Maresin 1 intervention reverses experimental pulmonary arterial hypertension in mice

Li, Hui; Li, Xinyu; Hao, Yu; Wu, Chenghua; Fu, Yuhao; Su, Nana; Chen, Houlin; Ying, Binyu; Wang, Haixing; Su, Lihuang; Cai, Haijian; He, Qinlian; Cai, Mengsi; Sun, Junwei; Lin, Jing; Scott, Aaron; Smith, Fanggao; Huang, Xiaoying; Jin, Shengwei

DOI:
10.1111/bph.15906

Title Page

Maresin 1 intervention Reverses Experimental Pulmonary Arterial Hypertension in mice

Running title: Maresin 1 reverses pulmonary hypertension in mice

Hui Li[1#], Xinyu Li[1#], Yu Hao[1#], Chenghua Wu[1], Yuhao Fu[1], Nana Su[1], Houlin Chen[1], Binyu Ying[1], Haixing Wang[1], Lihuang Su[2], Haijian Cai[2], Qinlian He[2], Mengsi Cai[2], Junwei Sun[2], Jing Lin[3], Aaron Scott[4,5], Fanggao Smith[1,4,5], Xiaoying Huang[2*], Shengwei Jin[1*]

[1]Department of Anaesthesia and Critical Care, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China;

[2]Division of Pulmonary Medicine, The First Affiliated Hospital of Wenzhou Medical University, Key Laboratory of Heart and Lung, Wenzhou, Zhejiang, China;

[3]Department of Anaesthesia, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China;

[4]The Birmingham Acute Care Research (BACR) Group, Institute of Inflammation and Ageing, University of Birmingham, Birmingham, United Kingdom;

[5]Academic Department of Anaesthesia, Critical Care, Pain and Resuscitation, Birmingham Heartlands Hospital, Heart of England National Health Service Foundation Trust, Birmingham, United Kingdom.

#These authors contributed equally to this work and share the first authorship.

Hui Li, MD, PhD (Orcid ID: 0000-0003-4212-9155)

Department of Anaesthesia and Critical Care,

Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University,

Wenzhou, Zhejiang, 325027, P. R. China,

Email: judy198406@sina.com

Xinyu Li

Department of Anaesthesia and Critical Care,

Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University,

Wenzhou, Zhejiang, 325027, P. R. China,
Email: 1501204817@qq.com
Yu Hao, MD, PhD (Orcid ID: 0000-0002-8351-1206)
Department of Anaesthesia and Critical Care,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University,
Wenzhou, Zhejiang, 325027, P. R. China,
Email: haoyu8403@163.com

*These authors contributed equally to this work and share the corresponding authorship.
Address correspondence to:
Shengwei Jin, MD, PhD (Orcid ID: 0000-0002-4734-8114)
Department of Anaesthesia and Critical Care,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University,
Wenzhou, Zhejiang, 325027, P. R. China,
Email: jinshengwei69@163.com
Xiaoying Huang, MD, PhD (Orcid ID: 0000-0003-4854-0922)
Division of Pulmonary Medicine,
The First Affiliated Hospital of Wenzhou Medical University,
Key Laboratory of Heart and Lung,
Wenzhou, Zhejiang, 325027, P. R. China,
Email: zjwzhxy@126.com

Word count： 5679

Bullet point summary

What is already known: Maresin 1 (MaR1) is a newly identified macrophage-derived lipid mediator that promotes the resolution of inflammation. However, its role in pulmonary arterial hypertension (PAH) remains largely unknown.

What this study adds: The serum levels of MaR1 decreased in PAH, post-treatment with MaR1 significantly attenuated PAH in mice.

Clinical significance: Approaches to enhance MaR1 or its related pathways provide potential therapeutic strategies for PAH.

Abstract

Background and Purpose: Pulmonary arterial hypertension (PAH) is a pulmonary vasculature obstructive disease that leads to right heart failure and death. Maresin 1 is an endogenous lipid mediator known to promote inflammation resolution. However, the effect of Maresin 1 on PAH remains unclear.

Experimental Approach: The serum Maresin 1 concentration was assessed using UPLC. A mouse model of PAH was established by combining the Sugen 5416 injection and hypoxia exposure (SuHx). After treatment with Maresin 1, the right ventricular systolic pressure (RVSP) and right ventricular function were measured by hemodynamic measurement and echocardiography, respectively. Vascular remodeling was evaluated by histological staining. Confocal and western blot were used to test related protein expression. In vitro, cell migration, proliferation and apoptosis assays were performed in primary rat pulmonary artery smooth muscle cells (PASMCs). Western blotting and siRNA transfection were used to clarify the mechanism of Maresin 1.

Key Results: Endogenous serum Maresin 1 was decreased in PAH patients and mice. Maresin 1 treatment decreased RVSP and attenuated the right ventricular dysfunction (RVD) in murine PAH model. Maresin 1 reversed abnormal changes in pulmonary vascular remodeling, attenuating endothelial to mesenchymal transformation (EndoMT) and enhancing apoptosis of α-SMA positive cells. Furthermore, Maresin 1 inhibited PASMC proliferation and promoted apoptosis by inhibiting STAT, AKT, ERK and FoxO1 phosphorylation via LGR6.

Conclusion and Implications: Maresin 1 improved abnormal pulmonary vascular remodeling and right ventricular dysfunction in PAH mice, targeting aberrant PASMC proliferation. This suggests Maresin 1 may have a potent therapeutic effect in vascular disease.

Keywords: pulmonary arterial hypertension; Maresin 1; pulmonary vascular remodeling; right ventricular dysfunction;

Abbreviations

MaR1: Maresin 1; PAH: pulmonary arterial hypertension; PH: pulmonary hypertension; RVSP: right ventricular systolic pressure; RVD: right ventricular dysfunction; RVH: right ventricular hypertrophy; UPLC: ultra-performance liquid chromatography tandem; EndoMT: endothelial to mesenchymal transformation; PVR: pathological vascular remodeling; SPMs: specialized pro-resolving mediators; AA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SPF: specific pathogen-free; SuHx: Sugen 5416/hypoxia; PFA: paraformaldehyde; PAT: pulmonary artery acceleration time; PET: pulmonary artery ejection time; TAPSE: tricuspid annulus plain systolic excursion; PE: polyethylene; mCAP: mean carotid arterial pressure; CH: Chronic hypoxia; RV: Right ventricular; PAs: Pulmonary Arteries; vWF: von Willebrand factor; α-SMA: α-smooth muscle actin; ANOVA: one-way analysis of variance; PMN: polymorphonuclear neutrophil.

