Supplemental Material

Data S1.

Supplemental Methods

Human subjects

After institutional review board approval, human explanted lung tissue from patients with PH (PH group, n=7) were obtained (Table S1). Discarded donor lung tissue or lung tissue from patients without evidence of PH undergoing lung transplants at UCLA Medical Center were used as controls for the study (CTRL group, n=8).

Human lung microarray data analysis for LDL-R and CD36

Gene expression data for LDL-R and CD36 from 13 Control human lungs and 18 PAH lungs was retrieved from GEO using the GSE15197 dataset[1]. A heatmap was generated using normalized expression values in the pheatmap package in R.

Oxidized LDL Assay

Human plasma was collected from PH patients (PH, n=9) and subjects without PH (CTRL, n=12) using EDTA as anticoagulant, and centrifuged for 15 minutes at 1,000×g at 2-8^{o}C within 30 minutes of collection. Samples were stored in aliquots at -80^{o}C until use. Plasma samples were diluted 1:500 in sterile 1X PBS for the assay. Plasma oxidized LDL (ox-LDL) were quantified using the ELISA assay for ox-LDL (Cloud-Clone Corp. cat#CEA527Hu) according to the manufacturer's instructions.

Real time PCR

Total RNA was purified from human lungs using the Trizol method. Two micrograms of total RNA were reverse transcribed to cDNA using the Omniscript RT kit (Qiagen), according to the manufacturer's instructions (Qiagen). Quantitative real-time reverse transcriptase-polymerase chain reaction (QRT-PCR) was performed using iTaq universal SYBR green supermix (Bio-Rad cat#1725121). The LDL-R and CD36 gene expression was assessed using gene-specific primers. GAPDH was used as a reference control for normalization. Levels of gene expression in each sample were determined with the comparative Ct method using Bio-Rad CFX manager 2.1 software.

The primer sequences were as follows:

1) LDL-R, F: 5′-CAACTGCGGGGACTGTTCA-3′, R: 5′ GGACAGGCATACTCATCGGAC-3′;

2) CD36, F: 5’-TGCAGTGTAGGACTTTCCTG-3’, R: TCCGGTCACAGCCCATTTT-3’

3) GAPDH, F: 5′-CACCATCTTCCAGGAGCGAG-3′; R: 5′-GACTCCACGACGTACTCAGC-3′.

For gene expression, thermocycler protocol was as follows: polymerase activation: 30 s at 95°C, then denaturation 5s at 95°C, and annealing/elongation 90s at 60°C for 40 cycles.

Animals and treatments

Middle aged male LDL-R KO mice (12-15 months old) were fed either chow (n=16) or Western diet (WD, n=27) for 12 weeks. Mice on WD were randomly divided into three

groups, WD (n=9), WD together with Apolipoprotein A1 mimetic (HDL mimetic) peptide D-4F (WF+4F group, n=11), or WD together with scramble peptide (WD+SCRM group, n=7) in drinking water for 12 weeks. A group of chow fed mice received 4F (chow+4F group, n=8, Fig. S1). Serial echocardiography was performed to monitor cardiopulmonary hemodynamics and the development of PH and RV dysfunction. Direct RV and LV catheterization was performed terminally and RV hypertrophy index was calculated as weight ratio of RV/(LV+IVS). Aorta, RV, LV and lung tissue were collected.

Echocardiography and cardiopulmonary hemodynamic measurements

Transthoracic echocardiography was performed to monitor cardiopulmonary hemodynamics using a Vevo 2100 high-resolution image system (VisualSonics, Toronto, Canada). Echocardiograms including B-mode, M-mode and pulsed wave Doppler images were obtained under isoflurane aesthesia. Ejection fraction (EF, %) was measured using M-mode echocardiographic images. A 30-MHz linear transducer was used to perform the pulmonary pulsed-wave Doppler echocardiography of PA flow. The probe was placed in a parasternal long-axis position to visualize the PA outflow tract. Pulsed flow Doppler imaging was then overlaid to observe the dynamics of blood flow through the PA valve. PA acceleration time (PAAT) was determined by calculating time taken from the start of flow to maximal velocity using echocardiogram software (Vevo 2100 version: 1.5.0).

