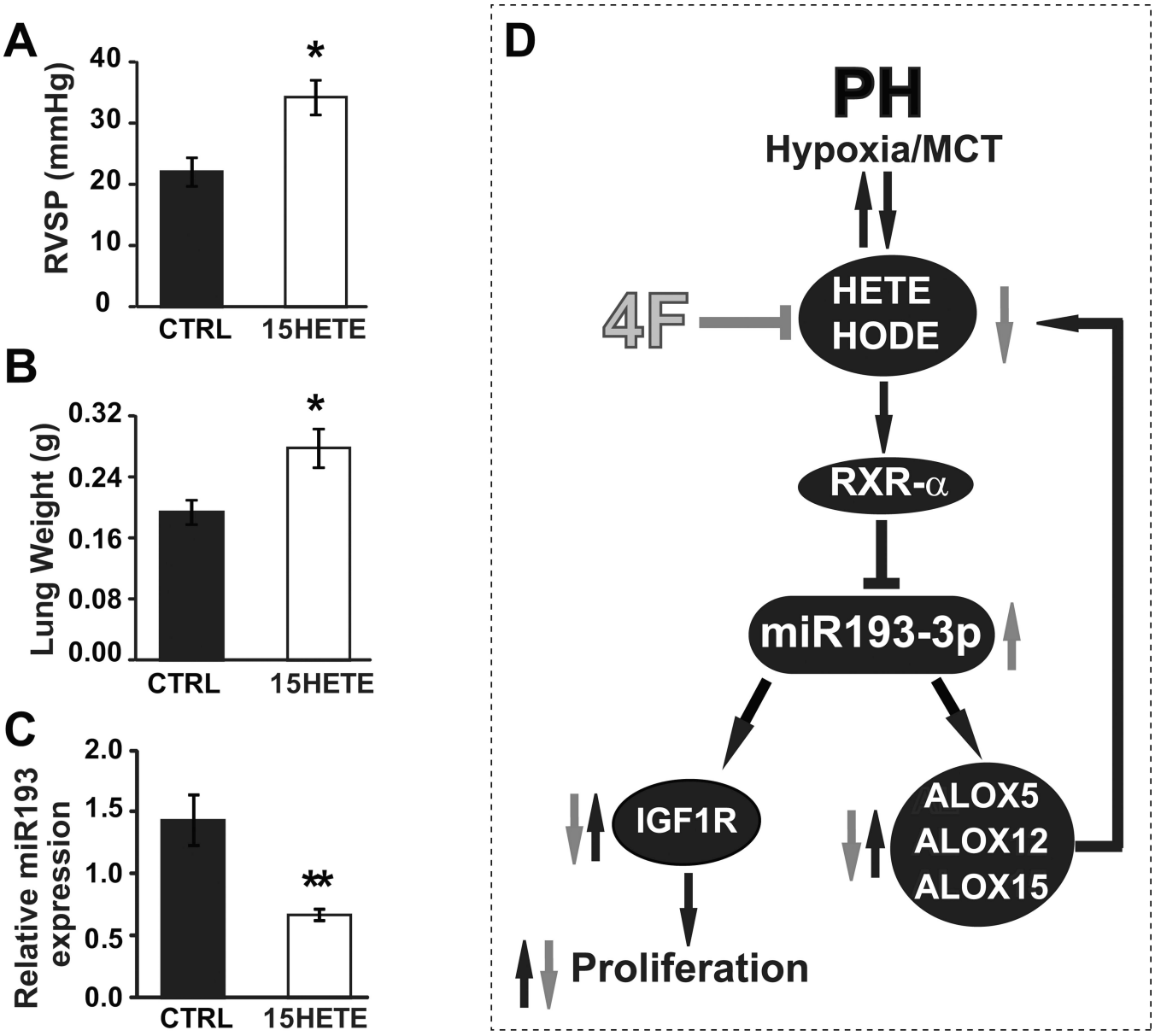

Figure 8.

Exogenous HETE therapy is sufficient to induce PH and decrease miR193 expression. 4F rescues advanced pulmonary hypertension by inducing miR193 through RXRα. Male mice were fed with 15-HETE-rich diet or regular chow for 3 weeks. **A–C,** RVSP (**A**), lung weight (**B**) and lung mir193 expression (**C**) in male mice fed with 15-HETE diet (white bars) or regular chow (black bars) for 3 weeks. For panels A–C **p*<0.05, ***p*<0.01 *vs.* CTRL, (N=5–8 mice/group). **D,** Hypothetical scheme: Pulmonary hypertension in experimental models results in elevated levels of oxidized lipids including multiple HETEs and HODEs, and exogenous 15-HETE is sufficient to induce PH. Sustained accumulation of HETEs and HODEs leads to an elevation in the expression of the transcription factor RXRα, which has a negative effect on the basal expression level of the miR193. 4F therapy by virtue of its ability to bind HETEs and HODEs removes the brake on the suppression of miR193 thus

permitting normalization of levels of this microRNA. Induction of miR193 inhibits the expression of its target genes including Insulin-like growth factor-1 (IGF1) receptor (IGF1R) and lipoxygenases, thus regulating proliferation of smooth muscle cells and establishing a mutually inhibitory loop between miR193 and HETEs and HODEs.