Introduction

Pulmonary arterial hypertension (PAH) is a chronic and progressive disease of the pulmonary arterial bed, defined by the mean pulmonary artery pressure of >20 mmHg and pulmonary vascular resistance ≥3 Wood Units via right-heart catheterization(Austin, West, Loyd & Hemnes, 2017; Thenappan, Ormiston, Ryan & Archer, 2018). The pulmonary vasculature suffers occlusive lesions, abnormal vasoconstriction and pathological vascular remodeling (PVR)(Vonk-Noordegraaf et al., 2013). Obstructive pulmonary vascular remodeling in PAH increases right ventricular afterload, leading to right ventricular dysfunction (RVD) and ultimately death(Vonk-Noordegraaf et al., 2013). Although current therapies improve quality of life and prognosis, PAH remains a life-limiting condition because current treatment options cannot change the chronic pathology of vascular cells in the pulmonary arteries(van der Feen, Bartelds, de Boer & Berger, 2017).

The vascular cells of pulmonary arteries include inner endothelial cells, media smooth muscle cells, and adventitial fibroblasts(Thenappan, Ormiston, Ryan & Archer, 2018; Zhang et al., 2017). It has been reported that endothelial cells show early apoptosis after an initial insult and then revert to a proliferative, apoptosis-resistant mesenchymal-like phenotype, in the process termed endothelial-to-mesenchymal transition (EndoMT), to maintain vascular integrity(Kovacic et al., 2019). A proliferative and apoptosis-resistant phenotype of pulmonary artery smooth muscle cell (PASMC), which results in medial thickening and occlusive vascular lesions. It is thus important to find an effective intervention to restrict cell proliferation and promote cell apoptosis for PAH

121 treatment(Gorelova, Berman & Al Ghouleh, 2020).

122 The specialized pro-resolving mediators (SPMs) are an endogenous family of chemical mediators
123 derived from polyunsaturated fatty acids including arachidonic acid (AA), eicosapentaenoic acid
124 (EPA), and docosahexaenoic acid (DHA) (Serhan et al., 2012). SPMs are temporally biosynthesized
125 in inflammatory exudates to control localized inflammation, stimulating multiple resolution
126 programs without immunosuppression and are organ protective(Serhan, 2014). Maresin 1 is a
127 member of SPM family, expressed in various tissues, such as adipose, lymphoid, brain, and nervous
128 tissues. It exerts protective actions, including limiting neutrophil infiltration and enhancing
129 phagocytosis and efferocytosis(Albuquerque-Souza et al., 2020; Fattori et al., 2019). A recent study
130 reported that Maresin 1 promoted phagocyte immune-resolvent actions by activating a GPCR,
131 leucine-rich repeat domain-containing G protein-coupled receptor 6 (LGR6), which is the only
132 known specific plasma receptor of Maresin 1(Chiang, Libreros, Norris, de la Rosa & Serhan, 2019).
133 However, the effect of Maresin 1 on PAH and RVH remains elusive.

134 Herein, we demonstrate that Maresin 1 expression level was subdued in patients with PAH and in
135 our murine model of induced PAH. Exogenous Maresin 1 improved pathological pulmonary arterial
136 remodeling and right heart dysfunction in Sugen 5416 / hypoxia (SuHx)-induced PAH mice model.
137 Mechanistically, Maresin 1 inhibited cell migration, decreased cell proliferation and promoted
138 apoptosis of PASMC via a mechanism that involved decreased phosphorylation of STAT3, AKT,
139 ERK and FoxO1 through LGR6 in vitro.

140

141 **Methods**

142 **Data availability statement**

143 The data that support the findings of this study are available from the corresponding author upon
144 reasonable request. Some data may not be made available because of privacy or ethical restrictions.

145 **Mice**

146 All study protocols were conducted as per the Guide for the Care and Use of Laboratory Animals.
147 Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill,
148 Emerson, Altman & Group, 2010) and with the recommendations made by the British Journal of
149 Pharmacology(Lilley et al., 2020). The Animal Studies Ethics Committee of Wenzhou Medical
150 University provided the ethical approval of this study (wydw2019-0698). Specific pathogen-free

(SPF) adult male mice C57BL/6 (6-8 wk) were bought from SLAC Laboratory Animal CO. (Shanghai, China). Before the experiments, the mice were kept in normal cages in a half-day light/dark cycle under moderated temperature (22–24 °C) and humidity (50–60%) parameters SPF environment in Wenzhou Medical University. The experimental mice freely accessed food, as well as water.

Sample preparation and LC-MS-MS-based Maresin 1 determination and analysis

The assays were performed as described previously(Jin et al., 2018). Serum was collected from whole blood for murine and human samples. Healthy volunteer samples were obtained from Physical Examination Center of the Second Affiliated Hospital of Wenzhou Medical University. PAH patient samples were obtained from the ICU of the Second Affiliated Hospital and Operation Room of the First Affiliated Hospital. Murine blood was collected via the retro-orbital plexus under terminal anesthetic, before sacrifice. Whole blood (murine/human) was allowed to clot for 30 mins then centrifuged at 560xg for 10mins and supernatant was collected for stored at -80°C. Maresin 1 was quantified in serum samples. Serum samples (humans 2.0 ml, mice 0.5 ml) were placed in ethyl acetate (2.5ml/0.5ml serum samples), which contained deuterium-labelled Maresin 1 (7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid, Cas# 1268720-28-0, Cayman Chemical) to each sample. Samples were then continuously vortexed for 3 min and sonicated for 15min before centrifugation at 560x g for 10 minutes to allow supernatant collection. Total lipid mediators were extracted from serum samples with a solid phase C18 column (Waters, USA) and the level of Maresin 1 analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Water containing 0.01% acetic acid was used as solvent A and MeOH containing 0.01% acetic acid was used as solvent B. We used the UPLC I-Class system (Waters, USA) equipped with an AB Sciex Instruments 6500 Q-TRAP mass spectrometer (Sciex, USA) and Analyst 1.6 software (Applied Biosystems, USA) to acquire and analyze data. Quantification of Maresin 1 was based on peak area of multiple reaction monitoring transitions and linear calibration curve of each compound.

Curative protocol in PAH models

The Sugen 5416/ hypoxia (SuHx)-induced PAH model mice were injected subcutaneously with 20 mg/kg Sugen 5416 (MCE), vascular endothelial growth factor receptor 2 blocker, which was dissolved in carboxyl methyl cellulose, once per week for 3 consecutive weeks (10% oxygen) and

subsequently exposure to chronic hypoxia (10% oxygen) for another 3 weeks. After 3 weeks the model of PAH pathology is established in these mice, after which mice were treated with an initial bolus of Maresin 1 (1 μg/mouse) intraperitoneally (i.p.) followed by booster injections (100 ng/mouse) every two days. This dosing scheme of Maresin1 has been used previously in similar models of chronic lung studies(Li et al., 2017; Martins et al., 2009). At times indicated, mice were anesthetized for hemodynamic and echocardiographic assay. After sacrifice, the heart and left lungs of mice were rinsed with PBS through the pulmonary arteries and then collected. The hearts were dissected, and the right ventricular hypertrophy were calculated as the ratio of the right ventricular free wall weight to the weight of the left ventricle plus septum (RV/LV+S). The left lungs were perfused with 4% paraformaldehyde solution for subsequent histological staining and analysis.