The right ventricular systolic pressure (RVSP) and left ventricular systolic pressure (LVSP) were measured directly by inserting a catheter (1.4 F Millar SPR-671, ADInstruments) connected to a pressure transducer (Power Lab, ADInstruments) into the

RV or LV just before sacrifice. Briefly, for cardiac catheterization, the mice were anesthetized with isoflurane. The animals were placed on a controlled warming pad to keep the body temperature constant at 37 °C. After a tracheotomy was performed, a cannula was inserted, and the animals were mechanically ventilated. After a midsternal thoracotomy, mice were placed under a stereomicroscope (Zeiss, Hamburg, Germany) and a pressure-conductance catheter (model 1.4 F Millar SPR-671) was introduced via the apex into the RV or LV and positioned towards the pulmonary or aortic valve respectively. The catheter was connected to a signal processor (ADInstruments) and pressures were recorded digitally. After recording the pressures, heart and lung tissues were removed rapidly under deep anesthesia for preservation of protein and RNA integrity.

Gross histologic analysis, tissue preparation and imaging

The right ventricular (RV) wall, the left ventricular (LV) wall, and the interventricular septum (IVS) were dissected. RV, LV, IVS and lungs were weighed. The ratio of the RV to LV plus septal weight [RV/(LV + IVS)] was calculated as the Fulton index of RV hypertrophy. Lungs were fixed in 4% paraformaldehyde (PFA) in 0.1 M Na_2HPO_4 and 23 mM $NaHPO_4$ (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose in 0.1 M Na_2HPO_4 and 23 mM $NaHPO_4$ (pH 7.4) overnight to cryoprotect the tissue, mounted using OCT, and transversal 4–6µm sections were obtained with a cryostat. Paraffin embedded control lung sections, obtained from the UCLA pathology lab, were sectioned at 5µm. Images were acquired using a confocal microscope (Nikon).

Lung tissue sections were stained with Masson's trichrome, Oil-Red-O, immunofluorescence (CD68, oxLDL) and immunohistochemistry (EO6).

Immunofluorescence and immunohistochemistry staining

Lung sections (5μm) were fixed in 4% paraformaldehyde. The sections were then washed with PBS+0.1% Tween20 three times and incubated with 5% normal goat serum in PBS for one hour to block the background. Following blocking, the sections were incubated with primary antibodies in PBS+2% normal goat serum at 4°C overnight. Sections were then washed with PBS three times, incubated with the secondary antibody in PBS+2% normal goat serum at room temperature for one hour. After washing the secondary antibody with PBS three times, the sections were mounted for imaging using Fluoromount-G with DAPI (Molecular Probes) (ThermoFisher cat#00-4959-52).

For immunohistochemistry, endogenous peroxidase activity was inhibited by incubating the lung sections with 0.3% H_2O_2 in PBS for 20 min at room temperature followed by washing with PBS thrice. The lung sections were then incubated with 10% normal goat serum in PBS containing 0.1% Triton for 30 min at room temperature to block the nonspecific binding. The sections were incubated with the appropriate primary antibodies in PBS+0.1% Triton+ 1% normal goat serum at 4^{o}C overnight, and washed 3 times with PBS+0.1% Triton. The sections were incubated with HRP-conjugated secondary antibody (1:200 dilution) in PBS+0.1% Triton+ 1% normal goat serum, for 1 h at room temperature, washed with PBS+0.1% Triton three times, and stained with 3,3'-diaminobenzidine (DAB) as a substrate (10X DAB solution + stable buffer) for 5-10 min. The DAB was rinsed with dH2O and stained with hematoxylin for 1 min, washed under running tap water for 10 min. Dehydration was then performed by incubating in 50%,

70%, 96%, 100% ethanol and xylene. Sections were mounted using Permount (Fisher Scientific). The images were acquired using a laser scanning confocal microscope (Nikon Eclipse E 400).

Masson's Trichrome and Oil-Red-O staining

Masson Trichrome staining was performed according to the manufacturer's protocol (Sigma), and images were acquired with a confocal microscope (Nikon).

