Maresin 1 intervention reverses experimental pulmonary arterial hypertension in mice

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Title Page

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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

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

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

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

Methods

Data availability statement

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

Mice

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

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

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

157 The assays were performed as described previously(Jin et al., 2018). Serum was collected from
158 whole blood for murine and human samples. Healthy volunteer samples were obtained from
159 Physical Examination Center of the Second Affiliated Hospital of Wenzhou Medical University.
160 PAH patient samples were obtained from the ICU of the Second Affiliated Hospital and Operation
161 Room of the First Affiliated Hospital. Murine blood was collected via the retro-orbital plexus under
162 terminal anesthetic, before sacrifice. Whole blood (murine/human) was allowed to clot for 30 mins
163 then centrifuged at 560xg for 10mins and supernatant was collected for stored at -80℃. Maresin 1
164 was quantified in serum samples. Serum samples (humans 2.0 ml, mice 0.5 ml) were placed in ethyl
165 acetate (2.5ml/0.5ml serum samples), which contained deuterium-labelled Maresin 1 (7R,14S-
166 dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid, Cas# 1268720-28-0, Cayman Chemical)
167 to each sample. Samples were then continuously vortexed for 3 min and sonicated for 15min before
168 centrifugation at 560x g for 10 minutes to allow supernatant collection. Total lipid mediators were
169 extracted from serum samples with a solid phase C18 column (Waters, USA) and the level of
170 Maresin 1 analyzed by ultra-performance liquid chromatography tandem mass spectrometry
171 (UPLC-MS/MS). Water containing 0.01% acetic acid was used as solvent A and MeOH containing
172 0.01% acetic acid was used as solvent B. We used the UPLC I-Class system (Waters, USA) equipped
173 with an AB Sciex Instruments 6500 Q-TRAP mass spectrometer (Sciex, USA) and Analyst 1.6
174 software (Applied Biosystems, USA) to acquire and analyze data. Quantification of Maresin 1 was
175 based on peak area of multiple reaction monitoring transitions and linear calibration curve of each
176 compound.

177 **Curative protocol in PAH models**

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

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

191 **Cell procedures**

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

200 **Small interfering RNA treatment of PASMC**

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

206 **Echocardiography**

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

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

216 **Hemodynamic assay**

217 After establishment of the PAH model, mice were anesthetized with 1% pentobarbital sodium
218 (60mg/kg, i.p). Two 15cm polyethylene catheters (inner diameter 0.5mm, external diameter 0.9mm),
219 prefilled with heparin in order to prevent coagulation, were connected to the pressure system (AD
220 Instruments, Colorado Springs, CO, Australia). Blunt dissection of neck muscle tissue was used to
221 expose the jugular vein and internal carotid. Finally, the right ventricle systolic pressure (RVSP)
222 was measured by polyethylene catheters, which were inserted into the right ventricle via the jugular
223 vein. Meanwhile, the mean carotid arterial pressure (mCAP) was monitored utilizing polyethylene
224 catheters inserted into the left carotid artery. All RVSP data were collected under a consistent mCAP.

225 **Immunohistochemistry**

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

237 **Endothelial-mesenchymal transition**

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

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

247 **Cell apoptosis assay**

248 Cell apoptosis was determined by the In Situ Cell Death Detection Kit (1:9, Roche Cat#
249 11684795910). In vivo, after modeling, mouse paraffin-embedded lungs were sectioned and
250 deparaffinized in xylene followed by rehydrating with gradient ethanol and water. Antigen retrieval
251 and then permeabilized with 0.5% Trion X-100 for 10 minutes. After blocking with 10% normal
252 goat serum, slides were incubated with anti-α-SMA antibody overnight at 4℃. The following day,
253 the slides were incubated with TUNEL mixed solution at 37℃ for 1 hour. Then nuclei were
254 counterstained with DAPI for 5 minutes at room temperature. After washing with PBS three times,
255 samples were imaged by confocal laser scanning microscope (Zeiss, Oberkochen, Germany).
256 In vitro, primary rat PASMCs were grown on coverslips in 24 well plates and fixed with 4%
257 paraformaldehyde for 20 min at room temperature after modeling. Cell membranes were ruptured
258 with 0.2% Triton X-100 for 10 min. the slides were incubated with TUNEL mixed solution at 37℃
259 for 1 hour. Nuclei were counterstained with DAPI for 5 minutes at room temperature. After washing
260 with PBS three times, the samples were imaged by confocal laser scanning microscope (Zeiss,
261 Oberkochen, Germany).