Cell procedures

Rat pulmonary artery smooth muscle cells (PASMCs) were obtained by dissection of secondary and tertiary order branches of the pulmonary arterial tree from euthanized rats. After the connective tissue, the adventitia, and the endothelial cells were removed with forceps under aseptic conditions, the media layer of vessels were cut into small pieces. And then these pieces were digested with 0.2% type 1 collagenase, incubating for 40 minutes at 37℃. Isolated cells were cultured in DMEM medium supplemented with 12% FBS, 1% penicillin, and 1% L-glutamine at 37℃. The cells were identified by immunofluorescence staining with anti-α-SMA antibody. Cultured PASMCs were used at passages 4 to 6.

Small interfering RNA treatment of PASMC

Normal rat PASMC were grown to approximately 60% confluence and then transfected using riboFECT CP Transfection Kit (Ribo, Guangzhou, CHN) and 100nM siRNAs targeting LGR6 (Ribo, Guangzhou, CHN) or a scrambled small interfering RNA as negative control. Normal culture medium was added after 48 hours, and medium was changed after 48 hours. Gene knockdown was confirmed by western blot.

Echocardiography

Transthoracic echocardiography was performed with a Visual Sonics Vevo 3100 small animal echocardiography machine (FUJIFILM Visual Sonics, Canada) with a 40 MHz ultrasound probe (Ms-400) for mice. Briefly, mice were anesthetized with isoflurane inhalation (1.5-3.0%, RuiWoDe Life Science, Shenzhen, China) and placed on a heated pad in a supine position. The fur on the chest

of the mice was removed with a chemical hair remover. The pulmonary artery acceleration time (PAT) and pulmonary artery ejection time (PET) were obtained from the modified parasternal long axis view using pulsed Doppler mode. Tricuspid annulus plane systolic excursion (TAPSE) was measured by M-mode from an apical four-chamber view. All data were collected under a stable and consistent heart rate in each group mice.

Hemodynamic assay

After establishment of the PAH model, mice were anesthetized with 1% pentobarbital sodium (60mg/kg, i.p). Two 15cm polyethylene catheters (inner diameter 0.5mm, external diameter 0.9mm), prefilled with heparin in order to prevent coagulation, were connected to the pressure system (AD Instruments, Colorado Springs, CO, Australia). Blunt dissection of neck muscle tissue was used to expose the jugular vein and internal carotid. Finally, the right ventricle systolic pressure (RVSP) was measured by polyethylene catheters, which were inserted into the right ventricle via the jugular vein. Meanwhile, the mean carotid arterial pressure (mCAP) was monitored utilizing polyethylene catheters inserted into the left carotid artery. All RVSP data were collected under a consistent mCAP.

Immunohistochemistry

Mouse paraffin-embedded lungs were sectioned and deparaffinized in xylene followed by rehydrating with gradient ethanol and water. The lung endogenous peroxidase activity was destroyed in methanol containing 30% H_2O_2 for 30 min at room temperature, followed by antigen retrieval by streaming in 10mM citric acid (pH 6.0) at 98 ℃ for 10 min followed by a 40 min cooling period. The lung sections were washed with PBST buffer and blocked with 10% normal Goat serum for 60 min at room temperature. Anti-α-SMA antibody (1:200, Abcam Cat# ab32575, RRID: AB_722538) was incubated with slides at 4 ℃ overnight, and then goat anti-rabbit IgG (H+L) secondary antibody (1:100, MultiSciences Cat# GAR007, RRID: AB_2827833) was incubated with lung for 1 hour at room temperature, followed by incubation with DAB solution（1:20, ZSGB-BIO Cat# ZLI-9019）for 5 min to detected the antibody. Cell nuclei were stained with hematoxylin. The slides were dehydrated and mounted with xylene-based mounting medium.

Endothelial-mesenchymal transition

For paraffin-embedded sections, the left lobe of lungs was perfused with 4%PFA followed by paraffin processing. Lung sections (5μm) were dewaxed and dehydrated. Antigen retrieval was performed by boiling the slides in 10 mmol/L sodium citrate (PH 6.0) at 98℃ for 10 minutes. After

cooling, the lung sections were blocked with 10% normal donkey serum for 1h at room temperature. Slides were then incubated with anti-α-SMA antibody (1:200 , Abcam Ca# ab32575) and anti-vWF antibody(1:100, Santa Cruz Biotechnology Cat# sc-365712, RRID: AB_10842026) overnight at 4℃, and with secondary antibody at room temperature for 1 hour. Cell nuclei were counterstained with DAPI for 5 min. Slides were then mounted on a slide and visualized using confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Cell apoptosis assay

Cell apoptosis was determined by the In Situ Cell Death Detection Kit (1:9, Roche Cat# 11684795910). In vivo, after modeling, mouse paraffin-embedded lungs were sectioned and deparaffinized in xylene followed by rehydrating with gradient ethanol and water. Antigen retrieval and then permeabilized with 0.5% Trion X-100 for 10 minutes. After blocking with 10% normal goat serum, slides were incubated with anti-α-SMA antibody overnight at 4℃. The following day, the slides were incubated with TUNEL mixed solution at 37℃ for 1 hour. Then nuclei were counterstained with DAPI for 5 minutes at room temperature. After washing with PBS three times, samples were imaged by confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

In vitro, primary rat PASMCs were grown on coverslips in 24 well plates and fixed with 4% paraformaldehyde for 20 min at room temperature after modeling. Cell membranes were ruptured with 0.2% Triton X-100 for 10 min. the slides were incubated with TUNEL mixed solution at 37℃ for 1 hour. Nuclei were counterstained with DAPI for 5 minutes at room temperature. After washing with PBS three times, the samples were imaged by confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Cell proliferation assay

Proliferation of rat PASMCs was determined by immunofluorescence staining for Ki67. PASMCs were grown on coverslips in 24 well plates after modeling, fixed with 4% paraformaldehyde for 20 min at room temperature. Cell membranes were ruptured with 0.2% Triton X-100 for 10 min. After blocked with 10% donkey serum for 30 min in 37℃, PASMCs were incubated with Ki67 antibody (1:100, Thermo Fisher Scientific Cat# 14-5698-82, RRID: AB_10854564) overnight at 4 ℃, then cells were incubated with Alexa Fluor 594 secondary antibody (1:200, Biyuntian, CHN) for 60 min. Then nuclear were stained with DAPI at room temperature for 5 min. Finally, images were visualized using confocal laser scanning microscope. (Zeiss, Oberkochen, Germany).