For Oil-Red-O staining, frozen sections cut at 4-6μm, were fixed in formalin, and briefly washed with running tap water for 1-10 mins. Sections were rinsed with 60% isopropanol and stained with freshly prepared Oil-Red-O working solution for 15 mins. Sections were rinsed with 60% isopropanol. Nuclei were lightly stained with 5 dips of haematoxylin. Sections were rinsed with distilled water and mounted in aqueous mounting medium. Images were acquired with a confocal microscope (Nikon).

Quantification of pulmonary vascular remodeling, lung fibrosis and lipid deposition

For assessment of pulmonary arteriolar wall thickness, only distal pulmonary arteries less than 100μm were quantified (3–4 vessels per mouse; number of mice: chow=7, WD=10, WD+Scramble=4, WD+4F=7) using ImageJ software. Stitched images of entire lung sections were obtained using 10x objective using a confocal microscope. Pulmonary arteriolar wall thickness was calculated by subtracting diameter of the lumen from total diameter of the vessel, divided by total diameter of the vessel. As the diameter of the vessel and lumen are not usually similar in different directions, pulmonary arteriolar wall

thickness was measured in two different directions and averaged. Assessment of percent fully muscular, partially muscular and non-muscular arteries was also made (Fig. 5).

Pulmonary fibrosis was quantified using a grid over stitched images of lungs that divided the field of view into 100 squares, each collagenous tissue (blue stain) filled square in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined (number of mice: chow=7, WD=11, WD+Scramble=7, WD+4F=6). Percent lipid deposition was quantified in lung sections using ImageJ software (number of mice: chow=4, WD=4, WD+Scramble=4, WD+4F=4).

Reagents

Primary antibodies used were anti-CD68 (Rat anti Mouse CD68, Bio-Rad cat# MCA1957), anti-ox-LDL (Rabbit polyclonal oxLDL orb10973 from Biorbyt), anti-E06 (Mouse Monoclonal Antibody, IgM Anti-Oxidized Phospholipid, Avanti Polar Lipids, Inc.) and anti-LOX1 (anti-OLR1; Rabbit polyclonal, Abcam cat#ab60178). Secondary antibodies used were goat anti-rat IgG secondary antibody Alexa Fluor 594, and goat anti-rabbit Alexa Fluor 594 secondary antibody.

Statistics

Unpaired T-tests and one-way ANOVA tests were used to compare between groups using GraphPad Prism for Windows. When significant differences were detected, individual mean values were compared by post-hoc tests (Tukey or Dunnett) that allowed for multiple comparisons. $P < 0.05$ was considered statistically significant. Values are expressed as mean ± SEM.

Table S1. Comparison between clinical characteristics of controls (CTRL) and patients with PH.

	CTRL, n=17	PH, n=9	*P*
Sex: female, n	2	5	0.004
Age, y	54±4	50±2	0.572
Diagnosis, (n)	-	iPAH (4), ILD-PH (3), CTD-PH (2)	-
NYHA Class, (n)	-	Class 3 (2), Class 4 (1)	-
Therapies, (n)	-	PDE5 inhibitor (6), ET1R inhibitor (5), Prostacyclin (5)	-
Combination therapy, n	-	6	-
6MWD, min	213±34 (n=6)	177±34 (n=6)	0.487
RVSP, mmHg	34±3 (n=6)	85±7 (n=6)	<0.001
mPAP, mmHg	24±1 (n=6)	55±6 (n=6)	<0.001
PVR, dyn•s•cm^{-5}	178±26 (n=4)	653±208 (n=4)	0.064
CO, L•min^{-1}	6.0±0.5 (n=6)	5.6±1.0 (n=5)	0.749
CI, L•min•cm^{-2}	2.8±0.3 (n=6)	3.1±0.8 (n=4)	0.640
LDL cholesterol, mg•dL^{-1}	98±12 (n=12)	77±7 (n=6)	0.285
HDL cholesterol, mg•dL^{-1}	48±5 (n=12)	41±2 (n=6)	0.367
Triglycerides, mg•dL^{-1}	118±18 (n=12)	97±15 (n=6)	0.445
Total cholesterol,mg•dL^{-1}	170±14 (n=12)	137±4 (n=6)	0.131
Body Mass Index	27±1.3(n=16)	23±1.1 (n=6)	0.102

Values are mean±SEM. 6MWD indicates 6-minutes walking distance; iPAH, idiopathic pulmonary arterial hypertension; ILD-PH, interstitial lung disease associated PH; CTD-PH, connective tissue disease associated PH; CI, cardiac index; CO, cardiac output; HDL,

high-density lipoprotein; LDL, low-density lipoprotein; WHO FC, World Health Organization functional class; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PVR, pulmonary vascular resistance; RVSP, right ventricular systolic pressure.