262 **Cell proliferation assay**

263 Proliferation of rat PASMCs was determined by immunofluorescence staining for Ki67. PASMCs
264 were grown on coverslips in 24 well plates after modeling, fixed with 4% paraformaldehyde for 20
265 min at room temperature. Cell membranes were ruptured with 0.2% Triton X-100 for 10 min. After
266 blocked with 10% donkey serum for 30 min in 37℃, PASMCs were incubated with Ki67 antibody
267 (1:100, Thermo Fisher Scientific Cat# 14-5698-82, RRID: AB_10854564) overnight at 4 ℃, then
268 cells were incubated with Alexa Fluor 594 secondary antibody (1:200, Biyuntian, CHN) for 60 min.
269 Then nuclear were stained with DAPI at room temperature for 5 min. Finally, images were
270 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 4th week to the 6th 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

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

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

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

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

375 The proliferation of apoptosis-resistant α-SMA positive cells is an important cause of pulmonary
376 artery occlusion in pulmonary hypertension. To examine the possible action of Maresin 1 on α-SMA
377 positive cell clearance in vivo, we examined the distribution of apoptotic cells in arterioles located
378 by α-SMA. Consistently, the arterioles in SuHx mice were characterized by increased α-SMA
379 expression. Apoptotic cells as indicated as TUNEL positive cells, were observed less in SuHx mice
380 pulmonary arterioles compared with the control group. Maresin 1 treatment restored α-SMA
381 positive cell apoptosis to control levels and decreased total α-SMA expression in these cells (Figure
382 5 a, b). Consistent with this, we observed that Bcl-2 was upregulated with little change in the level
383 of Bax protein, which resulted in a reduced Bax/Bcl-2 ratio in the lungs of the PAH mice model.
384 Cleaved-caspase-3, the downstream protein of the Bax/Bcl-2 pathway, was also decreased. Maresin
385 1 restored the Bax/Bcl-2 ratio and cleaved-caspase-3 levels to baseline in lung tissues from PAH
386 mice (Figure 5 c-e).

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

389 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.

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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.

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640 **Figure Legends**

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

642 exposed to hypoxia (10% oxygen) and injected with Sugen 5416 (SuHx, 20mg/kg) once a week for
643 3 weeks. (a) Representative images of RVSP via invasive hemodynamic assessment. Scatterplots
644 graphs for RVSP (b) and right ventricular hypertrophy (Fulton index (RV/LV+S)) (c), each plot
645 represents the value from an individual mouse, n=8 mice per group. Representative images (d) and
646 scatterplots graph (e) of TAPSE and representative images (f) and scatterplots graph (g) of
647 PAAT/PET via transthoracic echocardiography, each point represents the average of triplicate values
648 from an individual mouse, n=7 mice per group. Serum Maresin 1(MaR1) concentrations were
649 measured by UPLC. (h) Multiple reaction monitoring chromatogram shows the retention time for
650 MaR1 (m/z 359/177). Q1, M-H (parent ion) and Q3, diagnostic ion in the tandem mass spectrometry
651 (MS/MS) (daughter ion). (i) MS/MS spectrum and molecular structural formula of MaR1. (j)
652 Scatterplots graphs of MaR1 expressed in healthy and PAH subjects, n=8 for each group. (k)
653 Scatterplots graphs of MaR1 expressed in control and Suhx mice, n=5-8 for each group. (l-m) LGR6
654 protein levels in lungs in mice, n=6 for each group. Data are presented as the mean ± SEM, $^{**}P <$
655 0.01. Data in **b, c, e, g, j, k, m** were analyzed using two-tailed unpaired Student's t-test for two-
656 group comparisons.

657 **Figure 2 Maresin 1 reversed pulmonary arterial pressure and right ventricular dysfunction in**
658 **established experimental PAH model.** (a) Experiment protocol. Mice were randomly assigned to
659 three groups. (i) Control; (ii) SuHx [exposed to chronic hypoxia (10% oxygen) for 6 weeks and
660 injected with Sugen 5416 (20 mg/kg per week, s.c) during the first 3 weeks]; (iii) SuHx+MaR1
661 [SuHx mice were post-treated with MaR1 (1ug/mouse from the 4th week followed by boosted 100
662 ng/mouse every other day till 6weeks, i.p)]. (b) Evolution of body weight gain expressed as a
663 percentage of initial body weight. (c) Representative images of RVSP via invasive hemodynamic
664 assessment. (d) Scatterplots graphs for RVSP, each plot represents the value from an individual
665 mouse, n=8 mice per group. (e) Representative pictures of hearts in all three groups (scale bars,
666 5mm), the yellow dotted line represents the heart width of mice in the control group. (f) Assessment
667 of Fulton index (RV/LV+S). n=8 mice per group. (g) Representative HE staining of the hearts at the
668 papillary muscle level (scale bar, 1000μm), the solid yellow line represents the length of the RV
669 cross-section of control mice. n=5 mice per group (h) Scatterplots graphs for the length of RV cross-
670 section. Representative images (i) and scatterplots graph (j) of TAPSE and representative images (k)
671 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^{+}α-SMA^{+} cells in all α-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.