Western blotting

Lung tissue and pulmonary arterial smooth cells lysates were obtained using RIPA lysis, PMSF and buffer phosphatase inhibitor. Protein concentrations of the supernatants were determined using a BCA protein assay kit. Equal amounts of each sample (based on protein content) were loaded in each lane and separated by 8%, 10% or 12% SDS-PAGE, transferred onto a polyvinylidene fluoride membranes, and blocked with 10% skimmed milk for 2 hours at room temperature. The membrane was washed three times with TBST and incubated overnight at 4℃ with primary antibodies were used at indicated dilutions. Secondary antibodies coupled to Horseradish peroxidase-conjugated report were used to generate a chemiluminescent signal, using either goat anti-mouse or goat anti-rabbit as appropriate (1:3,000 dilution). Membranes were imaged with the Image Quant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The analysis of protein expression levels was quantified by scanning densitometry using the Quantity one analysis system.

The following antibodies were applied: anti-Bcl-2 antibody (1:1000, Abcam Cat# ab59348, RRID:AB_2064155), anti-Bax antibody (1:1000, Cell Signaling Technology Cat# 2772, RRID:AB_10695870), anti-cleaved-caspase3 antibody (1:1000, Cell Signaling Technology Cat# 9664, RRID:AB_2070042), anti-caspase3 antibody (1:1000, Cell Signaling Technology Cat# 9662, RRID:AB_331439), anti-phospho-stat3 antibody (1:2000, Cell Signaling Technology Cat# 9145, RRID:AB_2491009), anti-stat3 antibody (1:2000, Cell Signaling Technology Cat# 4904, RRID:AB_331269), anti-phospho-AKT antibody (1:1000, Cell Signaling Technology Cat# 4060, RRID:AB_2315049), anti-AKT antibody (1:1000, Cell Signaling Technology Cat# 4691, RRID:AB_915783), anti-phospho-MAPK/ERK antibody (1:2000, Cell Signaling Technology Cat# 4370, RRID:AB_2315112), anti-MAPK/ERK antibody (1:2000, Cell Signaling Technology Cat# 4695, RRID:AB_390779), anti-phospho-FoxO1/FoxO3a (1:1000, Cell Signaling Technology Cat# 9464, RRID:AB_329842), anti- FoxO1/FoxO3a (1:1000, Cell Signaling Technology Cat# 2880, RRID:AB_2106495), anti-LGR6 antibody (1:1000, Abcam Cat# ab126747, RRID:AB_11132458), anti-β-actin antibody(1:1000, Affinity Biosciences Cat# AF7018, RRID:AB_2839420).

Wound healing assay

PASMCs were seeded into 24-well plates after marking with black lines to make sure the same position of each photographing. We performed scratching experiments when the cell density reached 80% -90%. After washing with PBS, the cells were photographed under the microscope at the

indicated times. The value of each well is derived from the average of data obtained by the two independent researchers. Initial scratch area of well that were outside of the ± 30% cut off from the mean area of all wells, were discarded in subsequent experiments. Migration rate was calculated as (the area of the scratch at 0 hour − the area of the scratch at the indicated times)/the area of the scratch at 0 hour with ImageJ (RRID:SCR_003070). ~~The data of each well used for statistical analysis is derived from the average of the data obtained by the two independent researchers~~.

Statistical analysis

All studies comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology(Curtis et al., 2018). For *in vivo* experiments, "n" represents the number of mice in each group. For *in vitro* experiments, "n" represents the number of primary cultured cells isolated from different animals. Animals were randomized prior to treatment. All the animal experiments were designed to generate groups using randomization and blinded analysis. For the quantification of the PA vascular wall thickness, the percentage wall thickness was calculated as (wall outer circumference – wall inner circumference) / wall outer circumference. The entire vessel area was identified as the total area. The percentage wall area was calculated as (total area – lumen area) / total area. All western blotting and immunohistochemical procedures and analysis should comply with the recommendations detailed in the BJP editorial Alexander et al. (2018). Data are presented as the mean ± SEM. Data were analyzed using two-tailed unpaired Student's t-test for two-group comparisons and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Wound healing analysis (6 hour and 24 hour) were evaluated using Two-way ANOVA followed by multiple comparisons. GraphPad Prism (RRID:SCR_002798, San Diego, CA, version 8.0) was used for analyses and graphs. Results with a value of $p < 0.05$ were considered statistically significant.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

Results

Serum Maresin 1 concentration concentration is lower in PAH samples

Hypoxia combined with Sugen 5416 injection (SuHx) is an established method to trigger PAH in mice (Penumatsa, Warburton, Hill & Fanburg, 2019; Wu et al., 2017). We prepared SuHx (Sugen 5416, 20mg/kg, injected 3 times in 3 weeks, combined hypoxia exposure for 3 weeks)-induced PAH mice for our experiment. RVSP and Fulton Index were used to verify pathology was successfully established at week 3 (Figure 1 a-c). Furthermore, we found significant functional RV damage by echocardiography, with shortening of TAPSE and PAAT/PET ratio in this PAH model (Figure 1 d-g). Serum was collected from eight healthy and eight PAH subjects to evaluate the serum concentration of Maresin 1. There was no significant difference in age or sex distribution between healthy and PAH subjects (Supplemental Table 1). Importantly, we also found that serum Maresin 1 level was significantly lower in both PAH patients and Suhx mice compared to control subjects (Figure 1 h-k). LGR6, a specific receptor of Maresin 1, was also found to be downregulated in lungs of SuHx mice (Figure 1 l-m).

Maresin 1 reversed SuHx-induced PAH in mice

To determine the effect of Maresin 1 on SuHx-induced PAH mice, Maresin 1 was administered intraperitoneal every other day to the SuHx mice from the 4^{th} week to the 6^{th} week (Figure 2 a). Mice exposed with SuHx gained significantly less weight than controls. 3 weeks after Maresin 1 treatment, mice showed an increase in body weight, similar to the mice of the control group (Figure 2 b). Next, we measured the right ventricular systolic pressure (RVSP) and heart function to evaluate the development and progression of PAH. Our data showed that Maresin 1 inhibited SuHx-induced pulmonary artery pressure increase in mice significantly (Figure 2 c-d). Meanwhile, we observed that Maresin 1 decreased heart size and improved right ventricular hypertrophy of SuHx mice (Figure 2 e-h). Furthermore, Maresin 1 increased the TAPSE and PAAT/PET ratio in SuHx groups mice, indicating Maresin 1 improved PAH-induced right ventricular dysfunction (Figure 2 i-k).

Maresin 1 attenuated abnormal pulmonary vascular remodeling in SuHx-induced PAH mice

We next sought to understand how Maresin 1 reverses SuHx-induced PAH at the microscopic level. The PAs of the PAH mice model exhibit wall thickening and luminal narrowing via HE staining, while this was not seen in the Maresin 1 treatment group (Figure 3 a-c). Masson's trichrome and EVG staining results showed abnormal collagen deposition in the perivascular pulmonary arterioles

of SuHx mice. Maresin 1 intervention decreased perivascular collagen deposition in the PAH mouse model (Figure 3 d-g). Furthermore, α-SMA expression was increased in the lungs of SuHx mice; Maresin 1 intervention decreased α-SMA expression in pulmonary arterioles(Figure 3 h-j).