Table S2. Statistics.

Figure	Comparison	*P-value*
1A	CTRL vs. PH	<0.001
1B	CTRL vs. PH	<0.05
1C	CTRL vs. PH	<0.05
1E	CTRL vs. PH	<0.05
2B	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F WD+SCRM vs. WD+4F	<0.001 <0.001 <0.05 <0.01
2C	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F WD+SCRM vs. WD+4F	<0.001 <0.01 <0.001 <0.001
2D	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F	<0.001 <0.01 <0.05
2E	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F WD+SCRM vs. WD+4F	<0.001 <0.001 <0.001 <0.001
3B	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F WD+SCRM vs. WD+4F	<0.001 <0.001 <0.001 <0.001
3C	Chow vs. WD Chow vs. WD+SCRM	<0.05 <0.01
4A	0 Wk vs. 1 Wk 0 Wk vs. 3 Wk 0 Wk vs. 5 Wk 0 Wk vs. 6 Wk 0 Wk vs. 10 Wk 0 Wk vs. 12 Wk	<0.001 <0.001 <0.001 <0.001 <0.001 <0.001
4B	0 Wk vs. 10 Wk 0 Wk vs. 12 Wk	<0.05 <0.01

4C	Wk 1 vs. Wk 2	<0.001
5D	Chow vs. WD WD+SCRM vs. WD+4F	<0.01 <0.01
5F	Chow vs. WD WD+SCRM vs. WD+4F	<0.01 <0.01
5G	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F WD+SCRM vs. WD+4F	<0.001 <0.001 <0.001 <0.001
6C	Chow vs. WD Chow vs. WD+SCRM WD vs. WD+4F	<0.01 <0.01 <0.01
8A	Scramble vs. LDL	<0.01
8B	Scramble vs. LDL	<0.01
8C	Control vs. 20 ug/ml Control vs. 50 ug/ml Control vs. 100 ug/ml	<0.05 <0.05 <0.01

Figure S1. A. Experimental protocol. LDL-R KO mice were fed either regular chow diet, Western diet (WD) alone or WD in the presence of scrambled peptide, or HDL mimetic 4F peptide for 12 weeks. One group of LDL-R KO mice was fed with chow diet in the presence of 4F peptide. **B.** Body weight (g) and **C.** Lung weight (g) in LDL-receptor knockout mice fed with chow (n=8), Western diet (WD, n=12), WD+Scrambled peptide (n=7) and WD+4F peptide (n=8). **p<0.01; ***p<0.001.