Maresin 1 attenuated endothelial-to-mesenchymal transition in pulmonary arterioles in SuHx-induced PAH mice

To identify endothelial cells undergoing EndoMT in SuHx-induced PAH mice and examine the effect of Maresin 1 in this process, we tested the co-expression of von Willebrand factor (vWF) and α-SMA in pulmonary arterioles by confocal microscopy. In control pulmonary arterioles, vWF (Figure 4 a, red) was expressed in the innermost layer and did not co-localize α-SMA (green). However, α-SMA was expressed in the peripheral border of arterioles. In SuHx mice pulmonary arterioles, we observed marked co-localization of vWF and α-SMA (yellow in merged images). Following Maresin 1 treatment, merged images showed significantly less co-localization (Figure 4 a, b).

Maresin 1 enhanced apoptosis of α-SMA positive cells in pulmonary arterioles in SuHx-induced PAH mice

The proliferation of apoptosis-resistant α-SMA positive cells is an important cause of pulmonary artery occlusion in pulmonary hypertension. To examine the possible action of Maresin 1 on α-SMA positive cell clearance in vivo, we examined the distribution of apoptotic cells in arterioles located by α-SMA. Consistently, the arterioles in SuHx mice were characterized by increased α-SMA expression. Apoptotic cells as indicated as TUNEL positive cells, were observed less in SuHx mice pulmonary arterioles compared with the control group. Maresin 1 treatment restored α-SMA positive cell apoptosis to control levels and decreased total α-SMA expression in these cells (Figure 5 a, b). Consistent with this, we observed that Bcl-2 was upregulated with little change in the level of Bax protein, which resulted in a reduced Bax/Bcl-2 ratio in the lungs of the PAH mice model. Cleaved-caspase-3, the downstream protein of the Bax/Bcl-2 pathway, was also decreased. Maresin 1 restored the Bax/Bcl-2 ratio and cleaved-caspase-3 levels to baseline in lung tissues from PAH mice (Figure 5 c-e).

Maresin 1 inhibited hypoxia-induced PASMC migration, proliferation and promoted cell apoptosis

To gain insight into the mechanism underlying the effect of Maresin 1 on experimental PAH mice,

we employed an *in vitro* model using rat-derived PASMCs. We first characterized the effect of hypoxia on PASMC proliferation and migration via wound healing assay, examining migration of PASMCs at 6-hour and 24-hour timepoints. Maresin 1 treatment inhibited hypoxia-induced PASMC migration at 24 hours at the dose of 200nM (Figure 6 a). Cell proliferation and apoptosis were further examined by Ki67 staining and TUNEL staining respectively. Ki67 index, defined as the percentage of positive cells independent of intensity, was used to evaluate cellular proliferation. TUNEL-positive cell counts percentage in each group was used to evaluate cellular apoptosis. Compared to the control group and Maresin 1 group, there was a significant expansion of PASMC exposure to hypoxia without Maresin 1 treatment (Figure 6 b, d). Further, there were significantly more Ki67-positive cells and fewer TUNEL-positive cells in the hypoxia group, and Maresin 1 decreased hypoxia-induced Ki67-positive cells and increased TUNEL-positive cells (Figure 6 b-e).

Maresin 1 regulated PASMC through decreased phosphorylation of STAT3, AKT, ERK and FoxO1 via LGR6

Consistent with previous findings, Maresin 1 reversed hypoxia-induced Bax/Bcl-2 decrease (Supplemental Figure 1 a, b). Furthermore, we found that Maresin 1 inhibited phosphorylation of STAT3, AKT, ERK and FoxO1 which induced by hypoxia (Supplemental Figure 1 c-g). To explore a potential mechanism, we transfected siRNA of LGR6, a specific receptor of Maresin 1. PASMCs were transfected with LGR6 siRNA with different concentrations. Western blotting showed that LGR6 siRNA(100nM) was effective in reducing the expression of LGR6 (Supplemental Figure 2). Suppression of the Maresin 1 receptor significantly reduced the efficacy of Maresin 1 treatment in all parameters (Figure 7 a-f). Furthermore, in hypoxia-induced PASMC wound healing dysfunction, the protective effects of Maresin 1 on proliferation and migration were ~~significantly~~ attenuated by LRG6 receptor supression (Figure 7 g, h).

Discussion

In this study, we found that serum Maresin 1 concentration was decreased in PAH subjects, we determined that SuHx exposure for 3 weeks can induce PAH in mice with consequent serum Maresin 1 loss. After the disease model was established, exogenous Maresin 1 intervention reversed SuHx induced-PAH. We found that Maresin 1 decreased RVSP and improved right ventricular function. Treatment with Maresin 1 reduced right ventricular hypertrophy. Furthermore, we

observed Maresin 1 reversed abnormal proliferative changes in pulmonary vascular remodeling by attenuating EndoMT and enhancing apoptosis of α-SMA positive cells. Maresin 1 also inhibited PASMC proliferation through decreased phosphorylation of STAT3, AKT, ERK and FoxO1 via LGR6 in vitro. This could be a key mechanism whereby Maresin 1 exerts its protective effect in this murine PAH model.

Previous studies showed that hypoxia-induced PAH could be reversed upon returning to normoxia, to some extent, in both PAH patients and animal models(Sakao, Tatsumi & Voelkel, 2010). This suggests there are endogenous mechanisms to reverse vascular remodeling in hypoxic disease. In these experiments, Maresin 1, an endogenous lipid mediator, was decreased significantly when PAH was established by week 3, implying that it may be the potential mechanism in the development of PAH.

In some *in vivo* PAH treatment studies, interventions were administrated, followed by 3 weeks of hypoxia followed by reoxygenation for several weeks to exacerbation(Jia et al., 2020; Tu et al., 2019). Here we have maintained the mice in a continuous hypoxic environment for 6 weeks without reoxygenation to better understand the effect of Maresin 1 on chronic hypoxia-induced PAH models. In contrast to these prophylactic studies, we used Maresin 1 as a therapeutic intervention, we believe this approach is more clinically relevant to human disease pathology and treatment.

PAH has many manifestations, and the right heart function is closely related to the clinical prognosis of PAH patients(Howard et al., 2012), so, in addition to the gold standard cardiac catheters, other measurements are required to evaluate the right heart function. TAPSE reflects the movement of the base to apex shortening of the RV in systole, which is recommended by treatment guidelines as a prognostic indicator in PAH for the assessment of disease severity and response to therapy(Zelt, Chaudhary, Cadete, Mielniczuk & Stewart, 2019). Right ventricular dysfunction in pulmonary hypertension is mainly due to right cardiac hypertrophy and remodeling(2018; Asosingh et al., 2012; Oudit et al., 2008). In accordance with other studies(Tu et al., 2019), we observed myocardial hypertrophy occurred in SuHx-induced PAH mice. Treatment with Maresin 1 ameliorated RVH and improved RV function as evidenced by decreased TAPSE and PAAT/PET.

Unlike acute hypoxia-induced pulmonary vasoconstriction, chronic hypoxia causes increased pulmonary artery pressure mainly due to pulmonary vascular remodeling and vessel occlusion(Archer, Weir & Wilkins, 2010). Thus, our finding that Maresin 1 improved abnormal

pulmonary vascular remodeling is particularly important as it highlights it's potential as a novel PAH treatment option. Previous studies have identified that endothelial cells undergo an early excess of endothelial apoptosis and later resistance to apoptosis, promoting vascular obstruction in PAH(Sanchez-Duffhues et al., 2019; Thenappan, Ormiston, Ryan & Archer, 2018; Xue, Senchanthisai, Sowden, Pang, White & Berk, 2020). Consistent with these studies, we found that α-SMA increased in pulmonary arteries of SuHx mice. Furthermore, we observed greater co-expression levels of vWF and α-SMA in PAH groups by confocal microscopy, suggesting an important part of the increased expression of α-SMA was due to EndoMT. Local media α-SMA positive cells and EndoMT-derived α-SMA positive cells are all characterized by apoptosis resistance, the most important mechanical cause leading to lung vessel thickening and muscularization. Here we showed the potential therapeutic role of Maresin 1 in PAH by targeting pulmonary vascular remodeling, reducing EndoMT and restoring PASMC apoptosis to control levels.

Multiple signaling pathways are involved in mediating vascular remodeling. Indeed, activation of PI3K/AKT、ERK1/2 and STAT3 have been documented in PAH, and inhibition of these signaling pathways prevented PASMC proliferation induced by hypoxia(Courboulin et al., 2012; Song et al., 2018; Tantini et al., 2005). We demonstrated that Maresin 1 inhibited hypoxia-induced phosphorylation of AKT, ERK and STAT3, which could be a mechanism for PAH treatment. FoxO1 is centrally involved in the hyperproliferation and apoptosis-resistant phenotype of PASMCs(Savai et al., 2014). FoxO1 translocates from the nucleus to the cytoplasm, and phosphorylates by PI3K/AKT, leading to its inactivation and resulting in PASMC proliferation(Savai et al., 2014). It has been reported that hypoxia induced low-expression and nuclear import of FoxO1(Savai et al., 2014). Here, we demonstrated that hypoxia decreased FoxO1 expression and increased phosphorylated-FoxO1 expression, while Maresin 1 downregulated phosphorylated-FoxO1, improving hypoxia-induced PASMC anti-apoptosis.

In summary, serum Maresin 1 concentration is lower in PAH patients and in this murine PAH model. Our *in vivo* and *in vitro* results demonstrate that Maresin 1 shows great potential as a novel therapeutic agent for PAH, via anti-proliferation and pro-apoptotic effects on PASMCs by targeting phosphorylation of AKT, ERK, STAT3 and FoxO1 pathway following LGR6 activation (Figure 8).

Author contribution statement

S.J. and X.H. initiated the project and provided critical suggestions to the project; H.L. and Y.H. designed the experiments, analyzed the data, prepared the figures and wrote the manuscript; X.L. performed the animal experiments including echocardiography; H.W., C.W. and H.C. performed UPLC experiment; Y.F., Y. H. and N.S performed lung and heart staining and imaging; L.S., J.C., L.Q, M.C., and J.S. performed the hemodynamic assay and harvested animal samples; Y.H., B.Y. and J.L. provided human samples, A.S. and F.S. provided consultation and advice on the project and the manuscript. All authors approved for the submission of the manuscript.

Acknowledgements

The authors thank Professor Ming Li for technical assistance in our experiment. This work was supported in part by the grants from Wenzhou Major Science and Technology Innovation Project (2018ZY006), Clinical Research Fundation of the 2nd Affiliated Hospital of Wenzhou Medical University (SAHoWMU-CR2018-11-134), the Key R&D Program of Zhejiang Province (2019C03011), the Natural Science Foundation of Zhejiang Province (LQ20H150003), and the Wenzhou Science and Technology Bureau Project (Y20190118).

Conflict of interest disclosure

The authors declare that there is no conflict of interest.

Declaration of transparency and Scientific Rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Reference

(2018). 2018 AHA Late-Breaking Basic Science Abstracts. Circ Res 123**:** e69-e81.

Albuquerque-Souza E, Schulte F, Chen T, Hardt M, Hasturk H, Van Dyke TE*, et al.* (2020). Maresin-1 and Resolvin E1 Promote Regenerative Properties of Periodontal Ligament Stem Cells Under Inflammatory Conditions. Front Immunol 11**:** 585530.

Alexander SP, Kelly E, Mathie A, Peters JA, Veale EL, Armstrong JF*, et al.* (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Introduction and Other Protein Targets. Br J Pharmacol 178 Suppl 1**:** S1-S26.

Archer SL, Weir EK, & Wilkins MR (2010). Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. Circulation 121**:** 2045-2066.

Asosingh K, Farha S, Lichtin A, Graham B, George D, Aldred M*, et al.* (2012). Pulmonary vascular disease in mice xenografted with human BM progenitors from patients with pulmonary arterial hypertension. Blood 120**:** 1218-1227.

Austin ED, West J, Loyd JE, & Hemnes AR (2017). Translational Advances in the Field of Pulmonary Hypertension Molecular Medicine of Pulmonary Arterial Hypertension. From Population Genetics to Precision Medicine and Gene Editing. Am J Respir Crit Care Med 195**:** 23-31.

Chiang N, Libreros S, Norris PC, de la Rosa X, & Serhan CN (2019). Maresin 1 activates LGR6 receptor promoting phagocyte immunoresolvent functions. J Clin Invest 129**:** 5294-5311.

Courboulin A, Barrier M, Perreault T, Bonnet P, Tremblay VL, Paulin R*, et al.* (2012). Plumbagin reverses proliferation and resistance to apoptosis in experimental PAH. Eur Respir J 40**:** 618-629.

Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Giembycz MA*, et al.* (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Br J Pharmacol 175**:** 987-993.

Fattori V, Pinho-Ribeiro FA, Staurengo-Ferrari L, Borghi SM, Rossaneis AC, Casagrande R*, et al.* (2019). The specialised pro-resolving lipid mediator maresin 1 reduces inflammatory pain with a long-lasting analgesic effect. Br J Pharmacol 176**:** 1728-1744.

Gorelova A, Berman M, & Al Ghouleh I (2020). Endothelial-to-Mesenchymal Transition in Pulmonary Arterial Hypertension. Antioxid Redox Signal.

Howard LS, Grapsa J, Dawson D, Bellamy M, Chambers JB, Masani ND*, et al.* (2012). Echocardiographic assessment of pulmonary hypertension: standard operating procedure. Eur Respir Rev 21**:** 239-248.

Jia D, Bai P, Wan N, Liu J, Zhu Q, He Y*, et al.* (2020). Niacin Attenuates Pulmonary Hypertension

Through H-PGDS in Macrophages. Circ Res 127: 1323-1336.