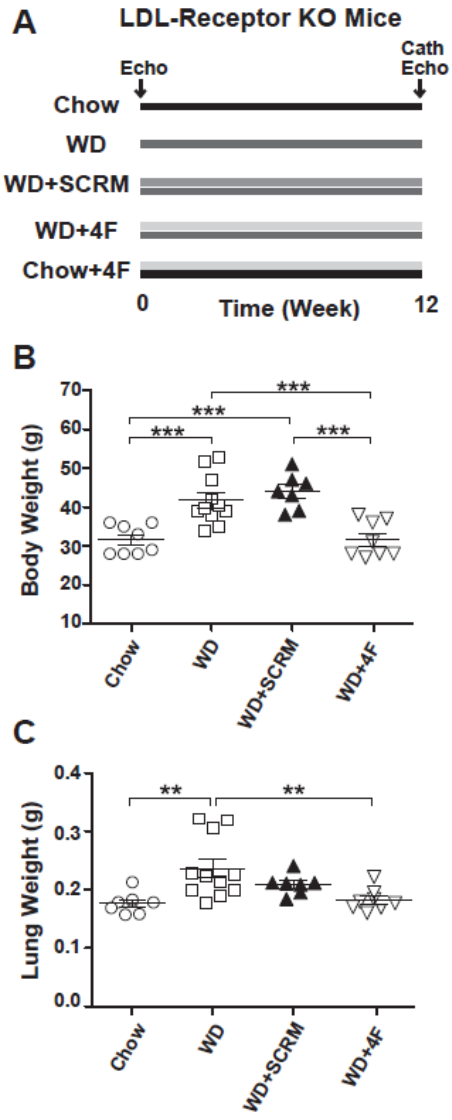
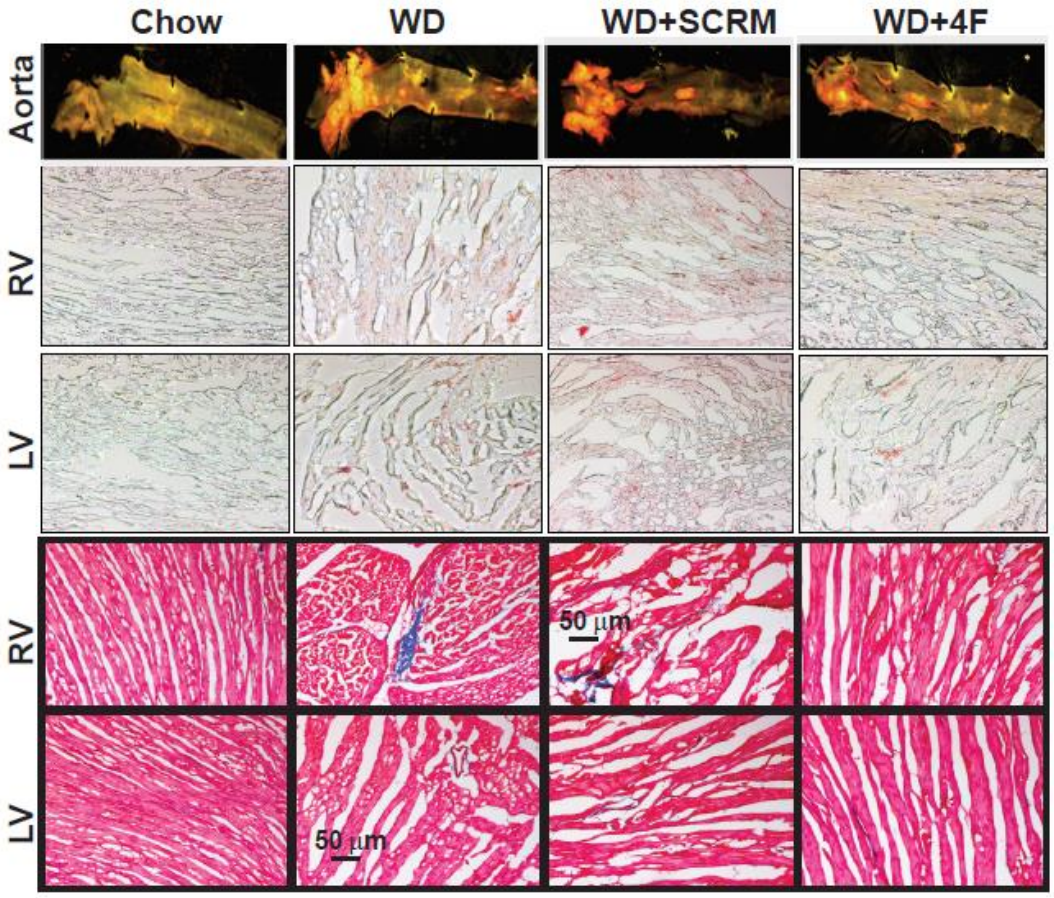


Figure S2. Oil Red O staining showing lipid deposition (red) in aorta (**A**), right ventricle (RV, **B**), and left ventricle (LV, **C**), and Masson's trichrome staining showing heart fibrosis (blue) in RV (**D**) and LV (**E**) in Chow, WD, WD+Scramble and WD+4F groups.



Supplemental Reference:

1. Rajkumar R, Konishi K, Richards TJ, Ishizawar DC, Wiechert AC, Kaminski N, Ahmad F. Genomewide RNA expression profiling in lung identifies distinct signatures in idiopathic pulmonary arterial hypertension and secondary pulmonary hypertension. *Am J Physiol Heart Circ Physiol.* 2010;298:H1235-1248.