Jin S, Chen H, Li Y, Zhong H, Sun W, Wang J, *et al.* (2018). Maresin 1 improves the Treg/Th17 imbalance in rheumatoid arthritis through miR-21. Ann Rheum Dis 77: 1644-1652.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, & Group NCRRGW (2010). Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577-1579.

Kovacic JC, Dimmeler S, Harvey RP, Finkel T, Aikawa E, Krenning G, *et al.* (2019). Endothelial to Mesenchymal Transition in Cardiovascular Disease: JACC State-of-the-Art Review. J Am Coll Cardiol 73: 190-209.

Li H, Hao Y, Zhang H, Ying W, Li D, Ge Y, *et al.* (2017). Posttreatment with Protectin DX ameliorates bleomycin-induced pulmonary fibrosis and lung dysfunction in mice. Sci Rep 7: 46754.

Lilley E, Stanford SC, Kendall DE, Alexander SPH, Cirino G, Docherty JR, *et al.* (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. Br J Pharmacol 177: 3611-3616.

Martins V, Valenca SS, Farias-Filho FA, Molinaro R, Simoes RL, Ferreira TP, *et al.* (2009). ATLa, an aspirin-triggered lipoxin A4 synthetic analog, prevents the inflammatory and fibrotic effects of bleomycin-induced pulmonary fibrosis. J Immunol 182: 5374-5381.

Oudit GY, Kassiri Z, Zhou J, Liu QC, Liu PP, Backx PH, *et al.* (2008). Loss of PTEN attenuates the development of pathological hypertrophy and heart failure in response to biomechanical stress. Cardiovasc Res 78: 505-514.

Penumatsa KC, Warburton RR, Hill NS, & Fanburg BL (2019). CrossTalk proposal: The mouse SuHx model is a good model of pulmonary arterial hypertension. J Physiol 597: 975-977.

Sakao S, Tatsumi K, & Voelkel NF (2010). Reversible or irreversible remodeling in pulmonary arterial hypertension. Am J Respir Cell Mol Biol 43: 629-634.

Sanchez-Duffhues G, Garcia de Vinuesa A, van de Pol V, Geerts ME, de Vries MR, Janson SG, *et al.* (2019). Inflammation induces endothelial-to-mesenchymal transition and promotes vascular calcification through downregulation of BMPR2. J Pathol 247: 333-346.

Savai R, Al-Tamari HM, Sedding D, Kojonazarov B, Muecke C, Teske R, *et al.* (2014). Pro-proliferative and inflammatory signaling converge on FoxO1 transcription factor in pulmonary hypertension. Nat Med 20: 1289-1300.

Serhan CN (2014). Pro-resolving lipid mediators are leads for resolution physiology. Nature 510: 92-101.

Serhan CN, Dalli J, Karamnov S, Choi A, Park CK, Xu ZZ*, et al.* (2012). Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. FASEB J 26**:** 1755-1765.

Song S, Carr SG, McDermott KM, Rodriguez M, Babicheva A, Balistrieri A*, et al.* (2018). STIM2 (Stromal Interaction Molecule 2)-Mediated Increase in Resting Cytosolic Free Ca(2+) Concentration Stimulates PASMC Proliferation in Pulmonary Arterial Hypertension. Hypertension 71**:** 518-529.

Tantini B, Manes A, Fiumana E, Pignatti C, Guarnieri C, Zannoli R*, et al.* (2005). Antiproliferative effect of sildenafil on human pulmonary artery smooth muscle cells. Basic Res Cardiol 100**:** 131-138.

Thenappan T, Ormiston ML, Ryan JJ, & Archer SL (2018). Pulmonary arterial hypertension: pathogenesis and clinical management. BMJ 360**:** j5492.

Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C*, et al.* (2019). Selective BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension. Circ Res 124**:** 846-855.

van der Feen DE, Bartelds B, de Boer RA, & Berger RMF (2017). Pulmonary arterial hypertension in congenital heart disease: translational opportunities to study the reversibility of pulmonary vascular disease. Eur Heart J 38**:** 2034-2041.

Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J*, et al.* (2013). Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. J Am Coll Cardiol 62**:** D22-33.

Wu K, Zhang Q, Wu X, Lu W, Tang H, Liang Z*, et al.* (2017). Chloroquine is a potent pulmonary vasodilator that attenuates hypoxia-induced pulmonary hypertension. Br J Pharmacol 174**:** 4155-4172.

Xue C, Senchanthisai S, Sowden M, Pang J, White J, & Berk BC (2020). Endothelial-to-Mesenchymal Transition and Inflammation Play Key Roles in Cyclophilin A-Induced Pulmonary Arterial Hypertension. Hypertension 76**:** 1113-1123.

Zelt JGE, Chaudhary KR, Cadete VJ, Mielniczuk LM, & Stewart DJ (2019). Medical Therapy for Heart Failure Associated With Pulmonary Hypertension. Circ Res 124**:** 1551-1567.

Zhang H, Wang D, Li M, Plecita-Hlavata L, D'Alessandro A, Tauber J*, et al.* (2017). Metabolic and Proliferative State of Vascular Adventitial Fibroblasts in Pulmonary Hypertension Is Regulated Through a MicroRNA-124/PTBP1 (Polypyrimidine Tract Binding Protein 1)/Pyruvate Kinase Muscle Axis. Circulation 136**:** 2468-2485.

Figure Legends

Figure 1 Establishment of pulmonary hypertension model in mice. Adult male mice were

exposed to hypoxia (10% oxygen) and injected with Sugen 5416 (SuHx, 20mg/kg) once a week for 3 weeks. (a) Representative images of RVSP via invasive hemodynamic assessment. Scatterplots graphs for RVSP (b) and right ventricular hypertrophy (Fulton index (RV/LV+S)) (c), each plot represents the value from an individual mouse, n=8 mice per group. Representative images (d) and scatterplots graph (e) of TAPSE and representative images (f) and scatterplots graph (g) of PAAT/PET via transthoracic echocardiography, each point represents the average of triplicate values from an individual mouse, n=7 mice per group. Serum Maresin 1(MaR1) concentrations were measured by UPLC. (h) Multiple reaction monitoring chromatogram shows the retention time for MaR1 (m/z 359/177). Q1, M-H (parent ion) and Q3, diagnostic ion in the tandem mass spectrometry (MS/MS) (daughter ion). (i) MS/MS spectrum and molecular structural formula of MaR1. (j) Scatterplots graphs of MaR1 expressed in healthy and PAH subjects, n=8 for each group. (k) Scatterplots graphs of MaR1 expressed in control and Suhx mice, n=5-8 for each group. (l-m) LGR6 protein levels in lungs in mice, n=6 for each group. Data are presented as the mean ± SEM, $^{**}P < 0.01$. Data in **b, c, e, g, j, k, m** were analyzed using two-tailed unpaired Student's t-test for two-group comparisons.

Figure 2 Maresin 1 reversed pulmonary arterial pressure and right ventricular dysfunction in established experimental PAH model. (a) Experiment protocol. Mice were randomly assigned to three groups. (i) Control; (ii) SuHx [exposed to chronic hypoxia (10% oxygen) for 6 weeks and injected with Sugen 5416 (20 mg/kg per week, s.c) during the first 3 weeks]; (iii) SuHx+MaR1 [SuHx mice were post-treated with MaR1 (1ug/mouse from the 4th week followed by boosted 100 ng/mouse every other day till 6weeks, i.p)]. (b) Evolution of body weight gain expressed as a percentage of initial body weight. (c) Representative images of RVSP via invasive hemodynamic assessment. (d) Scatterplots graphs for RVSP, each plot represents the value from an individual mouse, n=8 mice per group. (e) Representative pictures of hearts in all three groups (scale bars, 5mm), the yellow dotted line represents the heart width of mice in the control group. (f) Assessment of Fulton index (RV/LV+S). n=8 mice per group. (g) Representative HE staining of the hearts at the papillary muscle level (scale bar, 1000μm), the solid yellow line represents the length of the RV cross-section of control mice. n=5 mice per group (h) Scatterplots graphs for the length of RV cross-section. Representative images (i) and scatterplots graph (j) of TAPSE and representative images (k) and scatterplot graph (l) of PAAT/PET via transthoracic echocardiography, each point represents the

672 average of triplicate values from an individual mouse, n=6 mice per group. Data are presented as
673 the mean ± SEM, *$P < 0.05$, **$P < 0.01$. Data in **b, d, f, h, j, l** were analyzed using one-way ANOVA
674 followed by Tukey's post hoc test for multiple comparisons.
675 **Figure 3 Maresin 1 attenuated abnormal pulmonary vascular remodeling in SuHx-induced PAH**
676 **mice.** (a) Representative HE staining of small, peripheral pulmonary arteries. (b) Vessel wall area/total
677 area (WA/TA) of pulmonary arterioles. (c) Vessel wall thickness/total thickness (WT/TT) of pulmonary
678 arterioles. (d) Representative Masson trichrome staining of pulmonary arteries，collagen is stained blue.
679 (e) Scatterplots graphs for collagen volume. (f) Representative Elastica–van Gieson (EVG) staining of
680 pulmonary arteries, elastin fiber was stained dark gray and collagen in pink. (g) Scatterplot graphs for
681 collagen deposition area. (h)Representative immunohistochemical staining images of α–smooth muscle
682 actin (α-SMA) and statistical plot for WT/TT (i) and area per cell(j). Data are presented as the mean ±
683 SEM, each point represents the average of two arteries in each field from an individual mouse. n=6
684 mice per group, **$P < 0.01$. Scale bars, 50 μm. Data in **b, c, e, g, i, j** were analyzed using one-way
685 ANOVA followed by Tukey's post hoc test for multiple comparisons.
686 **Figure 4 Maresin 1 attenuated endothelial-to-mesenchymal transition in pulmonary arterioles in**
687 **SuHx-induced PAH mice** (a) Representative confocal images for vWF (red), α-SMA (green) and DAPI
688 (blue) of pulmonary arteries in lung sections from each group mice. Yellow indicates positive cell. (b)
689 Quantification of the total number of $vWF^+\alpha\text{-}SMA^+$ cells in all $\alpha\text{-}SMA^+$ cells. Data are presented as the
690 mean ± SEM, each point represents the average of two arteries in each field from an individual
691 mouse. n=6 mice per group. **$P < 0.01$. Scale bars, 50 μm. Data in **b** were analyzed using one-way
692 ANOVA followed by Tukey's post hoc test for multiple comparisons.
693 **Figure 5 Maresin 1 enhanced apoptosis of α-SMA positive cells in pulmonary arterioles in**
694 **SuHx-induced PAH mice** （a） Representative confocal images for α-SMA (red), TUNEL (green)
695 and DAPI (blue) of pulmonary arteries in lung sections from each group mice. Arrows indicate
696 positive cells. (b) Statistical graphs for TUNEL positive α-SMA+ cell count. (c) Bcl-2 (anti-
697 apoptotic), Bax (pro-apoptotic protein), cleaved-caspase3 and caspase3 protein levels in lung tissues.
698 Statistical graphs for Bax to Bcl-2 ratio (d) and cleaved-caspase3 toβ-actin (e) were shown. Data
699 are presented as the mean ± SEM. n=5-6 mice per group. *$P < 0.05$, **$P < 0.01$. Scale bars, 50 μ
700 m. Data in **b, d, e** were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple
701 comparisons.

Figure 6 Maresin 1 inhibited hypoxia-induced PASMC migration, proliferation and promoted cellular apoptosis. Primary rat pulmonary artery smooth muscle cells (PASMCs) were exposed to hypoxia or / and Maresin 1(MaR1, 200nM) for 24 hours. (a-b) Wound healing assay was used to evaluate the ability of cell migration (n=4, $^{**}P < 0.01$, compared to the normoxia group, ## $P < 0.01$, compared to the hypoxia group). (c-d) Cell proliferation assay. The cells were stained with DAPI (blue) and antibody for Ki67 (red). Representative confocal microscopy images of Ki67 staining and statistical analyses of Ki67-positive cells and total cells. (e-f) Cell apoptosis assay. Representative confocal microscopy images of TUNEL staining and statistical analyses of TUNEL-positive cells and total cells. Data are presented as the mean ± SEM. n=5 $^{**}P < 0.01$. Scale bars, 50 μm. Data in **b** were evaluated using two-way ANOVA followed by multiple comparisons. Data in **d, f** were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

Figure 7 Maresin 1 regulated PASMC through decreased phosphorylation of STAT3, AKT, ERK and FoxO1 via LGR6. PASMCs were transfected with LGR6 siRNA (100 nM) for 24 hours, followed by exposure to hypoxia (Hx) or / and Maresin 1(MaR1, 200nM) for another 24 hours. (a-f) The expression of STAT3, AKT, ERK, FoxO1, Bcl-2 and BAX in the presence of LGR6-siRNA. (g-h) Wound healing assay in the presence of LGR6-siRNA. Nx: normoxia, si-LGR6: LGR6 siRNA transfection, si-Scr: scrambled siRNA as negative control. Data are presented as the mean ± SEM. n=4-6. ## $P < 0.01$ compared to the control group, $$ $P < 0.01$ compared to the Hx group, & $P < 0.05$, && $P < 0.01$, compared to the Hx+MaR1 group. $^{**}P < 0.01$. Data in **b, c, d, e, f, h** were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

Figure 8 Maresin 1 improved abnormal pulmonary vascular remodeling and right ventricular dysfunction in the PAH mice model. Furthermore, Maresin 1 inhibited PASMC proliferation and migration, and promoted cell apoptosis through decreased hypoxia-induced phosphorylation of STAT3, AKT, ERK and FoxO1 via LGR6. Maresin 1 may have a potent therapeutic effect in vascular disease.